Heart Surgery Forum 2024; 27(3): E252–E263
doi: 10.59958/hsf.7149

Article

Danshen and Zhizi Compatibility Alleviates Heart Injury and Cardiac Ferroptosis in Myocardial Infarction in Rats by Cyclic Adenosine Monophosphate/Protein Kinase A Signaling

Jianqiao Zhao[1], Yifan Li[1], Hao Zhi[1], Wanying Hong[1], Jianping Shen[1,*]

[1]Department of Cardiology, Affiliated Hospital of Integrated Traditional Chinese and Western Medicine, Nanjing University of Chinese Medicine, 210028 Nanjing, Jiangsu, China
*Correspondence: shenjp@jsatcm.com (Jianping Shen)

Submitted: 19 December 2023 Revised: 31 January 2024 Accepted: 19 February 2024 Published: 17 March 2024

Abstract

Objective: To investigate the effect and mechanism of the Danshen and Zhizi Compatibility (DZ) on alleviating heart injury and cardiac ferroptosis in rats with myocardial infarction. **Methods**: A rat model of myocardial infarction was established by ligation of the left anterior descending artery. The rats were equally and randomly divided into 5 groups. The sham group underwent open-chest surgery without arterial ligation, while the other 4 groups underwent surgery, including 3 groups treated with low dose (4 g/kg/d), high dose (8 g/kg/d) DZ and high dose (8 g/kg/d) DZ supplemented with H-89 (0.5 mg/kg/d) respectively. The sham and myocardial infarction group received the same volume of saline. 14 days after surgery, the serum and heart tissues were harvested to detect cyclic adenosine monophosphate (cAMP) and protein kinase A (PKA) activity, heart injury and the level of ferroptosis. **Results**: G-protein coupled receptors (GPCRs) have a high binding affinity with the main components of DZ, which indicated that DZ probably contributed to ameliorating cardiac injury by activating downstream cyclic adenosine monophosphate (cAMP)/protein kinase A (PKA) signaling. Treatment with the high dose of DZ significantly increased cAMP concentration in the serum, PKA activity in the heart tissue and upregulated perilipin (PLIN)5 expression. DZ significantly attenuated heart injury, whereas H-89 reversed the protective effects of DZ. In addition, DZ administration inhibited ferroptosis as evidenced by reduced malondialdehyde (MDA), and 4-hydroxynonenal (4-HNE) levels. In addition, DZ increased glutathione (GSH) levels and Glutathione peroxidase (GPX)4 protein expression in heart tissue, whereas H-89 abrogated the regulatory effect of DZ. **Conclusion**: Our results demonstrated that DZ alleviated heart injury and cardiac ferroptosis in myocardial infarction through the cAMP-PKA signalling pathway.

Keywords

Danshen and Zhizi Compatibility; cAMP-PKA; ferroptosis; myocardial infarction

Introduction

Ischemic heart disease is the leading cause of disability and death worldwide threatening the physical health of human beings [1]. Cardiac ischemia can induce the death of cardiomyocytes. The damaged cardiac tissue is immediately replaced by fibrotic scar tissue thus eventually leading to heart failure [2]. Cyclic adenosine monophosphate (cAMP) was implicated as a secondary messenger that regulates cell growth and survival. In the heart, elevated cAMP signalled to protein kinase A (PKA), is the major downstream effector of cAMP to phosphorylate multiple substrates responsible for cardiac function [3]. In Langendorff-perfused hearts, adrenaline administration limited infarct size by activating the cAMP/PKA signaling pathway and facilitating mitochondrial KCa channels [4]. The adenosine A2a receptor was triggered to activate the cAMP-PKA signaling pathway to limit fibrotic scar after myocardial infarction (MI) [5]. The cAMP-PKA signaling pathway was triggered by numerous G-protein coupled receptors (GPCRs). For instance, overexpression of prostaglandin E isoform 4 improved cardiac function post-MI by increasing cAMP concentration [6]. In addition, β-adrenergic response was blunted to suppress inotropy [7]. Infarct size is reported to be limited by sitagliptin, pioglitazone, and simvastatin (a member of the statin family) by the cAMP/PKA pathway [8,9]. Many chemical compounds targeted the GPCR and thus transmitted the signaling through cAMP-PKA pathway as second messengers to protect the heart from injury.

In the theory of traditional Chinese medicine, Chinese herb pairs means two or more herbs based on clinical syndromes and herbs features can utilize their advantages to facilitate a pesticide effect. Herb-herb interaction can be complicated when the herbs are boiled for a long time [10]. It is crucial to comprehend the compatibility of medicine in curing diseases. The Danshen-Zhizi pair, the classic compatibility pair of Radix Salviae and Gardeniae Fructus in English, is widely used in the treatment of cardiovascular diseases. Numerous studies showed that Danshen worked to promote blood circulation and nourish cardiomyocytes. Studies indicated that Danshen exhib-

ited anti-inflammatory, antioxidant, and cardioprotective effects [11]. Danshen was found to suppress inflammatory injury in response to acute myocardial infarction-induced heart failure [12]. Zhizi also has anti-inflammatory and antioxidant effects on the myocardium. Geniposide, which is an important ingredient in Zhizi, suppressed thermogenesis via regulation of the PKA catalytic subunit [13]. However, the research based upon the integrative pair function in cardiac ischemia and whether it works through the PKA pathway, requires further experimental verification.

Ferroptosis is a unique iron-dependent form of non-apoptotic regulated cell death characterized by peroxidation of phospholipids with poly-unsaturated fatty acyl tail [14,15]. Intracellular excessive iron accumulation led to overproduction of reactive oxygen species with decreasing capacity for scavenging free radicals. Reactive oxygen species was able to alter membrane lipid peroxidation leading to disruption and cell death. In recent years, ferroptosis was recognized as a specific target for treating ischemic heart disease and is believed to be pay an important role in myocardial infarction [16]. Recent studies suggest that the presence of residual myocardial iron in patients with ST-segment elevation MI may contribute to adverse left ventricular remodelling after an MI [17]. Blocking iron overload using ferroptosis inhibitors may reduce lipid peroxidation and attenuate post-MI cardiac injury [18]. Reactive oxygen species (ROS), which play a key role in triggering ferroptosis, tend to increase post-MI injury due to compromized mitochondrial function [19]. Glutathione peroxidase (GPX)4 is a critical antioxidant enzyme that can significantly attenuate MI-induced ferroptosis. HIP-55 has been reported to play a role in alleviating MI-induced ferroptosis by targeting this enzyme [20]. In addition, our previous studies have shown that salvianolic acid B and geneposide conferred resistance against ferroptosis of cardiac heart tissue by activating the GRSF1/GPX4 and NRF2 pathways [21,22]. Since the components from drug pair shows resistance to ferroptosis and the compatibility triggers GPCR, we assumed that Danshen and Zhizi Compatibility (DZ) protected cardiomyocytes from myocardial infarction by inhibition of cardiac ferroptosis and activation of the cAMP-PKA signaling pathway.

Materials and Methods

Materials

We weighed 150 g Zhizi and 300 g Danshen (from Pharmacy of Jiangsu Province Hospital on Integration of Chinese and Western Medicine), added 200 mL water, soaked for 60 minutes, then boiled with high heat followed by using gentle heat to keep slightly boiling for 30 minutes. After using 4 layers of gauze to filter, 150 mL of water was added to the reside and repeated twice for 20 minutes each, followed by combining the filtrate to centrifuge to obtain the supernatant fixed to 500 mL, which is equivalent to 0.9 g/mL of Chinese herbal decoction.

Establishement of the Rat Myocardial Infarction Model

Sprague-Dawley rats scheduled for ligation of the left anterior descending artery were fasted for 12 hours prior to surgery without food or water. An intraperitoneal injection of 0.2% pentobarbital sodium (144-02-5, Shixing Industry, Wuhan, Hubei, China) at a dose of 30 mg/kg body weight was administered for anaesthesia. After anaesthesia, an endotracheal tube connected to a mechanical ventilator was inserted into the rat's trachea to assist breathing. The ventilator parameters are set to provide an appropriate tidal volume and respiratory rate for the size of the rat, typically around 10 mL/kg for the tidal volume and a respiratory rate to meet the metabolic needs of the animal, which is 70 breaths per minute.

Following aseptic skin preparation, the pectoral muscle is incised along the left third and fourth intercostal spaces, with anatomical dissection in successive layers exposing the thoracic cavity. The pericardium is opened below the junction of the pulmonary conus and the left atrial appendage, and the left atrial appendage is retracted with forceps, revealing a pinkish vessel identified as the left anterior descending artery (LAD). Using a 5-0 prolene suture with a needle, the LAD is ligated under the left atrial appendage with minimal myocardial penetration; the depth of penetration is approximately 1 mm, with the needle entry and exit points approximately 2 mm apart. After the chest cavity has been drained of fluids and residual gases expelled by compression, the muscle and skin are sutured in layers. After disinfection of the wound site, the rat is placed on a heated blanket at a constant temperature (37 °C).

After surgery, the rat is given an intramuscular injection of penicillin (040102659, Hongbaoshou Industry, Ruicheng, Shanxi, China) at a dose of 100,000 units, and this antibiotic treatment is continued for three consecutive days to prevent infection.

Animal Experiments

All animal experiments obeyed the National Institutes of Health's Guide for the Care of Laboratory Animals. The use of Laboratory Animals was approved by the Ethics Committee of Jiangsu Province Academia (NO. AEWC-20181205-65). 25 male Sprague–Dawley rats weighing 250 g to 300 g were randomly and equally divided into 5 group: the sham group (sham, n = 5), the model group (MI, n = 5), the low concentration compatibility group (MI + DZ-L, n =5), the high concentration compatibility group (MI + DZ-H, n = 5), the high concentration compatibility plus H-89 (PKA inhibitor, MCE, China) group (MI + DZ-H + H-89, n = 5). After anesthetization by injection of 0.2%

pentobarbital (30 mg/kg), rats in the latter 4 groups were subjected to the LAD ligation, while the rats in the sham group were treated with an open chest without arterial ligation. The low concentration compatibility rats were given decoction (4 g/kg/d) via intragastric administration. The high concentration compatibility rats were given decoction (8 g/kg/d) via intragastric administration. The high concentration compatibility plus H-89 rats were given decoction (8 g/kg/d) via intragastric administration and intraperitoneally injected with H-89 (0.5 mg/kg/d). 14 days after surgery, the rats were sacrificed for sampling. All treatments were administered 7 days prior to surgery until sacrifice except the 2 days after operation.

HE and Masson Staining

After the rats were sacrificed, the heart samples were harvested and fixed in formalin for 48 h. Then the samples were embedded in paraffin and sliced into pieces (7 μm). The slices were transferred to the glass slides and stained with eosin & hematoxylin (HE) and Masson's trichrome. All sections were observed and analyzed under the microscope.

cAMP Measurement

After perfusion by phosphate buffered saline (PBS) to eliminate remaining blood in the tissue, the heart was cut into pieces followed by the addition of PBS in the ratio of 1:9 (m/v). The lysates were centrifuged at 5000 g for 5 minutes after grinding. cAMP concentration in the supernatant was measured according to the instructions of the cAMP Elisa kit (JL10117, JiangLai Industrial, Shanghai, China). Protein concentrations of all samples were tested and the amount of cAMP was reported as ng/mg protein in the heart tissue.

Western Blot

All heart samples here lysated as homogenates for immunoblotting using RIPA (P0013B, Beyotime, Shanghai, China) supplemented with 1% phenylmethanesulfonylfluoride (PMSF). After separation in 12% Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis (SDS-PAGE) gels, the proteins were transferred to polyvinylidene fluoride (PVDF) membranes. The non-fat milk dissolved in TBST was utilized for unspecific binding with antibody. After washing with TBST buffer (G0004, Servicebio, Wuhan, Hubei, China) for 3 times, the membranes were incubated with primary antibody of GPX4 (T56959S, 1:500, Abmart, Shanghai, China), PLIN5 (1:1000, A20418, Abclonal, Wuhan, Hubei, China), phospho-PKA (1:500, HY-P80847, MCE, Shanghai, China) and PKA (1:500, HY-P80428, MCE, Shanghai, China) followed by secondary antibodies (1:5000, AS014, Abclonal, Wuhan, Hubei, China). GAPDH (1:5000, AC002, Abclonal, Wuhan, Hubei, China) was used as the internal reference. The images of blots were developed by enhanced chemi-luminescence detection reagents (FD8020, FDBio, Hangzhou, Zhejiang, China).

Immunohistochemistry and Immunofluorescence Analysis

The sections were cut to a thickness of 7 μm, followed by deparaffinization through xylene (10023418, China National Pharmaceutical Group Chemical Reagents Co., Ltd, Shanghai, China), then various concentrations of alcohol, and finally rinsed in water. To rejuvenate antigenicity, the heart tissue sections were subjected to a high microwave temperature for 15 minutes, followed by rinsing in phosphate buffered saline (PBS). Endogenous peroxidase was inhibited with a 3% hydrogen peroxide (AR1108, Boster, Wuhan, Hubei, China) solution to eliminate it from further possible effects in immunohistochemistry.

The next step involved placing sections in a wet box and titrating with primary antibodies including anti-phospho-PKA, anti-GPX4 and anti-perilipin (PLIN)5. An overnight incubation at 4 °C solidified the antibody-tissue binding. The following day, sections were extracted and rinsed in PBS. Horseradish peroxidase (HRP)-conjugated secondary antibodies (1:100, AS014, Abclonal, Wuhan, Hubei, China) were then applied. After a further rinsing cycle, 3,3′-Diaminobenzidine (DAB) (G1212 Servicebio, Wuhan, Hubei, China) chromogenic solution was added for immunohistochemical staining, which revealed positive protein expression as a brownish-yellow colour under light microscopy.

In parallel, with immunofluorescence staining, sections were marinated in a Abflor® 594-conjugated secondary antibody (1:100, AS039, Abclonal, Wuhan, Hubei, China) for 1 hour, then rinsed with PBS and finally stained with DAPI (BL105A, Biosharp, Hefei, Anhui, China) for 5 minutes. The resulting fluorescence intensity was visually examined using a fluorescence microscope to demonstrate protein localization.

MDA, GSH Measurement

The level of lipid peroxidation of the heart was demonstrated by measuring malondialdehyde (MDA) levels using a commercial MDA assay kit (1:100, RK05818, Abclonal, Wuhan, Hubei, China) following the manufacturer's instructions. The absorbance was at 532 nm to determine the concentration of MDA according to standard curves. Glutathione (GSH) activity was tested by commercial GSH assay kits (A006-2-1, Nanjing Jiangcheng , Nanjing, Jiangsu, China) according to the manufacturer's instructions. The optical density was measured with a microplate reader at a wavelength of 405 nm.

4-HNE Measurement

The blood was extracted from the aorta of rats and then centrifuged at 3000 rpm for 10 minutes. The resulting supernatant was collected and analyzed using an enzyme-linked immunosorbent assay with a 4-hydroxynonenal (4-HNE) rat Elisa kit (H268-1-2 Nanjing Jiancheng Bioengineering Institute, Nanjing, Jiangsu, China) following the manufacture's instruction.

LDH, CK-MB Measurement

The blood of the rats was drawn from the aorta followed by separation at 3000 g for 10 minutes. The upper serum was collected into a new Eppendorf Pipe (EP) tube. The contents of dehydrogenase (LDH) and creatine kinase-MB (CK-MB) were analyzed by an automatic biochemical analyzer (Chemray 800, Rayto, Shenzhen, Guangdong, China).

Ferrous Iron Concentration Measuremets

Myocardial tissue from each rat, weighing 100 mg, was homogenized using a homogenization reagent according to the instructions provided in the commercial kit (E-BC-K773-M, Elabscience, Wuhan, Hubei, China). After thorough homogenization, a color developing solution was added and the mixture was incubated at 37 °C in an incubator for 30 minutes. The optical density (OD) values of each well were then measured at 590 nm using an ELISA reader (1410101, Multiscan FC, Thermofisher, Waltham, Massachusetts, USA). The concentration of ferrous ions was determined using a standard curve.

Molecule Docking

Molecule docking was conducted using the program Autodock Vina. The chemical components of the decoction were analyzed by mass spectrum. Salvianolic acid B, geniposide, Tanshinone II and quercetin were chosen as major effective ingredients as they were widely studied and retrieved from the TCMSP websites. The complex crystal structure of ADORA1 (PDB code: 5n2s), ADORA2A (PDB code: 2ydo), ADORA2B (PDB code: 8hdo), ADRB1 (PDB code: 5f8u), ADRB2 (PDB code: 3ny8), ADRB3 (PDB code: 7xji), EP3 (PDB code: 6ak3) and EP4 (PDB code: 5ywy) were used as receptors. After removing H_2O, S, Cl and the ligand from the default structure in Pymol, both receptors and ligands were prepared by AutoDock Tools (v1.5.7 The Scripps Research Institute, La Jolla, California, USA). After setting the grid box wrapping the whole structures, the vina program was launched to presume the binding sites and binding energy [23].

Statistical Analysis

The statistical analysis was performed with GraphPad Prism 8.0 software (GraphPad Software, Inc., San Diego, CA, USA) and Microsoft Excel. All of the quantitative results were expressed as mean ± SD. The difference between two groups was demonstrated by student-*t* test, and data among multiple groups was compared using one-way analysis of variance (ANOVA). For further comparison between each group, post-hoc Tukey's multiple comparisons test was performed. $p < 0.05$ was considered statistically significant.

Results

Molecule Docking in DZ Major Chemical Components with GPCRs

Previous network pharmacology results showed that the effective major components of Danshen targeted genes enriched the cAMP signaling pathway [24] and Zhizi shared abundant targets with cardiovascular diseases [25]. There were 360 chemical components differentiated from the DZ decoction by HPLC/MS. Among them, the mass spectrum of salvianolic acid B, geneposide, tanshinone IIA and quercetin are shown in Fig. 1a. Before ensuring that the compatibility protected the heart from ischemia through the cAMP-PKA pathway, 4 main ingredients and 8 GPCRs were selected to perform the molecular docking to observe whether there was a proper docking between the main ingredients of DZ and the GPCR which can trigger the PKA downstream signals. A binding free energy lower than –1.2 kCal/mol was considered to be a stable molecule binding to the protein. The results showed that the components of DZ might have a tight junction (Fig. 1b) with GPCRs and indicated DZ might trigger cAMP-PKA signalling to confer cardioprotection.

DZ Activated cAMP-PKA Pathway

In order to ascertain whether DZ can prompt the activation of cAMP-PKA signaling, we evaluated cAMP concentrations within the rat serum. We found no evidence of impaired cAMP levels in the serum of MI rats. However, the increase of cAMP concentration following DZ administration was significant (Fig. 2a).

The presence and phosphorylated state of PKA (p-PKA) were examined by Western blotting. The findings revealed a marked reduction of p-PKA in MI rats, whereas DZ treatment led to an increase in the protein level of p-PKA (Fig. 2b–d). A similar tendency in p-PKA expression was also observed in the immunohistochemistry assays (Fig. 2e).

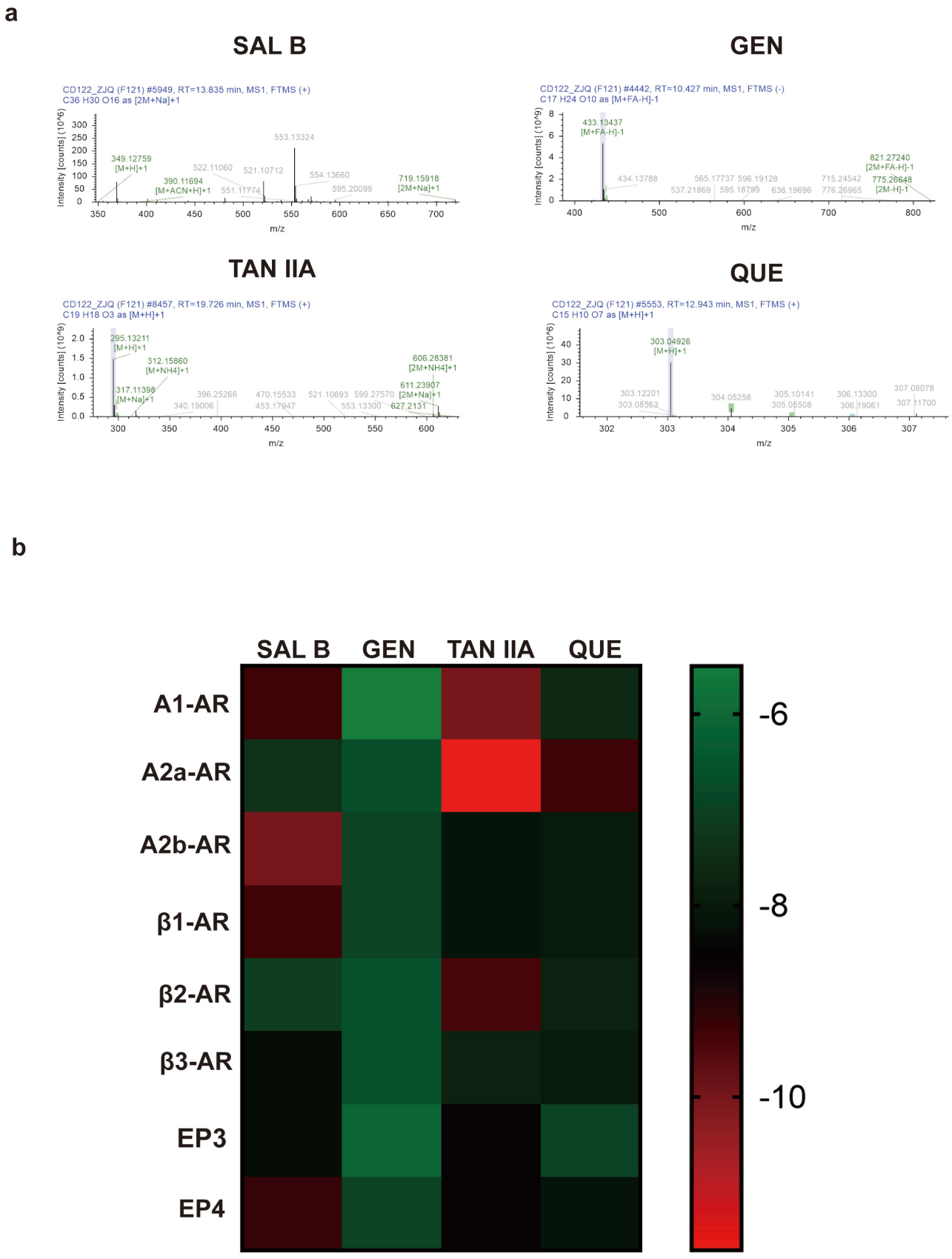

Fig. 1. Molecule docking between DZ components and GPCRs. (a) The primary conponents from DZ aligned by HPLC. (b) The lowest binding free energy predicted by docking between GPCRs and chemical components from DZ. (SAL B, Salvianolic acid B; GEN, Geniposide; TAN IIA, Tanshinone IIA; QUE, quercetin; DZ, Danshen and Zhizi Compatibility; GPCRs, Gprotein coupled receptors.)

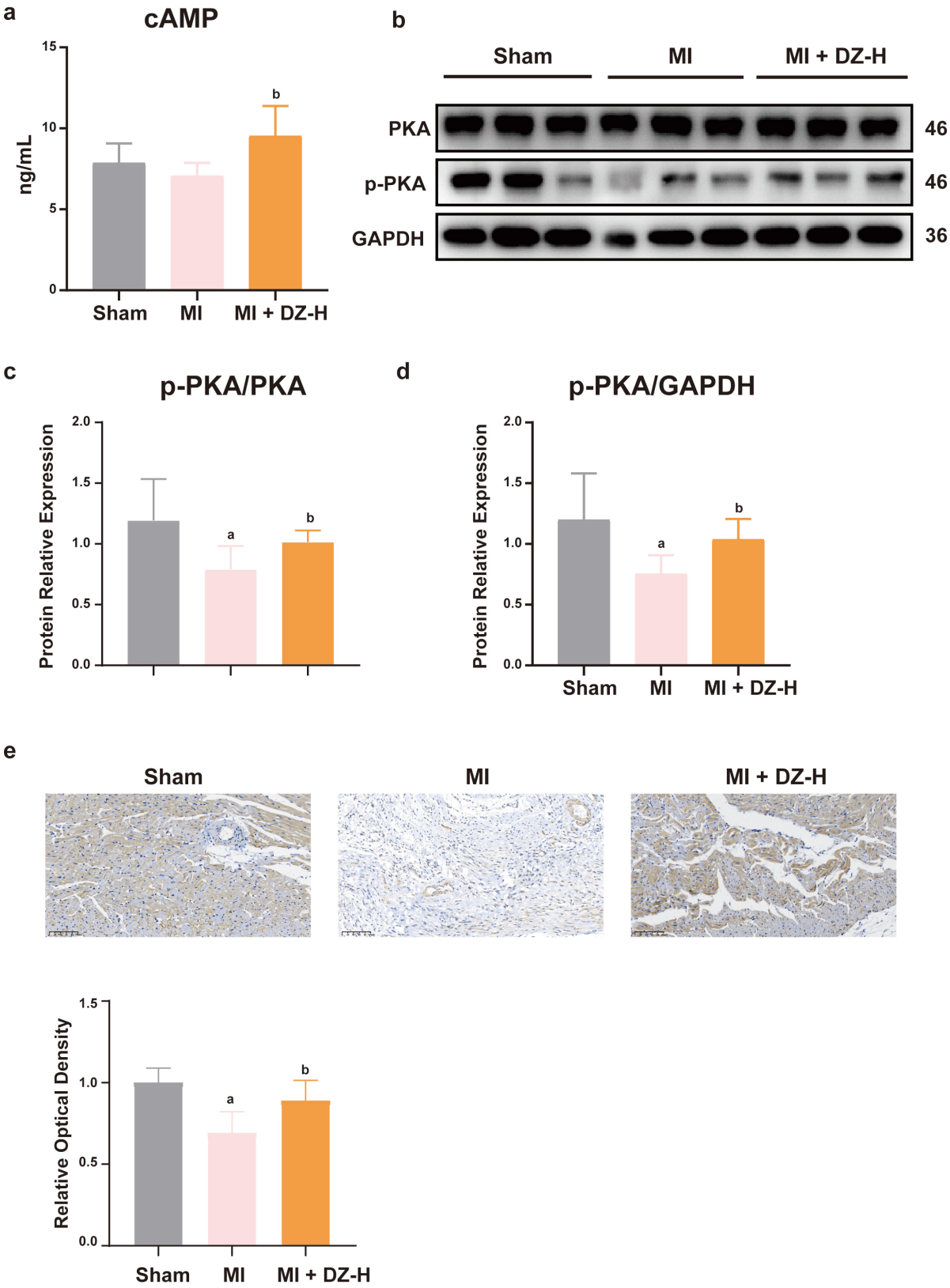

Fig. 2. DZ activated cAMP-PKA signaling of rats after myocardial infarction. (a) Serum concentration levels of cAMP in rats. (b–d) The relative expression of phospho-PKA examined by immunoblotting. (e) Phospho-PKA expression in rat heart section observed by immunohistochemistry. (n = 5; $^{a}p < 0.05$ *vs.* Sham; $^{b}p < 0.05$ *vs.* MI). cAMP, cyclic adenosine monophosphate; PKA, protein kinase A.

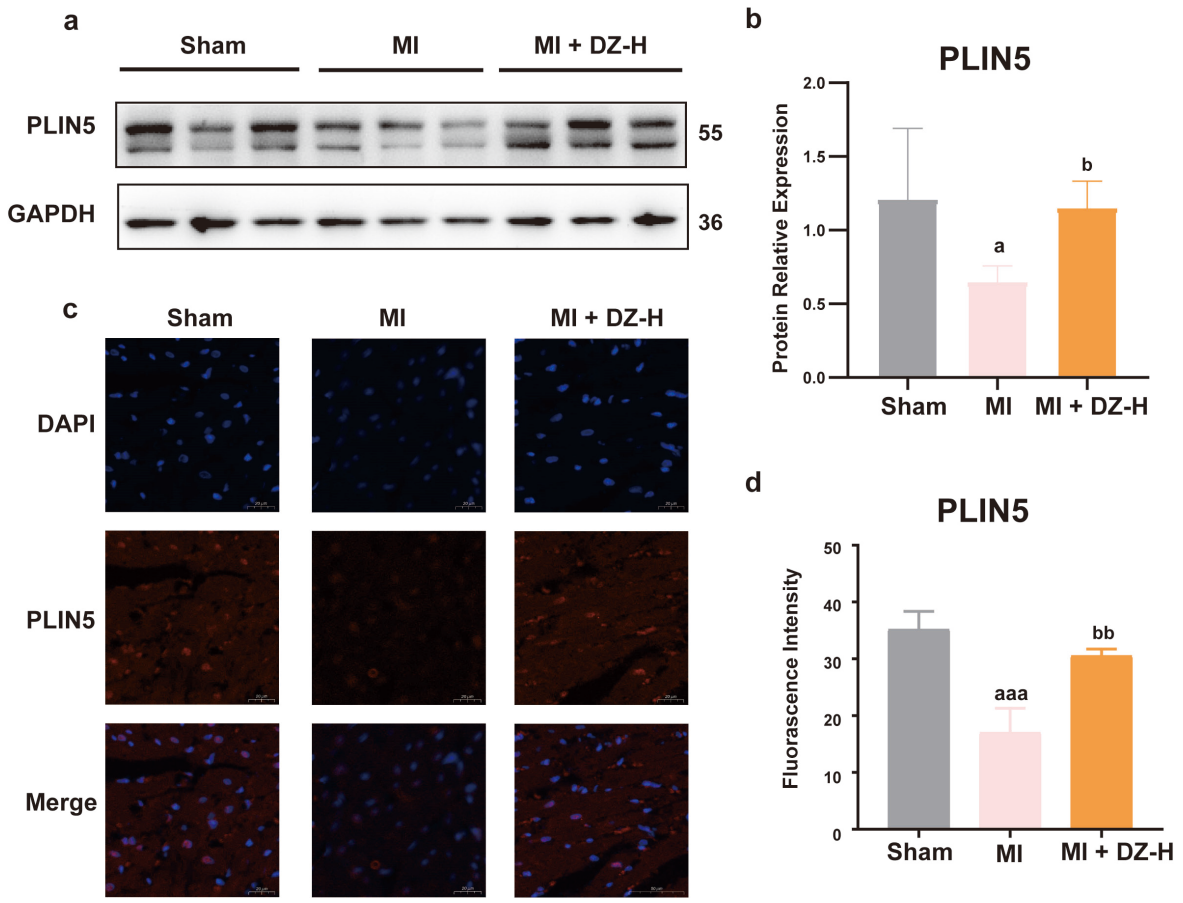

Fig. 3. DZ upregulates PLIN5 expression. (a,b) PLIN5 expression in heart tissue of rats examined by immunoblotting (n = 5) and (c,d) by immunofluorescence. (n = 3) ($^{a}p < 0.05$, $^{aaa}p < 0.001$ *vs.* Sham; $^{b}p < 0.05$, $^{bb}p < 0.01$ *vs.* MI). PLIN, perilipin.

DZ Upregulated PLIN5 Protein Expression

Recent studies suggest that perilipin (PLIN)5, a lipid droplet-associated protein, is intimately involved in the development of ferroptosis [26] and the attenuation of myocardial injury after myocardial infarction [27]. PLIN5 also serves as a switch for PKA activation [28]. We therefore investigated whether PLIN5 expression is regulated by DZ. We found that myocardial infarction induced a decrease in PLIN5 expression, an effect that was reversed by DZ administration (Fig. 3a,b). This expression pattern was consistently depicted by immunofluorescence staining (Fig. 3c,d). These results suggest that DZ increases the abundance of PLIN5, potentially enabling it to prevent ferroptosis in the context of myocardial infarction.

DZ Alleviated Cardiac Injury after Myocardial Infarction through Activating PKA

To investigate the therapeutic role of DZ in rats with an MI and whether it occurred through the cAMP-PKA pathway, hearts were harvested to observe morphological changes. As expected, MI rats showed an obvious infarct area on the anterior wall of the heart compared to the sham group. In the DZ treatment group, the infarct size was reduced in both the high and low dose groups. However, the supply of H-89 diminished the effect of high dose of DZ from limiting the infarct size (Fig. 4a). Cardiomyocytes from the sham rat group showed normal cellular structure when tissue sections were stained with HE. However, irregularly arranged myocardial cells, hyperplastic fibrous connective tissue and fibrous connective tissue and infiltration of immune cells were detected in the heart tissues of MI rats. Additionally, DZ treatment improved cell arrangement and a weaker inflammatory response in cardiomyocytes after HE staining. There was an augmentation of myocardial fibrosis on Masson staining in MI rats. As a result of DZ treatment, myocardial fibrosis and cardiac remodeling were markedly diminished. They were all inhibited by the addition of H-89 (Fig. 4b). CK-MB and LDH serum levels are indications of cardiac damage. MI rats showed higher levels of these myocardial enzymes which could be reduced by DZ treatment. Similar results also showed H-89 prevented the reduction in levels of DZ (Fig. 4c,d).

DZ Repressed Myocardial Ischemia Ferroptosis Mediated by cAMP-PKA Signaling

Ferroptosis has been reported to play a critical role in MI [16]. Previously, we have shown that cardiac damage

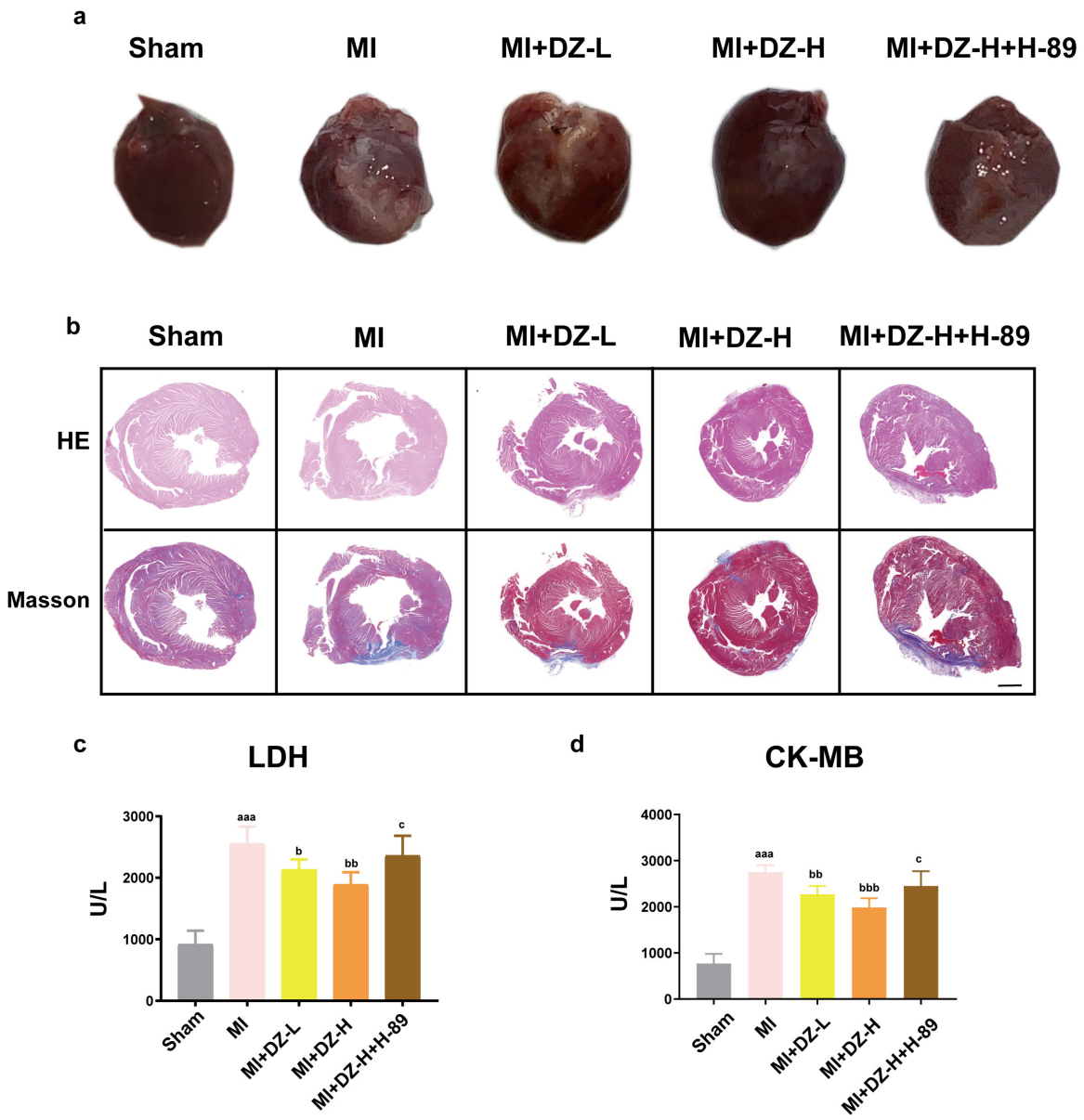

Fig. 4. DZ alleviated cardiac injury post myocardial infarction. (a) The representative heart images 14 days after the operation. (b) HE staining of cardiomyocyte injury as well as immune infiltration and masson staining of myocardial fibrosis in rat heart sections. (c,d) Serum levels of CK-MB, LDH from rats. (n = 5; Scale bar = 2 mm, $^{aaa}p < 0.001$ *vs*. Sham; $^{b}p < 0.05$, $^{bb}p < 0.01$, $^{bbb}p < 0.001$ *vs*. MI, $^{c}p < 0.05$ *vs*. MI + DZ-H). LDH, lactate dehydrogenase; CK-MB, creatine kinase-MB; MI, myocardial infarction.

induced by iron overload and lipid peroxidation can be attenuated and reversed by salvianolic acid B and geniposide, the main active components of DZ therapy [21,22]. We examined MDA, GSH, 4-HNE and the ferroptosis marker to evaluate whether DZ can attenuate myocardial infarction induced ferroptosis. First, GSH, MDA and 4-HNE were measured to assess the capacity of clearance of radicals and lipid peroxidation; both of which are known triggers of ferroptosis [15,29]. Our results indicated that DZ can significantly reduce the MDA and 4-HNE levels and reversed the decrease in GSH levels of MI in rats, effects that were abolished by H-89 (Fig. 5a–c). Iron contents were found to accumulate at higher concentration in MI rats compared to controls, however, high dose of DZ treatment reduced ferrous ion concentration and this effect was abolished with the addition of H-89 (Fig. 5d). GPX4 is a recognized marker for ferroptosis. We observed a decreased protein level of GPX4 in MI rats compared to controls. Notably, the change in GPX4 expression was significantly upregulated by DZ treatment, a reduction that was negated by H-89 (Fig. 5e,f). In addition, GPX4 showed a similar tendency observed by immunohistochemistry assay (Fig. 5g). Overall, these data suggest that DZ may interfere with ischemia-induced myocardial ferroptosis through the cAMP-PKA pathway.

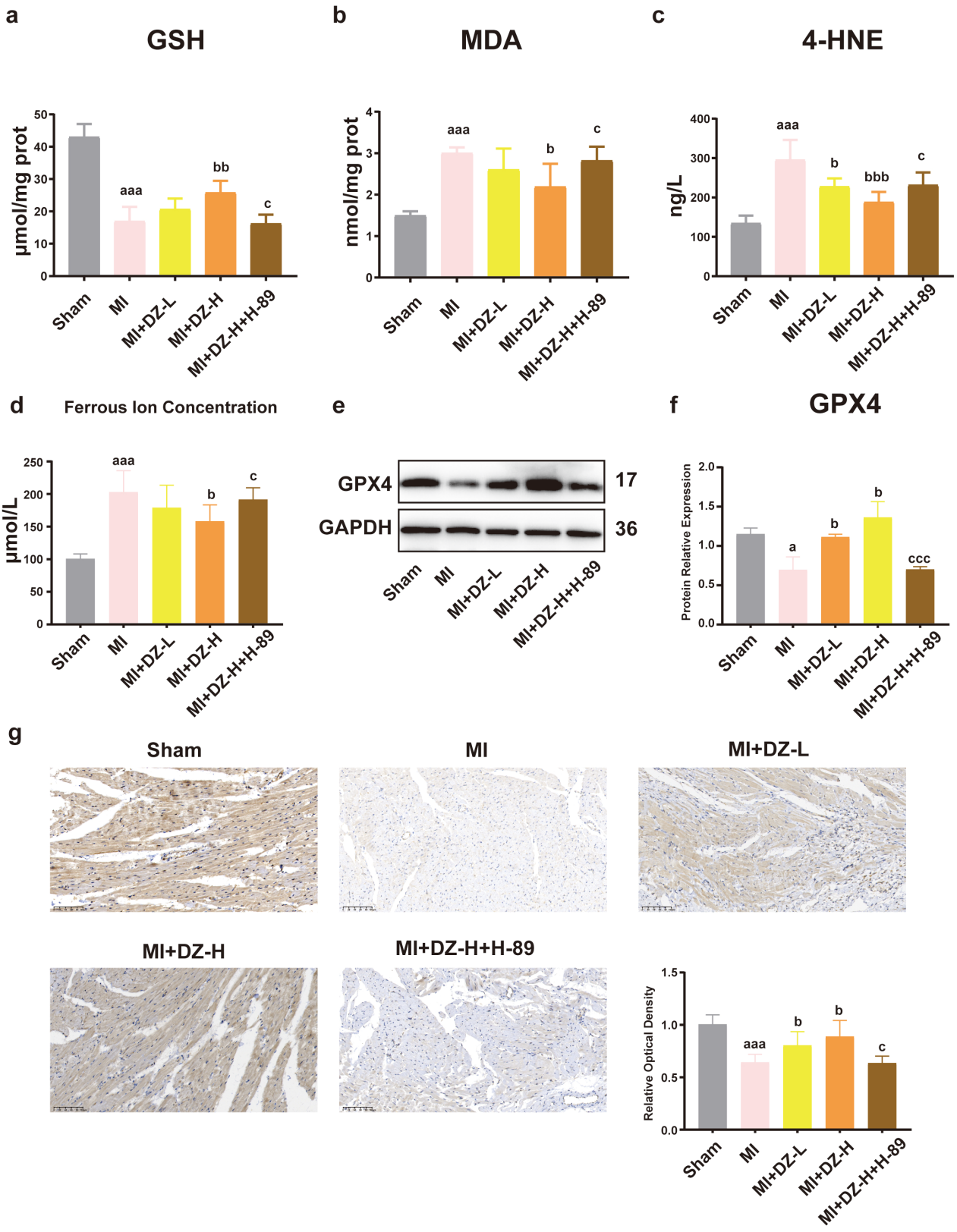

Fig. 5. DZ inhibited myocardial infarction induced ferroptosis. (a,b) GSH and MDA concentration from rat heart tissues. (c) 4-HNE level of the serum from rats. (d) Ferrous ion concentration from heart tissues of rats. (e,f) Protein expression of GPX4 in rat heart samples examined by western blotting. (g) GPX4 expression in rat heart section observed by immunohistochemistry. (n = 5; $^{a}p < 0.05$, $^{aaa}p < 0.001$ *vs*. Sham; $^{b}p < 0.05$, $^{bb}p < 0.01$, $^{bbb}p < 0.001$ *vs*. MI; $^{c}p < 0.05$, $^{ccc}p < 0.001$ *vs*. MI + DZ-H). GSH, Glutathione; MDA, Malondialdehyde; 4-HNE, 4-Hydroxynonenal; GPX, Glutathione Peroxidase.

Disscussion

Worldwide, ischemic heart disease remains the leading cause of death due to the dysfunction and loss of non-proliferative myocardial cells. Mechanistically, an increased production of oxygen radicals in mitochondria following cardiac ischemia reperfusion (IR) injury contributed to increased oxidative cell damage [30]. In our study, intragastric administration of DZ can significantly alleviate cardiac injury after myocardial infarction. DZ is a widely used traditional compatibilizer in clinical medicine in China, and was demonstrated to provide resistance to ferroptosis post-MI in a PKA-dependent manner. Therefore, it is better to use this pair of herbs to support the healing of acute myocardial infarction in clinical practice.

Different from other types of classical programmed cell death including apoptosis, pyroptosis and necrosis, ferroptosis is defined as the iron overload-dependent lipid peroxidation leading to regulatory cell death. Recent studies have shown that ferroptosis is intimately involved in myocardial infarction. By targeting iron deposition and the antioxidant defense system, many molecules and agents have been found to have a therapeutic effect on myocardial infarction [31]. In our previous studies, salvianolic acid B and geniposide were found to confer resistance to ferroptosis in ischemic myocardium. In this study, the results showed that cAMP levels and PKA activities in cardiac tissue were significantly higher after the administration of DZ compared to the model group. The use of PKA inhibitors after DZ treatment reduced the effect of the drug on alleviating myocardial infarction. The damaging effects of ferroptosis were also attenuated by administration of DZ and this effect was diminished by exposure to H-89. In summary, DZ can confer its resistance to ferroptosis and exert cardioprotection in myocardial infarction by activating cAMP-PKA signaling.

Classic signaling cascades involve the activation of cAMP by GPCRs including adrenergic and EP receptors, leading to increases in cAMP and PKA. It's recognized that the cAMP-PKA pathway has a protective effect against ischemic injury in many organs, including the brain, kidney and the heart [32]. After cAMP is activated, various intracellular downstream effectors are activated including PKA which regulate the response to specific stimuli. Adaptation to stress and the regulation of cardiac function are dependent on PKA. When PKA is activated, the downstream effector cAMP response element binding protein can be phosphorylated to suppress inflammatory-related genes to alleviate myocardial infarction [33]. Furthermore, the release of calcium was stimulated by PKA phosphorylation resulting in increased contractile force and a positive inotropic effect [34]. For the treatment of MI, elevated cAMP expression and PKA activities are reported to be beneficial in myocardial infarction and play a critical role in many cellular and physiological processes, although there is controversy as to whether the levels of PKA and cAMP are altered during MI [3]. It is one of the best characterized signaling pathways which can mediate homeostasis to protect cardiac cells from injury.

There are still many unsolved problems on this subject. G proteins vary depending on the types of GPCR. Coupled G-proteins, such as Gα and Gi, determine completely different cellular processes. The pattern of DZ to dynamically motivate GPCR, involving the dissociation of different types of G proteins, remained elusive. Additionally, the mechanism by which cAMP-PKA influences ferroptosis requires further investigation. One possible explanation would be that PKA is capable of phosphorylating downstream proteins which inhibit lipid peroxidation, thereby protecting cardiac cells from ferroptosis.

Our study also had several limitations throughout the research process. These include limitations related to the number of experimental rats, equipment limitations, and the potential increase in mortality associated with repeated blood sampling. These factors resulted in a limited assessment of cardiac injury and function. In future research, it would be prudent to use a more comprehensive range of assessment techniques, such as echocardiography, TTC staining and dynamic monitoring of serum cardiac biomarkers, to thoroughly evaluate the cardioprotective effects of DZ. In addition, there is a lack of toxicological evaluation of these medicines, particularly with regard to potential damage to various organs when administered singly. In the future, we aim to increase the number of experimental subjects and use a variety of experimental methods to support the conclusion that DZ can mitigate the damage caused by post-myocardial infarction.

Conclusion

The findings of this study suggest that DZ has therapeutic potential for the treatment of ischemia heart disease by mediating cAMP-PKA signaling. In addition, DZ can alleviate ferroptosis in myocardial infarction. Our study has provided an alternative therapy to facilitate the treatment of myocardial infarction, which may have important implications for clinical practice.

Availability of Data and Materials

Data to support the findings of this study are available on reasonable request from the corresponding author.

Author Contributions

JZ and JS conceived the study. JZ and YL conducted the rat modeling. JZ and WH administered drugs to the experimental animals. JZ and HZ performed the histological and molecular biological experiments. JZ analyzed the

data. JS supervised the study. All authors contributed to editorial changes in the manuscript. All authors read and approved the final manuscript. All authors have participated sufficiently in the work and agreed to be accountable for all aspects of the work.

Ethics Approval and Consent to Participate

The study was approved by the Ethics Committee of Jiangsu Province Academia (NO. AEWC-20181205-65).

Acknowledgment

Not applicable.

Funding

The study was funded by the National Nature Science Foundation of China (No. 82374203, 81973766).

Conflict of Interest

The authors declare no conflict of interest.

References

[1] Xu T, Ding W, Tariq MA, Wang Y, Wan Q, Li M, *et al*. Molecular mechanism and therapy application of necrosis during myocardial injury. Journal of Cellular and Molecular Medicine. 2018; 22: 2547–2557.

[2] Talman V, Ruskoaho H. Cardiac fibrosis in myocardial infarction-from repair and remodeling to regeneration. Cell and Tissue Research. 2016; 365: 563–581.

[3] Colombe AS, Pidoux G. Cardiac cAMP-PKA Signaling Compartmentalization in Myocardial Infarction. Cells. 2021; 10: 922.

[4] Nishida H, Sato T, Miyazaki M, Nakaya H. Infarct size limitation by adrenomedullin: protein kinase A but not PI3-kinase is linked to mitochondrial KCa channels. Cardiovascular Research. 2008; 77: 398–405.

[5] Xia Y, He F, Moukeila Yacouba MB, Zhou H, Li J, Xiong Y, *et al*. Adenosine A2a Receptor Regulates Autophagy Flux and Apoptosis to Alleviate Ischemia-Reperfusion Injury *via* the cAMP/PKA Signaling Pathway. Frontiers in Cardiovascular Medicine. 2022; 9: 755619.

[6] Bryson TD, Gu X, Khalil RM, Khan S, Zhu L, Xu J, *et al*. Overexpression of prostaglandin E2 EP4 receptor improves cardiac function after myocardial infarction. Journal of Molecular and Cellular Cardiology. 2018; 118: 1–12.

[7] Karam S, Margaria JP, Bourcier A, Mika D, Varin A, Bedioune I, *et al*. Cardiac Overexpression of PDE4B Blunts β-Adrenergic Response and Maladaptive Remodeling in Heart Failure. Circulation. 2020; 142: 161–174.

[8] Ye Y, Keyes KT, Zhang C, Perez-Polo JR, Lin Y, Birnbaum Y. The myocardial infarct size-limiting effect of sitagliptin is PKA-dependent, whereas the protective effect of pioglitazone is partially dependent on PKA. American Journal of Physiology. Heart and Circulatory Physiology. 2010; 298: H1454–H1465.

[9] Li XD, Yang YJ, Geng YJ, Zhao JL, Zhang HT, Cheng YT, *et al*. Phosphorylation of endothelial NOS contributes to simvastatin protection against myocardial no-reflow and infarction in reperfused swine hearts: partially via the PKA signaling pathway. Acta Pharmacologica Sinica. 2012; 33: 879–887.

[10] Qu C, Xu DQ, Yue SJ, Shen LF, Zhou GS, Chen YY, *et al*. Pharmacodynamics and pharmacokinetics of Danshen in isoproterenol-induced acute myocardial ischemic injury combined with Honghua. Journal of Ethnopharmacology. 2020; 247: 112284.

[11] Li ZM, Xu SW, Liu PQ. Salvia miltiorrhizaBurge (Danshen): a golden herbal medicine in cardiovascular therapeutics. Acta Pharmacologica Sinica. 2018; 39: 802–824.

[12] Wang X, Guo D, Li W, Zhang Q, Jiang Y, Wang Q, *et al*. Danshen (Salvia miltiorrhiza) restricts MD2/TLR4-MyD88 complex formation and signalling in acute myocardial infarction-induced heart failure. Journal of Cellular and Molecular Medicine. 2020; 24: 10677–10692.

[13] Li Y, Zhang K, Liu J, Liu S, Nie C, Yan Y, *et al*. Geniposide suppresses thermogenesis via regulating PKA catalytic subunit in adipocytes. Toxicology. 2021; 464: 153014.

[14] Dixon SJ, Lemberg KM, Lamprecht MR, Skouta R, Zaitsev EM, Gleason CE, *et al*. Ferroptosis: an iron-dependent form of non-apoptotic cell death. Cell. 2012; 149: 1060–1072.

[15] Stockwell BR. Ferroptosis turns 10: Emerging mechanisms, physiological functions, and therapeutic applications. Cell. 2022; 185: 2401–2421.

[16] Wang XD, Kang S. Ferroptosis in myocardial infarction: not a marker but a maker. Open Biology. 2021; 11: 200367.

[17] Han X, Zhang J, Liu J, Wang H, Du F, Zeng X, *et al*. Targeting ferroptosis: a novel insight against myocardial infarction and ischemia-reperfusion injuries. Apoptosis: an International Journal on Programmed Cell Death. 2023; 28: 108–123.

[18] Wu YT, Zhang GY, Hua Y, Fan HJ, Han X, Xu HL, *et al*. Ferrostatin-1 suppresses cardiomyocyte ferroptosis after myocardial infarction by activating Nrf2 signaling. The Journal of Pharmacy and Pharmacology. 2023; 75: 1467–1477.

[19] Zhang X, Hu C, Ma ZG, Hu M, Yuan XP, Yuan YP, *et al*. Tisp40 prevents cardiac ischemia/reperfusion injury through the hexosamine biosynthetic pathway in male mice. Nature Communications. 2023; 14: 3383.

[20] Jiang Y, Qiao Y, He D, Tian A, Li Z. Adaptor protein HIP-55-mediated signalosome protects against ferroptosis in myocardial infarction. Cell Death and Differentiation. 2023; 30: 825–838.

[21] Shen Y, Wang X, Shen X, Wang Y, Wang S, Zhang Y, *et al*. Geniposide Possesses the Protective Effect on Myocardial Injury by Inhibiting Oxidative Stress and Ferroptosis *via* Activation of the Grsf1/GPx4 Axis. Frontiers in Pharmacology. 2022; 13: 879870.

[22] Shen Y, Shen X, Wang S, Zhang Y, Wang Y, Ding Y, *et al*. Protective effects of Salvianolic acid B on rat ferroptosis in myocardial infarction through upregulating the Nrf2 signaling pathway. International Immunopharmacology. 2022; 112: 109257.

[23] Trott O, Olson AJ. AutoDock Vina: improving the speed and accuracy of docking with a new scoring function, efficient optimization, and multithreading. Journal of Computational Chemistry. 2010; 31: 455–461.

[24] Liu C, Du L, Zhang S, Wang H, Kong L, Du G. Network pharmacology and experimental study of phenolic acids in salvia miltiorrhiza bung in preventing ischemic stroke. Frontiers in Pharmacology. 2023; 14: 1108518.

[25] Tao W, Xu X, Wang X, Li B, Wang Y, Li Y, *et al*. Network pharmacology-based prediction of the active ingredients and potential targets of Chinese herbal Radix Curcumae formula for application to cardiovascular disease. Journal of Ethnopharmacology. 2013; 145: 1–10.

[26] Xu X, Qiu J, Li X, Chen J, Li Y, Huang X, *et al*. Perilipin5 protects against non-alcoholic steatohepatitis by increasing 11-Dodecenoic acid and inhibiting the occurrence of ferroptosis. Nutrition & Metabolism. 2023; 20: 29.

[27] Zheng P, Xie Z, Yuan Y, Sui W, Wang C, Gao X, *et al*. Plin5 alleviates myocardial ischaemia/reperfusion injury by reducing oxidative stress through inhibiting the lipolysis of lipid droplets. Scientific Reports. 2017; 7: 42574.

[28] Zhang X, Xu W, Xu R, Wang Z, Zhang X, Wang P, *et al*. Plin5 Bidirectionally Regulates Lipid Metabolism in Oxidative Tissues. Oxidative Medicine and Cellular Longevity. 2022; 2022: 4594956.

[29] Liu L, Yang S, Wang H. α-Lipoic acid alleviates ferroptosis in the MPP^+ -induced PC12 cells via activating the PI3K/Akt/Nrf2 pathway. Cell Biology International. 2021; 45: 422–431.

[30] Cadenas S. ROS and redox signaling in myocardial ischemia-reperfusion injury and cardioprotection. Free Radical Biology & Medicine. 2018; 117: 76–89.

[31] Wu X, Li Y, Zhang S, Zhou X. Ferroptosis as a novel therapeutic target for cardiovascular disease. Theranostics. 2021; 11: 3052–3059.

[32] Huang WT, Li CJ, Wu PJ, Chang YS, Lee TL, Weng CF. Expression and in vitro regulation of pituitary adenylate cyclase-activating polypeptide (pacap38) and its type I receptor (pac1-r) in the gonads of tilapia (Oreochromis mossambicus). Reproduction (Cambridge, England). 2009; 137: 449–467.

[33] Huang CK, Dai D, Xie H, Zhu Z, Hu J, Su M, *et al*. Lgr4 Governs a Pro-Inflammatory Program in Macrophages to Antagonize Post-Infarction Cardiac Repair. Circulation Research. 2020; 127: 953–973.

[34] Lai P, Nikolaev VO, De Jong KA. Understanding the Role of SERCA2a Microdomain Remodeling in Heart Failure Induced by Obesity and Type 2 Diabetes. Journal of Cardiovascular Development and Disease. 2022; 9: 163.