Ferroptosis Is Involved in Diabetes Myocardial Ischemia/Reperfusion Injury Through Endoplasmic Reticulum Stress

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Myocardial ischemic disease affects the prognosis in perioperative patients. Diabetes can aggravate myocardial injury. The purpose of this research is to investigate the effect of ferroptosis in the process of diabetes mellitus (DM) myocardial ischemia/reperfusion (I/R) injury (IRI). Endoplasmic reticulum stress (ERS) is investigated whether aggravates cardiomyocytes injury. Rat DM+I/R (DIR), cell high glucose (HG), hypoxia reoxygenation (H/R), and high-glucose H/R (HH/R) models were established. Ferroptosis inhibitor Ferrostatin-1, ferroptosis agonist Erastin, ERS inhibitor Salubrinal, and ERS agonist Tunicamycin were administered. Serum creatine kinase-MB (CK-MB), cell viability, lactate dehydrogenase (LDH), malondialdehyde (MDA), superoxide dismutase (SOD), reactive oxygen species (ROS), and cellular ferrous ion concentration were examined. The level of ACSL4, GPX4, ATF4, CHOP, BCL-2, and BAX was detected. Myocardial tissue pathological change was detected by hematoxylin-eosin staining. Cardiac function was monitored by invasive hemodynamic measurements. Evans Blue-triphenyltetrazolium chloride double staining was used to detect the myocardial infarct size. In DM+sham (DS) (or HG) and I/R (or H/R) models, cardiomyocytes were injured accompanied by increased level of ferroptosis and ERS. Moreover, the cell injury was more serious in rat DIR or cell HH/R models. Inhibition of ferroptosis in DIR model could reduce ERS and myocardial injury. Inhibition of ferroptosis in H9c2 cells HG, H/R, and HH/R models could reduce cell injury. Erastin could aggravate ERS and cell injury by stimulating ferroptosis in HH/R cell model. Meanwhile, inhibition of ERS could alleviate ferroptosis and cell injury. Ferroptosis is involved in DIR injury that is related to ERS. Moreover, inhibition of ferroptosis can alleviate DIR injury, which may provide a therapeutic regent for myocardial ischemic disease.

Keywords: ferroptosis, diabetes myocardial, ischemia/reperfusion, endoplasmic reticulum stress

Introduction

DIABETES IS A METABOLIC DISORDER caused by hyperglycemia and insufficient release of endogenous insulin or underutilization (Benninger *et al.*, 2011). Diabetes is a high-risk factor for perioperative complications and cardiovascular disease (Altunkaynak and Ozcelikay, 2016). In myocardial ischemia/reperfusion (I/R) injury (IRI), the ischemic heart gets injured irreversibly, although the heart restores blood flow reperfusion (Korkmaz-Icoz *et al.*, 2015). The pathological changes caused by metabolic abnormalities, such as coronary artery occlusion, decreased vascular compliance, and microvascular disease, usually occur in diabetic patients (Kurmus *et al.*, 2018). As a result, it leads to systolic diastolic dysfunction, ventricular hypertrophy, or myocardial fibrosis (Kurmus *et al.*, 2018).

The tolerance of damaged myocardium to ischemia–anoxia injury is much lower than normal myocardium (Kurmus *et al.*, 2018). Studies have shown that the incidence of

myocardial ischemia in diabetic patients is 2.45 to 2.99 times higher than that in nondiabetics (Ndumele *et al.*, 2016). The sensitivity of myocardial IRI in diabetic patients is higher than normal myocardium. This may increase oxidative stress caused by hyperglycemia and excessive reactive oxygen species (ROS) production (Khardori and Nguyen, 2012).

In 2012, Dixon *et al.* first proposed the concept that ferroptosis is a form of regulated cell death that is dependent on iron and ROS. Ferroptosis is resulted from the accumulation of cellular ROS that exceed the redox contents maintained by glutathione and the phospholipid hydroperoxidases (Dixon *et al.*, 2012). During the process of ferroptosis, the activity of cystine–glutamate antiporter (system Xc[−]) is inhibited, namely the amount of cystine entering into cells decreases, the amount of glutamate transporting out of cells decreases. The lipid peroxide will accumulate (Dixon *et al.*, 2012; Sui *et al.*, 2018). Then, glutathione peroxidase 4 (GPX4) is inactivated, causing cell death (Dixon *et al.*, 2012).

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Ferroptosis is characterized by the production of ROS and lipid peroxidation during fenton reaction. Ferroptosis is associated with a variety of pathological cell death in some degenerative pathological diseases (Stockwell *et al.*, 2017). Recent studies have shown that endoplasmic reticulum stress (ERS) plays an important role in the process of ferroptosis through the induction of unfolded protein. ERS is characterized by the activation of activating transcription factor 4 (ATF4)-C/EBP homologous protein (CHOP) pathway. Moreover, CHOP can further induce the cell injury during ERS (Zheng *et al.*, 2017; Lee *et al.*, 2018).

The relationship between ferroptosis and ERS includes the following three aspects. First, the occurrence of ferroptosis is accompanied by the production of ERS (Wang *et al.*, 2019a), which is verified in intestinal epithelial cells (Qi *et al.*, 2019) and some cancer cells, such as pancreatic ductal adenocarcinoma (Zhu *et al.*, 2017), cervical carcinoma (Sun *et al.*, 2015), and fibrosarcoma (Dixon *et al.*, 2014). Through in-depth mechanism research, it is found that the unfolded protein response (UPR) is caused by ferroptosis inducers. The PERK-eIF2 α -ATF4-CHOP pathway was subsequently activated, which triggers ERS (Lee *et al.*, 2018). Meanwhile, the ferroptosis could promote the cystine–glutamate antiporter system Xc⁻, which leads to ERS (Rahmani *et al.*, 2007; Sun *et al.*, 2015).

Second, ERS is a cellular response to endoplasmic reticulum dysfunction and can be triggered by ROS (Cao and Kaufman, 2014). More importantly, ROS is produced by the interaction between iron ions and NADPH oxidase in mitochondria during the process of ferroptosis (Shimada *et al.*, 2016). Third, ferroptosis agonists (sorafenib, erastin, sulfasalazine) could activate the ATF4-CHOP pathway (Lee *et al.*, 2018). CHOP-mediated ERS has been proved to play an important role in the I/R injury of rat myocardium (Zhang *et al.*, 2013).

The above studies suggest that ERS plays an important role in cell injury in the process of ferroptosis. However, whether ferroptosis is involved in diabetes myocardial IRI through ERS and inhibition of ferroptosis could alleviate myocardial IRI deserve further studies.

Materials and Methods

Materials

H9c2 rat cardiomyocyte cell line was purchased from Wuhan Punosi Life Science and Technology Co., Ltd (Wuhan, Hubei, China). Dulbecco's modified Eagle's medium (DMEM) low sugar medium was purchased from HyClone (Logan, UT). Fetal bovine serum was purchased from Gibco (Grand Island, NY). The cell counting kit-8 (CCK-8) was purchased from Dojindo (Kumamoto, Kyushu, Japan). The Annexin V-FITC/propidium iodide flow cytometry apoptosis kit was purchased from BD Biosciences (San Jose, CA). Lactate dehydrogenase (LDH), superoxide dismutase (SOD), ROS, and malondialdehyde (MDA) test kits were purchased from Nanjing Jiancheng Bioengineering Institute (Nanjing, Jiangsu, China).

Iron assay kit, ACSL4, and GPX4 primary antibodies were purchased from Abcam (Cambridge, MA). CHOP, ATF4, BCL-2, BAX, and β -actin primary antibodies were purchased from CST (Boston, MA). Second antibodies were purchased from LI-COR Biosciences (IRDye 800CW; LI-COR Corporate, Lincoln, NE). Cy3-labeled goat antirabbit second antibody

was purchased from Proteintech (Wuhan, Hubei, China). The Trizol, Prime-Script RT reagent kit and SYBR Premix Ex Taq kit were purchased from TAKARA (Dalian, Liaoning, China). Erastin (Era), Ferrostatin-1 (Fer-1) and Salubrinal (Sa) were purchased from Selleck (Houston, TX). Tunicamycin (Tu) was purchased from MedChemExpress (Manmouth Junction, NJ).

Animal model establishment and drug administration

Fifty specific pathogen free male Sprague-Dawley rats (weighing 210–240 g) were purchased from Beijing Huakang Biotechnology Co., Ltd (Beijing, China). The animal experiment was conducted in the animal experiment center at a temperature of 25°C $\pm$ 2°C, relative humidity of 50% $\pm$ 15%, and normal circadian rhythm (12 h dark/12 h light) in Renmin Hospital of Wuhan University. All the rats got free water and food. The study protocols were in accordance with the internationally accepted principles and Guidelines for the Care and Use of Laboratory Animals of Wuhan University [Approval No. SYXK (HUBEI) 2014-0080]. All the rats were randomly equally divided into five groups: normal+sham group (NS), I/R group (I/R), diabetes mellitus (DM)+sham (DS) group, DM+I/R group (DIR), and DM+I/R+Fer-1 group (DIR+Fer-1). Every group included 10 rats. The DS and DIR rat models were built as follows.

The DS, DIR, and DIR+Fer-1 group rats were injected with 1% streptozotocin 60 mg/kg once in the tail vein. After 3 days, if the fasting blood glucose level was higher than 16.7 mmol/L, the DS model was successfully built. The other group rats were injected with normal saline in the tail vein. Thereafter, the general conditions for normal and DM rats are recorded in Table 1. On the last day after 8 weeks, the NS group, DS group, I/R group, DIR group, and DIR+Fer-1 group rats were intraperitoneally injected with 1.5% sodium pentobarbital at a dose of 0.005 mL/g. They were given electrocardiogram (ECG) monitoring management. After the rats were subjected to endotracheal intubation, the tracheotomy catheter was connected to a microanimal ventilator for 80–90 times/min mechanical ventilation.

The heart rate (HR) and blood pressure were recorded. The rats' hearts were exposed by sternotomy along the left edge of sternum. The left anterior descending (LAD) coronary artery between the left atrial appendage and the pulmonary artery cone was sutured and then covered with saline gauze. If the left ventricle apex for myocardial blanched and ST segment of ECG was elevated, the LAD coronary artery was successfully ligatured. To restore the reperfusion of coronary arteries, the ligature was cut after 30 min of ischemia. At the same time, the anterior wall of left ventricle turning red and the elevated ST segment of electrocardiogram indicated the successful reperfusion. Then, the coronary arteries restored the reperfusion for 2 h.

Rats in the NS group were only threaded without ligation. Referred to the reported dosage (Zhang *et al.*, 2018), DIR+Fer-1 group rats were intraperitoneally injected with Fer-1 at a dose of 2 mg/kg until 2 h before building I/R model. The part of the cardiac apex and serum was collected for the follow-up experiments.

Determination of cardiac function

Invasive hemodynamic measurements were used to monitor the cardiac function. In brief, the heparin-saline-filled

TABLE 1. GENERAL CONDITION OF NORMAL AND DIABETES MELLITUS RATS

Group	Body weight (g)	Food intake g/(kg·day)	Water intake mL/(kg·day)	Heart weight index (%)	Blood glucose (mmol/L)
Normal (n=10)	479.6±30.5	70.2±6.9	114.5±9.6	3.6±0.3	6.5±0.7
DM (n=40)	283.7±27.0 ^a	121.4±10.7 ^a	280.9±22.4 ^a	4.8±0.5 ^a	27.8±2.9 ^a

Results are expressed as mean±standard deviation.

^a*P*<0.01, compared with normal rats.

DM, diabetes mellitus.

catheter was inserted into the right common carotid artery, and separately passed through the aortic arch, the left atrium, the mitral valve, and the left ventricle for measurement. The other end of the catheter was connected to a pressure transducer (Yixinda, Shenzhen, China). Measurements were taken at 10 min before ischemia and 2 h after reperfusion. HR, left ventricular systolic pressure (LVSP), maximal rate of increase of left ventricular developed pressure (+dp/dt), and maximal rate of decrease of left ventricular developed pressure (−dp/dt) were monitored by electrophysiograph purchased from BioPAC (Goleta, CA). Data analysis was performed by AcqKnowledge 4.0 software.

Triphenyltetrazolium chloride determination of myocardial infarction area

Myocardial infarction area was detected by Evans Blue and triphenyltetrazolium chloride (TTC) double staining. Six rats in each group were randomly selected, and the LAD coronary artery was ligated. The rats were rapidly injected 1 mL 5% Evans Blue from the femoral vein. After the myocardial blue staining and nonblue staining area was clearly distinguished through observation, the aortic arch was clipped by hemostasis. The heart was then quickly extracted, rinsed with 4°C PBS solution, and placed vertically. The heart was frozen at −20°C for 30 min. The frozen heart was placed in a special heart slice trough. Five pieces of heart slices ~1 mm thick were prepared, placed in the 1% TTC solution in a 37°C incubator for 15 min in the dark. The slices were then fixed in 4% paraformaldehyde for 30 min.

Each area of the myocardium was calculated by Image-Pro Plus 6.0 software. The nonischemic myocardium stained with Evans Blue was dark blue. For the ischemic myocardium stained with TTC staining, brick red myocardium was defined as the area at risk (AAR), and pale myocardium was defined as the infarct area (IA). The percentage of myocardial infarction area was calculated as the percentage of infarcted myocardium area to the ischemic myocardium area (IA/AAR%).

Cell model establishment and drug administration

H9c2 cells were cultured in a cell culture chamber containing 10% CO₂ at 37°C. When H9c2 cells grew to 70–80% density, the trypsin with ethylenediaminetetraacetic acid (EDTA) was used to digest the cells. The cells were planted in six-well plates. The cells were divided into normal group, high-glucose (HG) group, hypoxia reoxygenation group (H/R), high-glucose hypoxia reoxygenation group (HH/R), HH/R+Erastin group (HH/R+Era), HH/R+Erastin+Salubrinal group (HH/R+Era+Sa), HH/R+Salubrinal group (HH/R+Sa), HH/R+Ferrostatin-1 group (HH/R+Fer-1), HH/R+Ferrostatin-

1+Tunicamycin group (HH/R+Fer-1+Tu), and HH/R+Tunicamycin group (HH/R+Tu). After being synchronized with serum-free low-glucose DMEM for 24 h, HG medium (glycol concentration of 30 mmol/L) was added.

Then, the cells were cultured at 37°C for 24 h to establish HG model. The H/R model was established by hypoxia (volume fraction 94% N₂+ volume fraction 5% CO₂+ volume fraction 1% O₂) 4 h and reoxygenation (volume fraction 90% atmosphere+volume fraction 10% CO₂) 2 h. For the HH/R cell model, before the last 6 h for building HG cell model, the H/R cell model was beginning to establish. The above drugs respectively affected at a concentration of 10 μmol/L (Erastin) (Baba *et al.*, 2018; Bai *et al.*, 2018; Liu *et al.*, 2018), 10 μmol/L (Ferrostatin-1) (Baba *et al.*, 2018), 10 μmol/L (Tunicamycin) (Lee *et al.*, 2013; Wu *et al.*, 2014), 20 μmol/L (Salubrinal) (Liu *et al.*, 2012, 2016a; Wu *et al.*, 2014). Era and Fer-1 continuously acted for 24 h before building cell models. Tu and Sa were added 6 h before building cell models.

Cell viability detection

Cell suspension was prepared and inoculated into the 96-well plate at a density of 5×10³ cells/100 μL per well. After the cells were being attached, they were modeled and administered with the corresponding drugs. The control group was set with only adding DMEM. Each group had six duplicate holes. CCK-8 was added to the basal medium at a ratio of 1:10 to prepare CCK-8 working solution. Then, the culture solution was removed. CCK-8 working solution for 100 μL was added to each well. The cells were incubated for 1 h in the dark. At last, the optical density value for each well at 490 nm absorbance was measured by a microplate reader.

Creatine kinase-MB, ROS, SOD, MDA, LDH, and intracellular ferrous ion (Fe²⁺) determination

The cells and cell supernatant were collected to measure the levels of creatine kinase-MB (CK-MB), SOD, MDA, LDH, and intracellular Fe²⁺ by assay kits according to the instructions. Then, the intensity was observed by a microplate reader. Following the instructions of ROS assay kit, the cells were incubated with 2',7'-dichlorodihydrofluorescein diacetate (DCFH-DA) probes for 30 min in the dark. Then, the fluorescence intensity was observed under a fluorescence microplate. The stimulated light wavelength was 485 nm, and the emission light wavelength was 525 nm. ROS level (%) = fluorescence value of intervention group/control group×100% (Yang *et al.*, 2009).

Quantitative real-time PCR to detect mRNA expression

The primers were designed and synthesized by Wuhan Qingke Biotechnology Co., Ltd (Wuhan, Hubei, China). The

TABLE 2. THE PRIMERS OF QUANTITATIVE REAL-TIME PCR

<i>Gene</i>	<i>Forward (5'–3')</i>	<i>Reverse (5'–3')</i>
ACSL4	TCCAAGCCAGAAAACCTCAAGC	GGTGTACATGACAATGGCCAT
ATF4	GTTGGTCAGTGCCTCAGACA	CATTCCGAAACAGAGCATCGA
CHOP	CTGGAAGCCTGGTATGAGGAT	CAGGGTCAAGAGTAGTGAAGGT
β -Actin	TGCTATGTTGCCCTAGACTTCG	GTTGGCATAGAGGTCTTTACGG

primer sequences for ACSL4, ATF4, CHOP, and β -actin are listed in Table 2. Total RNA for H9c2 cells and myocardial tissue was extracted by Trizol. The RNA was then reverse transcribed into cDNA according to the Prime-Script RT reagent kit instruction. According to the instruction for the SYBR Premix Ex Taq kit, the polymerase chain reaction initiated at 95°C for 30 s, followed by 40 cycles of amplification of denaturation at 95°C for 5 s, annealing at 60°C for 34 s by using a StepOne Plus device (Applied Biosystems). The $2^{-\Delta\Delta CT}$ method was used to analyze the data.

Western blotting to detect protein expression

The H9c2 cells and myocardial tissue were homogenized with precooled RIPA lysis buffer. After being centrifuged (12,000 rpm/min) for 15 min at 4°C, the supernatant was taken to add loading buffer. Then, the extracts were boiled at 100°C for 5 min. Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) was used to separate the protein extracts. Protein was then transferred to a polyvinylidene fluoride (PVDF) membrane. The membranes were incubated with 5% skim milk for 1 h. The primary antibodies (ACSL4, 1:1000; GPX4, 1:1000; ATF4, 1:1000; CHOP, 1:1000; BCL-2, 1:1000; BAX, 1:1000; β -actin, 1:1000) were added to incubate with the membranes at 4°C overnight. Fluorescent secondary antibody was incubated for 1 h at room temperature. The blots were at last analyzed with Odyssey software (Li-Cor, Lincoln, NE).

Flow cytometry to detect apoptosis rate of each group

Each group of treated H9c2 cells was digested with trypsin. Then, the cells were centrifuged at 1000 rpm/min for 10 min, and the supernatant was discarded. The cells were washed twice with PBS and centrifuged to remove supernatant. Four hundred microliters buffer solution was added to make cell suspension. The cell suspension was then added with 5 μ L Annexin V and 5 μ L propidium iodide. After being incubated at room temperature for 20 min in the dark, the cells were detected by the flow cytometry (BD, San Jose, CA) for the apoptosis rate.

Hematoxylin-eosin staining and immunohistochemistry to detect myocardial tissue

The fresh myocardial tissues were fixed with 4% paraformaldehyde. After being embedded with paraffin, the paraffin blocks were cut into 3 mm slices. Then, they were detected with hematoxylin-eosin (HE) staining to assess the morphological changes and damage degree. The protein expression level of ACSL4 in the myocardial tissue slices was detected by immunohistochemistry.

Immunofluorescence staining to detect protein expression

The cells were fixed with 4% paraformaldehyde. After being blocked with normal goat serum, the rabbit antirat ACSL4 (1:200) and BAX (1:100) antibodies were incubated with the cells overnight at 4°C. Cy3-labeled goat antirabbit second antibody (1:50) was incubated for 1 h at 37°C in the dark. 4',6-Diamidino-2-phenylindole (DAPI) was added to incubate for 5 min. The magnification light microscope (Olympus, Tokyo, Japan) was used to take images at 200 $\times$ fields. The fluorescence intensity was analyzed with ImageJ software.

Statistical analysis

Statistical analysis was performed using SPSS 17.0 software. The measurement data were expressed as mean $\pm$ standard deviation. The comparison between two groups was analyzed with Student's *t*-test. The comparison among three or more groups was performed by one-way ANOVA (with *post hoc* analysis by Bonferroni correction). $p < 0.05$ indicated the statistical difference.

Results

Inhibition of ferroptosis during myocardial I/R in diabetic rats can reduce ERS and myocardial damage

The *in vivo* experiments first proved the effect of ferroptosis during myocardial I/R in diabetic rats. The pathological changes of diabetic rats' myocardial tissue with IRI were observed by HE staining. As shown in Figure 1A, the myocardial tissue structure in NS group was dense and tidy, the myocardial fibers were intact, and no myocardial fibers were broken. The myocardial tissue in DS and I/R groups showed mild disorder of cell arrangement and a few myocardial fibers were broken. In the DIR group, the myocardial tissue showed severe cell alignment disorder, the cells were swollen, and most of the myocardial fibers were broken. The degree of myocardial tissue lesions in DIR+Fer-1 group was significantly reduced compared with the DIR group.

As shown in Figure 1B and C, the expression of ACSL4 in rat myocardial tissue was detected by immunohistochemistry. Compared with NS group, the level of ACSL4 was increased in DS, I/R, and DIR group ($p < 0.05$). Compared with DS and I/R group, the ACSL4 protein level was increased in DIR group ($p < 0.05$). After being administered with Fer-1, the level of ACSL4 in the DIR+Fer-1 group was lower than that in the DIR group ($p < 0.05$).

As shown in Figure 1D for TTC staining, compared with NS group, the level of myocardial infarction area was increased in DS, I/R, and DIR group ($p < 0.05$). Compared with DS and I/R group, the myocardial infarction area was

FIG. 1. Inhibition of ferroptosis during myocardial ischemia-reperfusion in diabetic rats can reduce ERS and myocardial injury. (A) Histopathological changes of myocardium were detected by HE staining. (B) The expression of ACSL4 in myocardial tissue was detected by IHC. (C) The expression of ACSL4 in myocardial tissue detected by IHC was analyzed by histogram. (D) The myocardial infarction area was detected by TTC. (E) The CK-MB in serum was detected. (F) The mRNA level of ACSL4, ATF4, and CHOP in myocardial tissue was detected by qRT-PCR. (G) The protein expression of ACSL4, GPX4, ATF4, and CHOP in heart tissues was detected by western blot. [#] $p < 0.05$, compared with sham group. ^{*} $p < 0.05$, compared with DM group and I/R group. [▲] $p < 0.05$, compared with DM-I/R group. CK-MB, creatine kinase-MB; DM, diabetes mellitus group; ERS, endoplasmic reticulum stress; Fer-1, Ferrostatin-1; HE, hematoxylin-eosin; IHC, immunohistochemistry; I/R, ischemia-reperfusion group; qRT-PCR, quantitative real-time PCR; TTC, triphenyltetrazolium chloride.

increased in DIR group ($p < 0.05$). After being administered with Fer-1, the level of myocardial infarction area in DIR+Fer-1 group was lower than that in DIR group ($p < 0.05$).

As shown in Figure 1E–G, compared with NS group, the level of CK-MB, ACSL4, ATF4, and CHOP was increased

in DS group ($p < 0.05$), I/R group ($p < 0.05$), and DIR group ($p < 0.05$). The level of CK-MB, ACSL4, ATF4, and CHOP in DIR group was higher than that in DS group and I/R group ($p < 0.05$). Moreover, the expression of GPX4 protein in DS group ($p < 0.05$), I/R group ($p < 0.05$), and DIR group

($p < 0.05$) was decreased when compared with NS group. The level of GPX4 in DIR group was lower than that in DS group and I/R group ($p < 0.05$). After being administered with Fer-1, the level of CK-MB, ACSL4, ATF4, and CHOP in DIR+Fer-1 group was lower than that in DIR group ($p < 0.05$). But GPX4 protein level was increased ($p < 0.05$).

Left ventricular function was assessed by measuring hemodynamic parameters (Table 3). Compared with NS group, HR, LVSP, +dp/dt, and -dp/dt were elevated in DS group ($p < 0.05$). At the time of 10 min before ischemia, the hemodynamic parameters in DIR group were lower than those in the corresponding I/R group ($p < 0.05$). At the time of 2 h after reperfusion, the hemodynamic parameters in the I/R, DIR, and DIR+Fer-1 groups were significantly lower than those in the corresponding baseline ($p < 0.05$), the hemodynamic parameters in the I/R and DIR groups were significantly decreased than that in NS group ($p < 0.05$), and the hemodynamic parameters in DIR group were decreased than that in I/R group ($p < 0.05$).

However, compared with DIR group, Fer-1 treatment elevated the levels of HR, LVSP, +dp/dt, and -dp/dt in DIR+Fer-1 group ($p < 0.05$), suggesting that Fer-1 could improve cardiac function during myocardial I/R in diabetic rats.

Ferroptosis inhibitor Fer-1 can alleviate myocardial H9c2 cell damage during HG

It was first demonstrated that promoting ferroptosis by Era could aggravate cell injury in H9c2 cells. As shown in Figure 2A–H, compared with normal group, the cell viability and SOD levels of H9c2 cells in Era group were decreased ($p < 0.05$). LDH, MDA, ROS, and intracellular Fe^{2+} concentration values were increased ($p < 0.05$). The expression of ACSL4 was increased ($p < 0.05$), and the expressions of GPX4 were decreased ($p < 0.05$).

It was subsequently demonstrated that inhibition of ferroptosis could reduce cell injury in the process of H9c2 cell damage induced by HG. As shown in Figure 3A–N, compared with normal group, cell viability, SOD, GPX4,

and BCL-2 levels in HG group were decreased ($p < 0.05$). LDH, MDA, ROS, intracellular Fe^{2+} concentration, ACSL4, apoptosis rate, and BAX levels were elevated ($p < 0.05$). Compared with HG group, cell viability, SOD, GPX4, and BCL-2 in HG+Fer-1 group were increased ($p < 0.05$). LDH, MDA, ROS, intracellular Fe^{2+} concentration, ACSL4, apoptosis rate, and BAX levels were decreased ($p < 0.05$).

Ferroptosis inhibitor Fer-1 can alleviate myocardial H9c2 cell damage during hypoxia reoxygenation

It was then demonstrated that inhibition of ferroptosis could reduce cell injury in the process of H9c2 cell damage induced by H/R. As shown in Figure 4A–N, compared with normal group, cell viability, SOD, GPX4, and BCL-2 levels in H/R group were decreased ($p < 0.05$), LDH, MDA, ROS, intracellular Fe^{2+} concentration, ACSL4, apoptosis rate, and BAX levels were elevated ($p < 0.05$). Compared with H/R group, cell viability, SOD and GPX4 and BCL-2 levels in H/R+Fer-1 group were increased ($p < 0.05$). LDH, MDA, ROS, intracellular Fe^{2+} concentration, ACSL4, apoptosis rate, and BAX levels were decreased ($p < 0.05$).

Ferroptosis inhibitor Fer-1 can alleviate myocardial H9c2 cell damage during HH/R

Based on the above results, it was subsequently demonstrated that inhibition of ferroptosis could reduce cell injury induced by HH/R. As shown in Figure 5A–K, compared with normal group, the levels of cell viability, SOD, and GPX4 in HG group, H/R group, and HH/R group were decreased ($p < 0.05$). LDH, MDA, ROS, intracellular Fe^{2+} concentration, and ACSL4 levels were increased ($p < 0.05$).

However, compared with HG group and H/R group, cell viability, SOD, and GPX4 levels in HH/R group were decreased ($p < 0.05$). LDH, MDA, ROS, intracellular Fe^{2+} concentration, and ACSL4 levels were increased ($p < 0.05$).

TABLE 3. HEMODYNAMIC PARAMETERS AT BASELINE AND AFTER 2 H OF REPERFUSION

	HR (bpm)	LVSP (mmHg)	+dp/dt (mmHg/s)	dp/dt (mmHg/s)
NS	375 ± 25	130 ± 11	6053 ± 487	5018 ± 500
DS	327 ± 29 ^a	104 ± 9 ^a	4443 ± 301 ^a	3660 ± 510 ^a
10 Min before ischemia (baseline)				
I/R	368 ± 21	126 ± 14	6079 ± 570	5198 ± 490
DIR	333 ± 19 ^b	107 ± 10 ^b	4350 ± 328 ^b	3675 ± 469 ^b
DIR+Fer-1	340 ± 26	115 ± 11	4396 ± 287	3761 ± 401
2 H after reperfusion				
I/R	265 ± 25 ^{a,c}	90 ± 12 ^{a,c}	4540 ± 342 ^{a,c}	3450 ± 335 ^{a,c}
DIR	174 ± 18 ^{a-c}	62 ± 10 ^{a-c}	2707 ± 452 ^{a-c}	2051 ± 272 ^{a-c}
DIR+Fer-1	210 ± 17 ^{c,d}	82 ± 10 ^{c,d}	4026 ± 338 ^{c,d}	2837 ± 347 ^{c,d}

Results are expressed as mean ± standard deviation, $n = 8$. Normal+sham (NS), diabetes mellitus+sham (DS), ischemia reperfusion (I/R), diabetes mellitus+I/R (DIR), diabetes mellitus+I/R+Fer-1 (DIR+Fer-1).

^a $p < 0.05$ versus NS group.

^b $p < 0.05$ versus their corresponding I/R group.

^c $p < 0.05$ versus their corresponding baseline.

^d $p < 0.05$ versus their corresponding DIR group.

HR, heart rate; LVSP, left ventricular systolic pressure.

FIG. 2. Promoting ferroptosis by Era could aggravate cell injury. (A) Cell viability was detected by CCK-8. (B–F) LDH, MDA, SOD, cellular ROS production, and Fe²⁺ concentration levels were detected. (G–H) The protein expression of ACSL4 and GPX4 in H9c2 cell was detected by western blot. [#]*p* < 0.05, compared with normal group. CCK-8, cell counting kit-8; Era, Erastin; LDH, lactate dehydrogenase; MDA, malondialdehyde; ROS, reactive oxygen species; SOD, superoxide dismutase.

After being administered with Fer-1, cell viability, SOD and GPX4 levels in HH/R+Fer-1 group were increased compared with HH/R group (*p* < 0.05). LDH, MDA, ROS, intracellular Fe²⁺ concentration, and ACSL4 levels were decreased (*p* < 0.05).

Ferroptosis inhibitor can reduce ERS and injury in myocardial H9c2 cells during HH/R

To further validate the role of ERS in ferroptosis-induced cardiomyocyte injury. ERS inhibitor Sa and agonist Tu are used in cell experiments. As shown in Figure 6A–M, compared with HH/R group, the levels of cell viability, SOD, and GPX4 in HH/R+Fer-1 group were increased (*p* < 0.05). The levels of LDH, MDA, ROS, intracellular Fe²⁺ concentration, ACSL4, ATF4, and CHOP were decreased (*p* < 0.05). Compared with HH/R+Fer-1 group, the levels of cell viability, SOD, and GPX4 in HH/R+Fer-1+Tu group were decreased (*p* < 0.05). The levels of LDH, MDA, ROS, intracellular Fe²⁺

concentration, ACSL4, ATF4, and CHOP were increased (*p* < 0.05). Compared with HH/R+Fer-1+Tu group, the levels of cell viability, SOD, and GPX4 in HH/R+Tu group were decreased (*p* < 0.05). The levels of LDH, MDA, ROS, intracellular Fe²⁺ concentration, ACSL4, ATF4, and CHOP were increased (*p* < 0.05).

Ferroptosis agonist can aggravate ERS and injury in myocardial H9c2 cells during HH/R

As shown in Figure 7A–M, compared with HH/R group, the levels of cell viability, SOD, and GPX4 in HH/R+Era group were decreased (*p* < 0.05). The levels of LDH, MDA, ROS, intracellular Fe²⁺ concentration, ACSL4, ATF4, and CHOP were increased (*p* < 0.05). Compared with HH/R+Era group, the levels of cell viability, SOD, and GPX4 in HH/R+Era+Sa group were increased (*p* < 0.05). The levels of LDH, MDA, ROS, intracellular Fe²⁺ concentration, ACSL4, ATF4, and CHOP were decreased (*p* < 0.05). Compared with

FIG. 3. Inhibition of ferroptosis could reduce cell injury in the process of H9c2 cell damage induced by HG. (A) Cell viability was detected by CCK-8. (B–F) LDH, MDA, SOD, cellular ROS production, and Fe²⁺ concentration levels were detected. (G–I) The protein expression of ACSL4, GPX4, BAX, and BCL-2 in H9c2 cell was detected by western blot. (J) The mRNA level of ACSL4 in H9c2 cell was detected by qRT-PCR. (K, L) The expression of BAX was detected by immunofluorescence. (M, N) The apoptosis rate of H9c2 cells was determined by flow cytometry. [#]*p* < 0.05, compared with normal group. ^{*}*p* < 0.05, compared with HG group. HG, high glucose.

FIG. 4. Ferroptosis inhibitor Fer-1 can alleviate myocardial H9c2 cell damage during H/R. (A) Cell viability was detected by CCK-8. (B–F) LDH, MDA, SOD, cellular ROS production, and Fe²⁺ concentration levels were detected. (G–I) The protein expression of ACSL4, GPX4, BAX, and BCL-2 in H9c2 cell was detected by western blot. (J) The mRNA of ACSL4 in H9c2 cell was detected by qRT-PCR. (K, L) The expression of BAX was detected by immunofluorescence. (M, N) The apoptosis rate of H9c2 cells was determined by flow cytometry. [#]*p* < 0.05, compared with normal group. ^{*}*p* < 0.05, compared with H/R group. H/R, hypoxia reoxygenation.

FIG. 5. Ferroptosis inhibitor Fer-1 can alleviate myocardial H9c2 cell damage during HH/R. (A) Cell viability was detected by CCK-8. (B–F) LDH, MDA, SOD, cellular ROS production, and Fe²⁺ concentration levels were detected. (G, H) The protein expression of ACSL4 and GPX4 in H9c2 cell was detected by western blot. (I, J) The expression of ACSL4 was detected by immunofluorescence. (K) The mRNA level of ACSL4 in H9c2 cell was detected by qRT-PCR. [#]*p* < 0.05, compared with normal group. **p* < 0.05, compared with HG group and H/R group. [▲]*p* < 0.05, compared with HH/R group. HH/R, high-glucose hypoxia reoxygenation.

FIG. 6. Ferroptosis inhibitor can reduce the degree of ERS and myocardial injury in H9c2 cell during HH/R. (A) Cell viability was detected by CCK-8. (B–F) LDH, MDA, SOD, cellular ROS production, and Fe²⁺ concentration levels were detected. (G–J) The protein expression of ACSL4, GPX4, ATF4, and CHOP in H9c2 cell was detected by western blot. (K) The mRNA level of ACSL4, ATF4, and CHOP in H9c2 cells was detected by RT-PCR. (L, M) The expression of ACSL4 was detected by immunofluorescence. [#]*p* < 0.05, compared with HH/R group. ^{*}*p* < 0.05, compared with HH/R+Fer-1 group. [▲]*p* < 0.05, compared with HH/R+Fer-1+Tu. Tu, Tunicamycin group.

FIG. 7. Ferroptosis agonist can aggravate the degree of ERS and myocardial injury in H9c2 cell during HH/R. (A) Cell viability was detected by CCK-8. (B–F) LDH, MDA, SOD, cellular ROS production, and Fe²⁺ concentration levels were detected. (G–J) The protein expression of ACSL4, GPX4, ATF4, and CHOP in H9c2 cells was detected by western blot. (K) The mRNA of ACSL4, ATF4, and CHOP in H9c2 cell was detected by RT-PCR. (L, M) The expression of ACSL4 was detected by immunofluorescence. [#]*p* < 0.05, compared with HH/R group. ^{*}*p* < 0.05, compared with HH/R+Era group. [▲]*p* < 0.05, compared with HH/R+Era+Sa. Sa, Salubrinal.

HH/R+Era+Sa group, the levels of cell viability, SOD, and GPX4 in HH/R+Sa group were increased ($p < 0.05$). The levels of LDH, MDA, ROS, intracellular Fe^{2+} concentration, ATF4, CHOP, and ACSL4 were decreased ($p < 0.05$).

Discussion

Epidemiological investigations show that ischemic heart disease is the main cardiovascular complication and cause of death for diabetic patients (Donahoe *et al.*, 2007). Compared with nondiabetic patients, diabetic patients with severe acute myocardial occlusion have more severe disease, more rapid progression, higher mortality, higher perioperative complications, and poorer prognosis (Ngaage *et al.*, 2009; Cavallero *et al.*, 2010; Keller *et al.*, 2010). A large number of studies have shown that diabetic myocardial IRI is closely related to ERS and ROS (Runkel *et al.*, 2013). More importantly, ERS and ROS production are mutually causal.

In diabetic state, the increased ROS is one of the pathological features of diabetic cardiomyopathy (Newsholme *et al.*, 2016). As the pathophysiological characteristics of diabetic patients, increased ROS production and oxidative stress could significantly reduce myocardial tolerance to IRI (Liu *et al.*, 2016b; Zhao *et al.*, 2017). Hyperglycemia caused by diabetes can produce ROS through advanced glycation end products, polyol pathway, and *de novo* synthesis of triose metabolism (Shen, 2010). ROS can directly lead to oxidative damage of tissues and organs (Polimeno *et al.*, 2013). The damaged cell activates the UPR. Then, the UPR causes ERS due to the changes of redox status, calcium levels, and the decreased function of chaperone protein (Tabas and Ron, 2011).

ERS destroys Ca^{2+} homeostasis in endoplasmic reticulum, further causing mitochondrial calcium overload and increasing ROS production (De Stefani *et al.*, 2012). The accumulation of ROS activates the downstream caspase family proteins through cascade amplification, which initiates the cell injury process (Wang *et al.*, 2013). Moreover, the key ERS molecules (ATF4 and CHOP) are upregulated, and CHOP can further induce the expression of proapoptotic proteins during ERS (Zheng *et al.*, 2017; Lee *et al.*, 2018). Therefore, control of the damage of myocardial myocytes caused by ERS and ROS is crucial for the treatment of myocardial IRI.

As a new type of cell death discovered in recent years, ferroptosis is mostly characterized by overloaded iron-dependent lipid peroxidation and ROS (Dixon *et al.*, 2012). Ferroptosis is regulated by iron metabolism (Manz *et al.*, 2016), system Xc-/GPX4 (Yang *et al.*, 2014), and lipid metabolism (Kagan *et al.*, 2017) pathways. It will further increase the ferroptosis of the marker protein ACSL4. Research studies have shown that antioxidant vitamin E can inhibit ferroptosis (Yang *et al.*, 2016; Kagan *et al.*, 2017). Ferroptosis has been proved to be involved in the development of various diseases, especially cardiovascular diseases (Bai *et al.*, 2018; Kobayashi *et al.*, 2018).

In recent years, it has been found that inhibition of ferroptosis can significantly reduce liver, kidney, brain, and heart defects in mouse models (Gao *et al.*, 2015; Ponikowski *et al.*, 2016). The ferroptosis agonist can significantly reduce the expression of GPX4 in cardiomyocytes, causing dysregulation of iron metabolism and lipid peroxidation in cardiomyocytes,

leading to heart failure in rats (Ponikowski *et al.*, 2016). Therefore, the regulation of ferroptosis can affect the level of oxidative stress in cardiomyocytes. Therefore, we hypothesized that myocardial IRI regulated by ferroptosis can change the oxidative stress level of cardiomyocytes, causing the myocardial cell damage through the interaction of ROS with ERS.

In this study, to simulate the effects of I/R on myocardial cells in diabetic patients, rat DS, I/R, and DIR models were, respectively, established. For the *in vivo* results, the degree of myocardial tissue injury for DIR group was more serious than DS and I/R group. Therefore, it could be demonstrated that diabetes was a risk factor for myocardial IRI. The experiments subsequently proved that after being administered with ferroptosis inhibitor Fer-1, the degree of ERS and injury in DIR rats were decreased. However, Fer-1 was intraperitoneally injected one time before building I/R model. It only verified the effect of Fer-1 on I/R during DS. But whether the effect of inhibiting ferroptosis on both I/R and diabetes was still unknown. And whether ERS involved in this process was also unknown. Therefore, the mechanism was verified in H9c2 cells in detail.

For the *in vitro* experiments, it was demonstrated that promoting ferroptosis by Era could directly decrease the cell viability, SOD and GPX4 level, increase LDH, MDA, ROS, intracellular Fe^{2+} , and ACSL4 levels. To further validate the effects of ferroptosis on cardiomyocytes during HH/R, H9c2 cells were stimulated with HG and H/R, respectively; then treated with Fer-1. It was found that inhibition of ferroptosis could reduce cell injury in the process of H9c2 cell damage induced by HG or H/R. The mechanism of HG- and H/R-induced injury in H9c2 cells associated with ROS (Qiu *et al.*, 2019). Moreover, hyperglycemia stimulates ROS production and induces oxidative stress (Xu *et al.*, 2014), which is greater in the presence of DM after reperfusion injury, and contributes to the exacerbation of myocardial IRI (Liu *et al.*, 2005).

The decreased SOD can lead to lipid peroxidation and ferroptosis. This involves a complex oxidation feedback regulation mechanism. As an important antioxidant mechanism in the body, SOD can remove ROS in cells and maintain the balance of ROS (Gemma *et al.*, 2007). In the process of ferroptosis, the level of ROS is increased, so the SOD in the cells will be consumed and its level will be reduced. When the production of ROS is prolonged, the endogenous reserves of antioxidants become insufficient, leading to cell damage (Gemma *et al.*, 2007; Peng *et al.*, 2014). Therefore, the decrease of SOD level was accompanied by the occurrence of ferroptosis in this experiment.

Moreover, factors that induce ferroptosis can induce apoptosis. The research studies show that ferroptosis is accompanied by the occurrence of apoptosis in tumor necrosis factor alpha (TNF- α)/D-galactosamine-induced hepatocyte damage (Wang *et al.*, 2019b, 2019c), radiation-induced lung injury (Kim *et al.*, 2017; Li *et al.*, 2019a), and intestinal I/R injury (Du *et al.*, 2019; Li *et al.*, 2019b). The associated factor that links ferroptosis and apoptosis is most likely ERS (Hong *et al.*, 2017). Therefore, the proapoptotic protein BAX, anti-apoptotic protein BCL-2, H9c2 cell apoptosis rate that reflected the degree of apoptosis were detected in this experiment. The BAX protein level and apoptosis rate in HG or H/R group were increased when compared with normal group; whereas the BCL-2 protein level was decreased.

FIG. 8. Ferroptosis participates in the pathological process of diabetes myocardial IRI. The occurrence of ferroptosis is accompanied by the production of ERS. ERS is a cellular response to endoplasmic reticulum dysfunction and can be triggered by ROS. ERS could interact with ROS in the process of ferroptosis, and both of them could cause cardiomyocyte injury. DIR, DM+I/R; IRI, I/R injury.

After the cells were administered with Fer-1, the BAX protein level and apoptosis rate were decreased when compared with HG or H/R group; whereas the BCL-2 protein level was elevated. Based on the above results, it was subsequently demonstrated that inhibition of ferroptosis could reduce cell injury in the process of H9c2 cell damage induced by HH/R. To further validate the role of ERS in ferroptosis-induced cardiomyocyte injury, ERS inhibitor and agonist were used in cell experiments.

Compared with the ERS agonist group alone, the combination of ferroptosis inhibitor and ERS agonist could reduce the occurrence of ERS, oxidative stress damage, and cardiomyocytes injury. Compared with the ERS inhibitor group alone, the combination of ferroptosis agonist and ERS inhibitor could aggravate the occurrence of ERS, oxidative stress damage, and cardiomyocytes injury. The above results suggested that ferroptosis could aggravate diabetes associated with ERS pathway.

Conclusion

In conclusion, as shown in Figure 8, ferroptosis participates in the pathological process of diabetes myocardial IRI, inhibition of ferroptosis could alleviate diabetes myocardial IRI. It was deserved that the essential of ferroptosis was oxidative stress injury. Our current results suggested that ERS could interact with ROS in the process of ferroptosis. And both of them could cause cardiomyocyte injury. Ferroptosis was involved in diabetes myocardial IRI through ERS pathway. The occurrence of ferroptosis is accompanied by the production of ERS and activation of ATF4-CHOP pathway. ERS is a cellular response to endoplasmic reticulum dysfunction and can be triggered by ROS, which is produced by the interaction between iron ions and NADPH oxidase during ferroptosis.

However, the ERS inhibitor or agonist was not used *in vivo*. It was only preliminarily verified that ferroptosis could affect ERS and cardiomyocyte injury. Moreover, in

our next experiment, the ERS process and key pathogenic targets in DIR animal model would be further studied. In all, this experiment provided the effective means to prevent and reduce the death of cardiomyocytes to improving and restoring the cardiac function of ischemic cardiomyopathy.

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