Zhishi xiebai guizhi decoction ameliorates mitochondrial dysfunction induced by myocardial ischemia-reperfusion injury in mice via the Autophagy-ACBP-TSPO axis

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Research Article
Abstract

Background

Myocardial ischemia-reperfusion injury (MIRI) is often associated with oxidative stress, mitochondrial damage, programmed cell death, and autophagy. Protecting the mitochondrial function of myocardial tissue is critical for cardiac function. However, there is currently no effective treatment for MIRI. We aimed to determine whether zhishi xiebai guizhi decoction (ZXGD) attenuates MIRI through the autophagy-ACBP-TSPO axis.

Methods

A model of MIRI was established in mice by ligating the left anterior descending coronary artery for 30 min and reperfusion for 2 h. The myocardial injury was assessed by TTC-Evans blue and hematoxylin and eosin (HE) stains. The potential protein targets were identified using network analysis and molecular docking. Mitochondrial membrane potential and ATP production were detected using JC-1 assay and ATP assay kit, respectively. The target proteins were detected by western blotting, immunofluorescence analysis, and immunohistochemistry.

Results

ZXGD markedly attenuated myocardial damage, and enhanced cardiac function and mitochondrial morphology in mice accompanied with ischemia-reperfusion. It was found that AKT1-mTOR-mediated autophagy was possibly involved in the pretreatment of ZXGD on MIRI by network analysis and molecular docking. Experiments in vivo confirmed that ZXGD could inhibit myocardium autophagy, partly through activating the AKT1-mTOR signaling pathway. Furthermore, we revealed that ZXGD could promote ACBP expression and ACBP-TSPO binding in the myocardium, which might result from the regulation of autophagy.

Conclusion

ZXGD pretreatment significantly ameliorates MIRI by activating the autophagy-ACBP-TSPO axis in mice.

1. Introduction

A common cause of cardiovascular death in the world is myocardial infarction [1], for which percutaneous coronary intervention is considered the most effective and direct treatment [2]. However, MIRI can be caused by the process of reperfusion to the ischemic heart muscle, and protecting against MIRI remains a challenge [3]. The main mechanisms of MIRI include oxidative stress, calcium overload,
apoptosis, and mitochondrial dysfunction [4]. Many cardioprotective mechanisms against MIRI have been proposed [5]. However, there is currently no specific treatment for MIRI in the pharmacopeia.

Under normal physiological conditions, mitochondria are primarily responsible for cellular functions, a role that is particularly important in the heart. Therefore, Mitochondrial damage is a critical contributor to MIRI, and over-opening, during MIRI, of the mitochondrial permeability transition pore (mPTP) generates excess ROS [6]. This reduces the mitochondrial membrane potential (Δψm) and disrupts mitochondrial morphology, ultimately contributing to cell death. When there is damage to the mitochondria, autophagy can sustain intracellular stability by removing damaged mitochondria. However, autophagy can be a double-edged sword. Excessive autophagy is frequently linked to cell death [7]. Acyl coenzyme A binding protein (ACBP) can be secreted extracellularly via an autophagy-dependent paracrine pathway and inhibition of autophagy leads to an increase in intracellular levels of ACBP [8]. Additionally, the mitochondrial translocator protein (TSPO), one of the classical receptors for ACBP, is an essential protein indispensable for the maintenance of mitochondrial function in myocardial tissue [9]. Although numerous ligands of TSPO play a protective role in MIRI [10], the role of ACBP, regulated by autophagy, in MIRI remains unelucidated.

Traditional Chinese medicine is widely used in clinical settings and is effective in treating cardiovascular diseases [11]. Zhishi xiebai guizhi decoction (ZXGD) was first mentioned in Jin Gui Yao Lue and is commonly used to treat cardiovascular diseases such as unstable angina [12]. ZXGD attenuates hypoxia/reoxygenation injury by alleviating oxidative stress, and mitochondrial dysfunction, inhibiting apoptosis, and improving cellular energy acquisition, which helps to control the incidence of coronary heart diseases [13, 14]. However, the regulation effect of ZXGD on MIRI requires further investigation. Here, we developed a mouse model of MIRI to investigate the possible mechanisms of ZXGD on autophagy and mitochondrial damage induced by MIRI in mice.

2. Materials and Methods

2.1 Chemicals and reagents

From Merck Life Science (Darmstadt, Germany), we purchased polyvinylidene fluoride (PVDF) membrane (0.2 µm) and Evans blue (CAS: 314-13-6). 2,3,5-Triphenyl-2H-tetrazolium chloride (TTC) (CAS#298-96-4) was purchased from Solarbio (Beijing, China). From Jiancheng Bioengineering Institute (Nanjing, China), cardiac troponin (CTnT) and lactate dehydrogenase (LDH) assay kits were purchased. Targetmol (Shanghai, China) provided 3-Methyladenine (3-MA) (CAT# T1879) and Rapamycin (CAT# T1537). Primary antibodies against ACBP (CAT#ab231910) and TSPO (CAT#ab92291) were purchased from Abcom (Shanghai, China). Primary antibodies against Beclin1, ATG3, ATG7, and LC3A/B were obtained from Servicebio (Wuhan, China) and AKT1, p-AKT1, mTOR, p-mTOR, ULK1, and p-ULK1 were obtained from Huabio (Hangzhou, China). Affinity (Jiangsu, China) provided HRP-conjugated secondary antibodies (CAT# S000

2.2 Preparation of ZXGD and drugs
As shown in Table 1, ZXGD consists of five Chinese medicinal herbs. We checked all the plant names with The Plant List (http://www.theplantlist.org). All the herbs were purchased from Nanfang Hospital (Guangdong, China). The herbs were extracted twice by refluxing in boiling water (500 ml) for 30 minutes each time, after soaking in distilled water for 1 hour. The resulting suspension was concentrated appropriately and stored at 4°C for further experiments. The dosage of ZXGD for mice was based on the body surface conversion factor of mice [15]. Based on previous literature [16, 17], 3-MA and rapamycin were dissolved in a solution containing 2% DMSO, 30% PEG300, and 5% Tween 80, and formulated at 30 and 4 mg/kg, respectively. The above reagents were used immediately after preparation.

<table>
<thead>
<tr>
<th>Chinese herbal name</th>
<th>Botanical plant name</th>
<th>Medicinal parts</th>
<th>Production area</th>
<th>Grams</th>
<th>% (dry weight in ZXGD)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Xiebai</td>
<td>Allium chinense G.Don</td>
<td>Rhizomes</td>
<td>Hubei, China</td>
<td>9</td>
<td>18.4</td>
</tr>
<tr>
<td>Gualou</td>
<td>Trichosanthes kirilowii Maxim.</td>
<td>pericarp</td>
<td>Zhejiang, China</td>
<td>12</td>
<td>24.5</td>
</tr>
<tr>
<td>Zhishi</td>
<td>Citrus aurantium L.</td>
<td>fruit</td>
<td>Hunan, China</td>
<td>10</td>
<td>20.5</td>
</tr>
<tr>
<td>Houpo</td>
<td>Magnolia officinalis Rehder &amp; E.H.Wilson</td>
<td>bark</td>
<td>Sichuan, China</td>
<td>12</td>
<td>24.4</td>
</tr>
<tr>
<td>Guizhi</td>
<td>Cinnamomum cassia (L.) J.Presl.</td>
<td>branch</td>
<td>Guangdong, China</td>
<td>6</td>
<td>12.2</td>
</tr>
</tbody>
</table>

2.3 Animals and groups

Male C57B/L6 mice (seven weeks old), around 25 g, were obtained from the Experimental Animal Centre of Southern Medical University (Guangzhou, China, approval number: L2022063) and kept in rooms with 12-hour cycles of light and darkness and had unlimited access to food and water. Throughout the experimental period, the health of the animals was monitored daily. We randomly distributed Eighty-four mice into seven groups of 12 mice as follows: sham group, I/R group, ZL group (3.82 g/kg ZXGD), ZM group (7.65 g/kg ZXGD), ZH group (15.3 g/kg ZXGD), MA group (7.65 g/kg ZXGD + 30 mg/kg 3-MA), and RA group (7.65 g/kg ZXGD + 4 mg/kg rapamycin). In the ZXGD groups, mice were given different doses of ZXGD by gavage twice a day for 1 week before surgery, while both the sham and I/R groups were administered an equivalent amount of normal saline. In addition to daily intragastric administration of 7.65 g/kg ZXGD, the MA and RA groups received intraperitoneal injections of 3-MA and rapamycin, respectively (dosage as above).

2.4 MIRI model

The care and use of laboratory animals followed NIH guidelines during all experimental procedures. All animal experiments were approved by the Animal Ethics Committee. We established a mouse MIRI model
based on previous literature [18]. In short, we anesthetized mice by intraperitoneal injection of 50mg/kg of sodium pentobarbital, used an animal ventilator (SAR-830, IITC Inc) to establish mechanical ventilation, made an incision over the heart of the mouse, bluntly stripped off the left pectoral muscle with forceps, cut a small opening in the left 4–5 intercostal space, and fixed the open area to expose the heart fully. We identified the course of the cardiac artery, ligated the left anterior descending thereof with a 6−0 suture needle, tied a slipknot, left the thread on the outside of the wound, and sutured the wound. After 30 min of ligation and 2 h of reperfusion, the hearts were removed to await subsequent processing. The hearts of mice in the sham group were threaded only, but not subjected to coronary artery ligation.

2.5 TTC-Evans blue staining

At the end of the reperfusion, we ligated the coronary artery again, dissected the aorta bluntly, and infused the heart with 5% Evans blue from the aorta in a retrograde fashion. The heart was frozen at -20 °C for 30 min, and then we cut the heart into 2mm slices perpendicular to the long axis. The heart slices were subsequently submerged in a 4% paraformaldehyde solution for a duration of 1 h, following a 30-min incubation in a 2% TTC at a temperature of 26 °C. The infarcted region was identified by its unstained white appearance, while the unaffected region exhibited a blue stain, and the region at risk displayed a red stain. The heart slices were captured in photographs, and these images were subsequently subjected to analysis using the Image Pro Plus software.

2.6 Echocardiography

Cardiac function was assessed in each group of mice (anesthetized by 1.5% isoflurane) at the appropriate time points using the Vevo2100 high-frequency ultrasound imaging system. Left ventricular diameter at diastole (LVDd) and left ventricular diameter at systole (LVDs) were measured by M-mode echocardiography of the short-axis section of the heart and then calculated ejection fraction and fractional shortening.

2.7 Qualitative analysis of ZXGD-containing serum components

ZXGD aqueous decoction was obtained as in 2.2. We randomly selected 3 mice each from the sham group and the ZM group, and each group was treated as before. After blood was collected from the abdominal aorta, serum was obtained and stored at -80°C. All samples were detected by UHPLC-QE-MS at Biotree Biomedical Technology Co, Ltd (Shanghai, China).

2.8 Network analysis and functional enrichment analysis

We screened molecular targets of the active ingredients in the serum of mice through the TCMSP database. On the other hand, a search of the Genecard, Disgenet, OMIM, and Drugbank databases retrieved MIRI-related targets. The common targets in drug and disease were screened using Venny 2.1. Protein-protein interaction (PPI) data were obtained using the STRING database and visualized by Cytoscape 3.7.1.
The GO analysis and KEGG pathway enrichment analysis data were obtained by entering the gene symbols of the key targets into the Matescape database and selecting Homo sapiens as the study subject. Data were visualized via the bioinformatics website (http://www.bioinformatics.com.cn/).

2.9 Molecular docking

Download protein and ligand files from the RCSB Protein Data Bank and PubChem. After removing water molecules and adding hydrogen atoms in AutoDock software, the AutoDock vina plug-in was used to perform molecular docking. The molecular conformation with more hydrogen bonding and lower binding energy was selected after molecular docking.

2.10 Immunofluorescence staining

After deparaffinization and hydration, mice myocardial tissue sections (5 µm-thick paraffin sections) were soaked in boiled citric acid antigen repair buffer to repair the antigen in a microwave for 10 min. After cooling to 26 °C, the slides were blocked in 5% donkey serum with 0.1% Triton-X100 for 2 h at 26 °C. These were then incubated in primary antibody (diluted in the 2.5% donkey serum) at 4 °C overnight. All primary antibodies were diluted at a ratio of 1:200. The following day, after rinsing with PBST thrice, 5 min/time, the slides were treated with secondary antibodies, including Alexa Fluor488-conjugated donkey anti-goat IgG (1:200) and Alexa Fluor594-conjugated donkey anti-rabbit IgG (1:200), and incubated for 2 h in the absence of light. Next, slides were covered with DAPI-aqueous, fluoroshield (CAT#ab104139, Abcom) for 5 min. A KEYENCE BZ-X800E All-in-One Fluorescence Microscope was used to capture images of the slides covered with coverslips.

2.11 Immunohistochemical (IHC)

After deparaffinization, hydration, and antigen repair (same method as 2.10), sections were immersed in a 3% hydrogen peroxide solution for 10 min, followed by washes 3 times with PBS. The slides were then blocked in 5% goat serum in PBS plus 0.1% Triton-X100 for 2 h at 26 °C. After further overnight incubation with the primary antibody, horseradish peroxide conjugated goat anti-rabbit IgG was used for 2 h at 26°C. After being treated with dianminobenzidine reagent for the same amount of time and hematoxylin for 5 min, protein expression was observed using a microscope.

2.12 Western blotting

At the end of the refusion, proteins in the ischemia-reperfusion area were extracted. The BCA Method was used to assay the protein concentration. 30 µg of protein per well were separated by SDS-PAGE. The proteins were transferred to PVDF membranes. We blocked the membranes using 5% skim milk for 1 h and then incubated with primary antibodies for 12 h at 4°C. The membranes underwent three wash cycles using TBST for 5 minutes each time. Following this, the membranes were incubated in the secondary antibody (1:5,000) for 1 h at RT and then visualized using a multimodal imaging platform (ChemiDoc XRS + System, BIO-RED). Image J software was used to determine their intensities, which were used to ascertain the target protein expression.

2.13 Statistical analysis
Statistical analysis was performed using SPSS 20.0 software (Chicago, IL, USA). All data are presented as the mean ± standard deviation (SD). One-way ANOVA followed by Bonferroni post hoc analysis for multiple groups was used to assess statistically significant differences. \( P < 0.05 \) was considered statistically significant. \#P < 0.05, \##P < 0.01, and \###P < 0.001 (compared to the sham group); \*P < 0.05, \**P < 0.01, and \***P < 0.001 (compared to the I/R group); \▲P < 0.05, \▲▲P < 0.01, and \▲▲▲P < 0.001 (compared to the ZM group).

3. Results

3.1. ZXGD could protect the heart against ischemia-reperfusion injury

To elucidate the effect of ZXGD in mice, we observed the myocardial tissue using HE and TTC-Evans blue stains, while the serum concentration of CTnT and LDH was determined. HE stains showed well-organized and clear myocardium in the sham group. Myocardial edema and erythrocyte sludge in the I/R group were found around the myocardial vessels. ZXGD pretreatment significantly improved these pathological changes in MIRI mice (Fig. 1A). The infarct area was substantially increased in the I/R group, while ZXGD could significantly improve the myocardial injury (Fig. 1B and C). The CTnT and LDH levels were markedly elevated in the I/R group, whereas ZXGD could reduce both serum levels (Fig. 1D and E). These results showed that ZXGD pretreatment could significantly attenuate MIRI in mice.

3.2. ZXGD could improve heart function and maintain the stability of the mitochondrial morphology in mice

We monitored the cardiac function of mice after I/R by echocardiography. LVDs were substantially elevated and LVEF and LVFS were markedly downregulated in the I/R group. However, ZXGD reversed this phenomenon (Fig. 2A-D). Nevertheless, the impact of ZXGD on LVDd was not statistically significant (Fig. 2E). This suggested that ZXGD could protect cardiac function in MIRI mice.

To observe the mitochondrial morphology in the myocardium, transmission electron microscopy was performed. In the sham group, mitochondrial membrane structure and mitochondrial cristae were intact. However, the myocardial tissues, in the I/R group, showed disrupted mitochondrial membrane structure (red arrow); loosely arranged and broken mitochondrial cristae and newborn autophagic vesicles (black arrow) could be observed on the damaged mitochondria. Notably, ZXGD partially maintained the stability of the mitochondrial morphology, compared with that in the I/R group. (Fig. 2F). Therefore, we speculated that the therapeutic effect of ZXGD on MIRI may be related to autophagy and mitochondrial function.

3.3. Network analysis of ZXGD treating MIRI

To further clarify the relevant therapeutic targets of ZXGD, we identified 491 and 111 active ingredients in ZXGD and the serum of ZXGD-treated mice, respectively, by UPLC-QE-MS (Fig. 3A). We identified 204 molecular targets associated with 111 compounds in the serum using the TCMSP database. Subsequent
comparison with the 1761 disease targets associated with MIRI retrieved from disease databases revealed 103 common targets (Fig. 3B). After constructing the PPI network with 103 common targets, it was found that AKT1 may be the most critical target of ZXGD on MIRI (Fig. 3C). GO enrichment analysis revealed that these targets regulate key biological processes associated with MIRI, including responses to nutrient and oxygen levels, regulation of blood flow, and programmed cell death. In addition, the top 10 GO molecular functions included heme binding, protein homodimerization activity, ubiquitin protein ligase binding, protein kinase regulator activity, etc (Fig. 3E). We screened the top 20 pathways using KEGG pathway enrichment analysis (Fig. 3D). Focusing on the role of AKT1 in these pathways, we found that AKT1 was able to regulate mTOR in the cancer-related pathway, the endocrine resistance-related pathway, the HIF-1 signaling pathway, and the diabetic cardiomyopathy-related pathway. Notably, these molecular targets were also closely associated with mitophagy. Considering that mTOR could inhibit mitophagy by inhibiting the assembly of autophagosomes, we focused primarily on autophagy regulated by the AKT1-mTOR signaling pathway in MIRI.

3.4. The main components of ZXGD in the serum could directly target AKT1 and autophagy-related proteins

To further explore whether ZXGD active ingredients could act on AKT1 and autophagy-related proteins, we found that 37 active ingredients were both in ZXGD and in the serum of ZXGD-treated mice (Fig. 4A). From these 37 compounds, we selected the 10 most abundant and subjected them to molecular docking with AKT1, mTOR, ULK1, ATG3, ATG7, Beclin1, p62, and LC3B proteins (Table 2). As shown in Fig. 4B-J, the results of docking the identified components with the most representative molecules of autophagy-related molecular targets were as follows: AKT1 to naringenin (-7.7 kcal/mol), mTOR to naringenin (-7.7 kcal/mol), ULK1 to naringenin (-7.5 kcal/mol), ATG7 to irigenin (-7.8 kcal/mol); Beclin1 to naringenin (-7.3 kcal/mol); p62 to naringenin (-5.9 kcal/mol); ATG3 to naringenin (-7.4 kcal/mol) and LC3B to naringenin (-6.9 kcal/mol) (the yellow dashed lines indicated that each component was hydrogen-bonded to the corresponding molecular target point; The red dashed line represented the pi-cation interaction). Therefore, the active components of ZXGD might act directly on AKT1, mTOR, ULK1, ATG3, ATG7, Beclin1, LC3B, and p62, and regulate the AKT1-mTOR signaling pathway and autophagy levels in the myocardium.
### Table 2
Top 10 active ingredients in ZXGD that are absorbed directly into the bloodstream

<table>
<thead>
<tr>
<th>Name</th>
<th>EnChIKey</th>
<th>Formula</th>
<th>rtmed</th>
<th>type</th>
<th>rank</th>
</tr>
</thead>
<tbody>
<tr>
<td>Feruloyl putrescine</td>
<td>SFUVCMKSYKHYLD-UHFFFAOYSA-N</td>
<td>C14H20N2O3</td>
<td>38.619</td>
<td>pos</td>
<td>1</td>
</tr>
<tr>
<td>Fumaric acid</td>
<td>VZCYOOQTPOCHFL-OWOJBTEDSA-N</td>
<td>C4H4O4</td>
<td>32.803</td>
<td>neg</td>
<td>2</td>
</tr>
<tr>
<td>Antiarol</td>
<td>VTCDZPUMZAZMSB-UHFFFAOYSA-N</td>
<td>C9H12O4</td>
<td>104.60</td>
<td>pos</td>
<td>3</td>
</tr>
<tr>
<td>Naringenin</td>
<td>FTVWIRXFELQPLP-UHFFFAOYSA-N</td>
<td>C15H12O5</td>
<td>241.84</td>
<td>pos</td>
<td>4</td>
</tr>
<tr>
<td>Loliolide</td>
<td>XEVQXKKKAVVSMW-UHFFFAOYSA-N</td>
<td>C11H16O3</td>
<td>132.3</td>
<td>pos</td>
<td>5</td>
</tr>
<tr>
<td>Isomyristicin</td>
<td>DHUZAAUGHUHIDS-ONEGZNNKSA-N</td>
<td>C11H12O3</td>
<td>89.912</td>
<td>pos</td>
<td>6</td>
</tr>
<tr>
<td>Irigenin</td>
<td>TUGWPJJTONLKL-OHFFFAOYSA-N</td>
<td>C18H16O8</td>
<td>305.88</td>
<td>neg</td>
<td>7</td>
</tr>
<tr>
<td>2,6-DIHYDROXYBENZOIC ACID</td>
<td>AKEUNKRJATALU-UHFFFAOYSA-N</td>
<td>C7H6O4</td>
<td>79.490</td>
<td>neg</td>
<td>8</td>
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<tr>
<td>Coniferyl aldehyde</td>
<td>DKZBBWMURDFHNE-UHFFFAOYSA-N</td>
<td>C10H10O3</td>
<td>139.736</td>
<td>pos</td>
<td>9</td>
</tr>
<tr>
<td>5,7-Dihydroxychromone</td>
<td>NYCXYKOXLNBYID-UHFFFAOYSA-N</td>
<td>C9H6O4</td>
<td>72.5857</td>
<td>neg</td>
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</tbody>
</table>

3.5. **ZXGD pretreatment could downregulate myocardial autophagy in mice, partly through activation of the AKT1-mTOR signaling pathway**

Based on the core targets screened from the network analysis and the molecular docking results, it was hypothesized that ZXGD could mitigate MIRI by AKT1-mTOR signaling pathway and autophagy-related proteins. We discovered that myocardial p-AKT1 and p-mTOR content was substantially downregulated in the I/R group, whereas it was upregulated by the ZXGD intervention (Fig. 5A-C). The I/R group showed significant increases in p-ULK1, Beclin1, LC3B/LC3A, ATG3, and ATG7 expression, while p62 expression decreased. The trends of all the above proteins were markedly reversed after different doses of ZXGD (Fig. 5D-J). This suggested that ZXGD could inhibit autophagy through the AKT1-mTOR signaling pathway or by directly regulating autophagy-related proteins.

3.6. **ZXGD could upregulate myocardial ACBP and promote the binding of ACBP to TSPO**
ACBP, as one of the traditional ligands for TSPO, may function to activate TSPO to regulate mitochondrial membrane potential and ATP production [19]. Additionally, inhibition of autophagy results in the buildup of ACBP within cells [20]. We therefore hypothesized that reduced levels of autophagy in myocardial tissue would also lead to intracellular aggregation of ACBP. Intriguingly, we found that myocardial ACBP expression was markedly reduced in the I/R group, whereas ZXGD suppresses this trend (Fig. 6A and C). Then, we found that the Pearson correlation coefficient of the I/R group was significantly reduced. However, ZXGD was observed to reverse this decline (Fig. 6B and D). Therefore, ZXGD could enhance Levels of ACBP in myocardial tissues and facilitate the interaction between ACBP and TSPO. It might be related to the regulation of autophagy by ZXGD.

3.7. 3-MA and rapamycin markedly regulated autophagy levels in myocardial tissue

Considering the broad regulatory role of AKT1 on other cellular processes, we used rapamycin and 3-MA to investigate the role of autophagy in the regulation of cardiac function and its intrinsic mechanisms in mice with MIRI. In the MA group, the expression of p-ULK1 Beclin1, LC3B/LC3A, ATG3, and ATG7 was significantly downregulated, related to the ZM group, while the expression of p62 was significantly decreased. In contrast, rapamycin not only specifically inhibited p-mTOR, but also reversed the regulation of these proteins by ZXGD (Fig. 7A-I). This suggested that 3-MA and rapamycin successfully inhibited or activated autophagy levels in myocardial tissue, respectively.

3.8. Rapamycin could reverse the protective effect of ZXGD on cardiac function and myocardial mitochondria

To investigate whether the regulation of autophagy by ZXGD was essential for its protection of cardiac function, we measured cardiac function in mice with MIRI treated with 3-MA and rapamycin. 3-MA treatment significantly increased LVEF and LVFS in the mice of MIRI compared to the ZM group, while rapamycin reversed this effect of ZXGD (Fig. 8A-C). Further, we found that mitochondrial membrane potential (Δψm) and ATP content were markedly reduced in the I/R group. Notably, ZXGD pretreatment protected the mitochondrial function of mice after I/R, and 3-MA enhanced this effect. In contrast, the function of ZXGD was reversed by rapamycin (Fig. 8D and E). It suggested that inhibiting excessive autophagy could protect cardiac mitochondrial function in mice.

3.9. Autophagy inhibition might have a protective effect on mitochondrial function by enhancing ACBP-TSPO interactions

Here, we found that ACBP content was further increased in the myocardium of the MA group, while significantly reduced in the RA group (Fig. 9A and D). Moreover, further inhibition of autophagy by 3-MA led to an increase in Pearson’s coefficient of ACBP and TSPO, based on the effect of ZXGD, whereas the opposite was observed for the RA group (Fig. 9B and C). These results suggested that ZXGD might
protect mitochondrial function and maintain normal cardiac function by inhibiting excessive autophagy and promoting the binding of ACBP to TSPO in mice.

4. Discussion

Currently, there is no effective treatment for MIRI [21], and its therapeutic methods and related mechanisms still need to be further elucidated. Here, we identified the therapeutic effect of ZXGD in mice with MIRI and explored its underlying mechanism. Luckily, we firstly revealed that ZXGD could protect myocardial mitochondrial function through the autophagy-ACBP-TSPO axis. This may provide insights into the clinical management of MIRI.

The development of MIRI involves several different mechanisms, including oxidative stress, apoptosis, and mitochondrial damage [22]. In past studies, ZXGD and the herbs contained therein have been proven to be involved in the treating mechanism of MIRI. ZXGD had an exceptional ability to attenuate cardiomyocyte injury induced by hypoxia/reoxygenation, concerning oxidative stress, cell apoptosis, mitochondrial dysfunction, and energy acquisition [23]. Among them, the herb combination of Gualou and Xiebai has a positive effect on chronic myocardial ischemia in rats via the regulation of energy homeostasis and apoptosis [24]. Moreover, naringenin, the active ingredient of Zhishi, reduces apoptosis, inflammation, and oxidative stress while improving mitochondrial energy metabolism, ultimately mitigating myocardial ischemia injury and promoting angiogenesis of ischemic myocardium [25]. Stabilizing the mitochondrial structure in MIRI is crucial to reducing ROS production [26, 27]. Here, we found that ZXGD could ameliorate the impairment of cardiac function caused by ischemia-reperfusion, and this effect was closely related to the protection of the morphology and function of mitochondria.

Autophagy assumes a pivotal function in the elimination of misfolded proteins and the preservation of intracellular homeostasis, thereby conferring advantageous implications for numerous pathological conditions, and is a biological process that involves multiple proteins working together including mTOR, ULK1, ATG3, ATG7, p62, LC3A and LC3B [28, 29]. However, autophagy is not always beneficial. On the one hand, excessive autophagy itself not only could lead to cell death (autophagic cell death), but also be closely linked to other types of cell death [30, 31]. Increased autophagy leads to the degradation of ferritin, lipid droplets, circadian proteins, and GPX4, which induces the onset of ferroptosis, and this phenomenon is reversed in autophagy-deficient cells [32, 33]. During the reperfusion phase of MIRI, the increase in autophagy levels is mainly mediated by beclin1 [34]. Phosphorylation of beclin1 leads to its direct binding to SLC7A11 and inhibits System Xc activity, thereby inducing ferroptosis [35]. On the other hand, excessive autophagy leads to excessive mitochondrial clearance, which induces cell death. Healthy mitochondria are essential for cardiac function during MIRI. Excessive mitophagy could lead to excessive mitochondrial clearance and exacerbate mitochondrial dysfunction, which is detrimental to the cell, especially cardiomyocytes [36, 37]. It has been proposed that autophagy may enable excessive degradation of certain cell survival factors and organelles to induce cell death [38]. Therefore, we determined by network analysis and molecular docking that the major components of ZXGD may regulate myocardial tissue autophagy levels by modulating the AKT1-mTOR signaling pathway and
autophagy-related proteins. Among these compounds, irigenin and naringenin have an excellent affinity to AKT1 and autophagy-related proteins and are, therefore, considered important compounds for the treatment of MIRI by ZXGD. AKT1 has been shown to play a critical role in MIRI in previous studies [39]. AKT1 activation has a positive regulatory role in both mitochondrial damage and apoptosis during MIRI [40, 41]. However, few studies have focused on the role of AKT1-mTOR pathway-mediated autophagy in MIRI. To avoid the effects of AKT1 on other pathways, we used 3-MA and rapamycin to modulate autophagy levels in MIRI mice and verified that inhibition of autophagy could protect the myocardial mitochondrial function of the mice with MIRI.

Autophagy is not only capable of influencing the degradation of intracellular substances, but it is also closely related to cellular secretory functions (autophagy-dependent secretion) [42]. Past studies have shown that autophagy is necessary for the secretion of ACBP [43, 44]. Interestingly, we found elevated ACBP content in the myocardium of ZXGD-intervened mice, validating this idea. In addition, ACBP is essential for the regulation of normal mitochondrial function [45].

Certain proteins within the mitochondria have a crucial role in regulating the mitochondrial damage that results from MIRI. Among them, TSPO is important in maintaining normal mitochondrial function. The knockdown of TSPO leads to increased mitochondrial fragmentation, increased glucose uptake and lactate conversion, decreased oxidative phosphorylation, and increased glycolysis [46]. Additionally, TSPO has a significant association with the generation of ROS and the reinstatement of mitochondrial membrane potential [47]. 40-Chlorodiazepine (a ligand of TSPO) reduces mitochondrial sterol and oxysterol concentrations and improves mitochondrial function during MIRI in a rat model of high cholesterol [48]. Coordinated control of fatty acid uptake, beta-oxidation, and mitochondrial oxidative phosphorylation is essential for normal cardiac activity [49], and ACBP, a traditional receptor for TSPO, plays an essential role in mitochondrial energy metabolism. LCACoAs could be directly donated by ACBP to carnitine palmitoyl transferase I (CPTI), thereby enhancing mitochondrial β-oxidation [50]. However, the effect of ACBP action on TSPO on mitochondrial function still needs further elucidation [51]. Notably, when the autophagy-dependent paracrine pathway of ACBP is inhibited, it increases its intracellular content, closely related to mTOR [8, 44]. Similarly, we found that ZXGD could exert mitochondrial protection by inhibition of autophagy, which promotes ACBP aggregation in tissues and activates the ACBP-TSPO signaling. The enhancement of the ACBP-TSPO signaling may play an essential role in the maintenance of mitochondrial function in the myocardium during MIRI. This provides new evidence for the protective effect of the ACBP-TSPO signaling axis on mitochondrial function.

In the present study, we found that ZXGD could activate the autophagy-ACBP-TSPO axis to improve myocardial mitochondrial function in mice with MIRI, partly through activating the AKT1-mTOR pathway. This finding not only reveals the relationship between autophagy and mitochondrial function during MIRI but also provides new evidence of ZXGD in the therapy of cardiovascular diseases.

5. Conclusion
In conclusion, we found that ZXGD could activate the autophagy-ACBP-TSPO axis to improve myocardial mitochondrial function in mice with MIRI, partly through activating the AKT1-mTOR signaling pathway. This research partly explains how ZXGD reduces MIRI and proves its use in clinics.

List Of Abbreviations

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<thead>
<tr>
<th>Abbreviation</th>
<th>Definition</th>
<th>Description</th>
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<tbody>
<tr>
<td>ACBP</td>
<td>Acyl coenzyme A binding protein</td>
<td>LVDD</td>
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<tr>
<td>ANOVA</td>
<td>Analysis of Variance</td>
<td>LVFS</td>
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<tr>
<td>ATG3</td>
<td>Autophagy-Related Protein 3</td>
<td>LVDs</td>
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<td>ATG5</td>
<td>Autophagy-Related Protein 5</td>
<td>LVEF</td>
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<tr>
<td>ATG7</td>
<td>Autophagy-Related Protein 7</td>
<td>MIRI</td>
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<tr>
<td>AKT1</td>
<td>RAC-alpha serine/threonine-protein kinase</td>
<td>mTOR</td>
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<tr>
<td>CTnT</td>
<td>Cardiac troponin</td>
<td>mPTP</td>
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<tr>
<td>DAPI</td>
<td>4,6-diamidino-2-phenylindole</td>
<td>PEG 300</td>
</tr>
<tr>
<td>DMSO</td>
<td>Dimethyl sulfoxide</td>
<td>PVDF</td>
</tr>
<tr>
<td>GO</td>
<td>Gene Ontology</td>
<td>TTC</td>
</tr>
<tr>
<td>HE</td>
<td>Hematoxylin eosin</td>
<td>TSPO</td>
</tr>
<tr>
<td>I/R</td>
<td>Ischemia/reperfusion</td>
<td>TBST</td>
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<tr>
<td>KEGG</td>
<td>Kyoto Encyclopedia of Genes and Genomes</td>
<td>Tween 80</td>
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<tr>
<td>LC3A</td>
<td>MAP1LC3-I</td>
<td>3-MA</td>
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<tr>
<td>LC3B</td>
<td>MAP1LC3-II</td>
<td>UHPLC-QE-MS</td>
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<tr>
<td>LDH</td>
<td>Lactate dehydrogenase</td>
<td>ZXGD</td>
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Declarations

Ethics approval and consent to participate
All animal studies were approved by the Experimental Animal Centre of Southern Medical University (Guangzhou, China, approval number: L2022063)

Consent for publication

All authors consent to the publication of this work in Chinese Medicine.

Availability of data and materials

All datasets used are available from the corresponding author upon request

Competing interests

All of the authors declared there are no conflicts of interest for this manuscript.

Authors' contributions

Lin-ling Liu designed the research concept, conducted the experiments, and wrote the manuscript. Jing Jiang and Xiao-bing Cui were responsible for the experiments and statistics. Ya-xin Zhang, Ping Zeng, Xueqin Fu, and Yu-yan Gu were responsible for the experimental methodology. Yu-hua Jia and Rong Li participated in the discussion. Sai-bo Cheng, Wen Jin, and Feng-hua Zhou provided funding and improved the manuscript. All authors have reviewed and approved the manuscript.

Acknowledgments

Not applicable

Funding

This project was supported by grants from the National Natural Science Foundation of China (Grant No. 82374192, 82074424, 82074295); Science and Technology Program of Guangzhou, China (202201011647); Natural Science Foundation of Guangdong, China (2023A1515010451); Traditional Chinese Medicine Bureau of Guangdong Province, China (20222137, 20231184); Guangdong Deepening Medical Pair-Up Assistance Program To Ganzi Tibetan Autonomous Prefecture Hospital (Grant No. 2019181).

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**Figures**

**Figure 1**

ZXGD could protect the heart against ischemia-reperfusion injury
(A) HE stains of cardiac tissue sections. (B) Representative images of TTC-Evans blue-stained heart coronal sections of mice. (C) Area of infarct/area of risk calculated by TTC-Evans blue staining after MIRI ($n = 3$). (D and E) The content of CTnT and LDH of serum ($n = 3$).

**Figure 2**

** ZXGD could improve heart function and maintain the stability of the mitochondrial morphology in mice **

(A) Representative echocardiographic images of mice. (B-E) LVEF, LVFS, LVDs, and LVDd ($n = 3$). (F) Representative photographs were visualized using transmission electron microscopy.
Figure 3

Network analysis of ZXGD treating MIRI

(A) The UPLC chromatograms of mice serum containing ZXGD components. (B) Venn diagram of 103 common targets of serum containing ZXGD components and MIRI. (C) PPI network analysis of 103
common targets obtained from (B). (D) KEGG pathway analysis of the common targets. (E) The top 10 significantly enriched terms in biological processes, molecular functions, and cellular components.

Figure 4

The main components of ZXGD in the serum could directly target AKT1 and autophagy-related proteins.
(A) Venn diagram of the active ingredients in the aqueous decoction of ZXGD and the serum of mice serum containing ZXGD components. (B) Heat map showing the binding affinity of various components with AKT1 and autophagy-related targets. (C) AKT1-Naringenin. (D) mTOR-Naringenin. (E) ULK1-Naringenin. (F) p62-Naringenin. (G) ATG7-Irigenin. (H) ATG3-Naringenin. (I) Beclin1-Naringenin. (J) LC3B-Naringenin.
ZXGD pretreatment could downregulate myocardial autophagy in mice, partly through activation of the AKT1-mTOR signaling pathway

(A) Expression of AKT1, p-AKT1, mTOR, p-mTOR, ULK1, and p-ULK1. (B-D) Quantified analysis of (A) \( (n = 3) \). (E) Expression of autophagy relative proteins in cardiac tissue. (F-J) Quantified analysis of (D) \( (n = 3) \).

**Figure 6**

ZXGD could upregulate myocardial ACBP and promote the binding of ACBP to TSPO
(A) Immunohistochemical results of ACBP. (B) Results of immunofluorescence co-staining of ACBO and TSPO. (C) Quantitative results of (A) (n = 3). (D) Quantitative results of (B) (n = 3).

Figure 7

3-MA and rapamycin markedly regulated autophagy levels in myocardial tissue
(A) Expression of p-ULK1, ULK11, p-mTOR, and mTOR. (B and C) Quantified analysis of (A) \( (n = 3) \). (D) Expression of ATG3, ATG7, Beclin1, p62, and LC3B/LC3A in cardiac tissue. (E-I) Quantified analysis of (D) \( (n = 3) \).

Figure 8

**Rapamycin could reverse the protective effect of ZXGD on cardiac function and myocardial mitochondria**

(A) Representative echocardiographic images of mice. (B) LVEF \( (n = 3) \). (C) LVFS \( (n = 3) \). (D) Mitochondrial membrane potential (Δψm) assay \( (n = 3) \). (E) The content of ATP \( (n = 3) \).
Figure 9

Autophagy inhibition might have a protective effect on mitochondrial function by enhancing ACBP-TSPO interactions

(A) Immunohistochemical results of ACBP. (B) Results of immunofluorescence co-staining of ACBO and TSPO. (C) Pearson’s correlation of (B) \( (n = 3) \). (D) Mean gray value of ACBP of (A) \( (n = 3) \).