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RESEARCH ARTICLE

Evaluating the Impact of Trimetadizine on Myocardial Ischemia-Reperfusion Injury through Mitochondrial ATP Pathway in Rats

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ABSTRACT

Myocardial ischemia/reperfusion injury (MIRI) refers to heart muscle failure due to cellular and tissue structural damage following coronary revascularization. Myocardial I/R injury is increasing in cats and dogs, kept as pets. The occurrence of MIRI is complicated, and various pathways are possible. Therefore, it is urgent to further explore the mechanism of disease occurrence and development and find key regulatory factors to achieve the goal for reducing MIRI. In this study, MIRI model was established in SD rats through coronary artery ligation and randomized the rats into the model group (MIRI), the trimetazidine group (TMZ), the trimetazidine combined with diazepam treatment group (TMZ+DZ), and the trimetazidine combined with 5-hydroxytryptamine treatment group (TMZ+5-HD). Experimental results indicated that TMZ+DZ significantly reduced myocardial cell apoptosis and inflammation levels in MIRI rats, lowered Creatine Kinase (CK), Creatine Kinase MB Isoenzyme (CK-MB), Lactate Dehydrogenase (LDH), and ROS levels, and decreased myocardial infarct size and mass. While TMZ and TMZ+5-HD also improved these indicators, the therapeutic effect of TMZ+DZ was notably superior to the other two treatment groups. The results suggest that the combination therapy of trimetazidine and diazepam ameliorated myocardial damage in MIRI rats, providing experimental evidence for the treatment of MIRI with the combination therapy of trimetazidine and diazepam.

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INTRODUCTION

The cardiovascular (CVS) system is the most vital system in maintaining the lives of humans and animals (Figueiro and Pedler, 2023). Cardiovascular diseases are prevalent in modern times due to rapid lifestyle changes among humans (Figueiro and Pedler, 2023). The ways humans feed animals have also developed with the rapid evolution of human lifestyles, leading to companion animals facing similar issues (Ardente, 2023). Domesticated cats, dogs, and other companion animals are also experiencing similar cardiovascular issues due to improper feeding practices (Fascetti and Delaney, 2023). Clinical reports show that the canine and feline populations are also suffering from CVS diseases, including sudden heart arrest and cardiac reperfusion/ ischemia (I/R) (Fascetti and Delaney, 2023). Reports are scarce about death in dogs and cats due to I/R injury, but clinicians report that there are higher chances of dogs and cats dying due to I/R injury compared to the number of cases reported. Although experimentally Myocardial ischemia-reperfusion injury (MIRI) -induced dogs and cats have similar symptoms to humans, there is a need for proper diagnosis and treatment of the animals with MIRI (Hoehne *et al.*, 2023).

The main treatment for myocardial ischemia is timely reperfusion; however, reperfusion therapeutic measures such as pharmacological thrombolysis, aortic bypass surgery, or percutaneous coronary intervention often result in reperfusion injury (Mukhopadhyay *et al.*, 2014; Mizushima, 2017; Shi *et al.*, 2019). Numerous studies indicate that MIRI exacerbates the area of myocardial infarction and cardiac dysfunction in patients. (Simsek and Boersma, 2011; Wang *et al.*, 2017; Göbel *et al.*, 2018). The current clinical prevention and treatment of MIRI is mainly through inhibition of inflammatory response, anti-oxidation, reduction of calcium overload and impaired

energy metabolism, and protection of vascular endothelial cells (Wang *et al.*, 2017; Feng *et al.*, 2018). Therefore, reducing myocardial ischemia-reperfusion injury can improve the clinical efficacy of acute myocardial infarction treatment, making it a subject of ongoing and significant interest in research.

Specify the inflammatory: responses induced during myocardial ischemia and reperfusion, inducing the production and release of large amounts of pro-inflammatory factors (Hashmi and Al-Salam, 2015). A variety of inflammatory factors are involved in the pathological process of MIRI. Tumor necrosis factor-α causes myocardial injury by promoting adhesion and interaction between endothelial cells and leukocytes, resulting in a significant increase in granulocyte infiltration into the ischemia/reperfusion region; interleukin-6 (IL-6) has long been considered being involved in the acute phase response of cellular inflammatory factor, with significantly elevated expression in I/R myocardium.

Clinical studies have confirmed that trimetazidine (TMZ), a piperazine derivative, is a novel drug for optimizing myocardial energy metabolism, improving oxygen utilization in ischemic myocardium, and providing protection to cardiomyocytes, and has received more attention in recent years (Lebuffe *et al.*, 2003; Zhang *et al.*, 2017). TMZ is capable of optimizing the process of oxygen utilization within the mitochondrial matrix while simultaneously enhancing the activity of pyruvate dehydrogenase. This is beneficial for catalyzing the β-oxidation of fatty acids, thereby reducing the β-oxidation supply of free fatty acids, enhancing the aerobic oxidation capacity of glucose, generating ATP to the greatest extent, and optimizing the energy supply of cardiac myocytes. On the other hand, TMZ reduces myocardial cell apoptosis and inhibits ventricular remodeling by decreasing the release of oxygen-free radicals, endothelin-1, and creatine phosphokinase, thereby enhancing the contractile function of failing hearts. Additionally, TMZ possesses anti-inflammatory and anti-oxidative stress effects (Kraft *et al.*, 1978). Meanwhile, some studies have confirmed that ATP-sensitive potassium channels (KATP) play a crucial role in protecting the myocardium from the effects of MIRI (Sun *et al.*, 2017). While it was traditionally thought that there was only one channel in the myocardial cell membrane, namely the cytoplasmic ATP-sensitive potassium channel (sarcKATP), it was later discovered that there is another channel in the mitochondria, referred to as the mitochondrial ATP-sensitive potassium channel (mitoKATP). The latter plays a more prominent role in inhibiting MIRI than the former (Kahraman *et al.*, 2018). Therefore, this study aims to investigate whether the protective effect of trimetazidine on rat MIRI is related to the mitoKATP signaling pathway.

MATERIALS AND METHODS

Experimental design: The study was approved by the Ethics Committee of Central Hospital Affiliated to Shandong First Medical University (No.20230130-002). Rats were housed in a specific pathogen-free environment at the Experimental Animal Center. They were kept in a temperature-controlled room, maintained at 25 ± 2°C, with a 12-hour dark/light cycle. In this study, 40 adult male Sprague Dawley (SD) rats were randomly divided into five groups: sham surgery group (sham), model group (MIRI), trimetazidine group (TMZ), trimetazidine (4mg/kg) combined with mitoKATP signaling pathway opener diazepam (80μg/kg) (TMZ+DZ) group and trimetazidine glucose solution (4 mg/kg) combined with mitoKATP signaling pathway inhibitor 5-hydroxyquinoline (10 mg/kg) group (TMZ+5-HT). All drugs were injected through the tail vein. The equal amount of glucose was given into the tail vein of rats in the unadministered group. For the MIRI model, rats were anesthetized with methoxamine and ketamine, followed by tracheal intubation and mechanical ventilation from an animal respirator. A three-lead electrocardiogram (ECG) was used to monitor the onset of myocardial ischemia, as well as typical ECG changes. During the surgical procedure, a microcatheter was inserted into the left ventricle via the right carotid artery to assess cardiac function. Myocardial ischemia was induced by ligating the left anterior descending coronary artery for 30 minutes, followed by 2 hours of reperfusion. The sham operation group underwent the same surgical procedure without ligation. The whitening of the left ventricular apex and anterior wall indicated successful model induction, while the recovery and reddening of these areas indicated successful reperfusion. Then collect relevant test data.

Detection of apoptotic cardiomyocytes by TUNEL method: Frozen heart tissue was sectioned into 50 micrometers (μm) thick slices. Following the guidelines of the colorimetric TdT-mediated dUTP Nick-End Labeling (TUNEL) apoptosis assay kit (C1086, Beyotime, China), these sections were initially stained with biotin-dUTP and streptavidin-HRP. Finally, the substrate was added, counter-stained with DAPI (C1002, Beyotime, China), and images were taken under a fluorescence microscope.

Analysis of inflammatory cytokines IL-6 and TNF-α in myocardial tissue: Blood samples were taken from anesthetized SD rats. Serum samples were separated by centrifugation (4°C, 15 minutes, 1500 rpm), processed as supernatant, and stored at -80°C. ELISAs for interleukin-6 (IL-6) (E-EL-R0015, Elabscience, China) and tumor necrosis factor-alpha (TNF-α) (E-EL-R2856, Elabscience, China) were performed using commercially available kits.

Analysis of serum myocardial enzymes: Blood samples were taken from anesthetized SD rats. Serum samples were separated by centrifugation (4°C, 15 minutes, 1500 rpm), processed as supernatant, and stored at -80°C. ELISAs for CK (E-EL-R0274, Elabscience, China), CK-MB (E-EL-R1327, Elabscience, China), and LDH (E-EL-R2547, Elabscience, China) were performed using commercially available kits.

H&E staining: For histological research, heart samples from SD rats were collected and fixed in 4% paraformaldehyde, embedded in paraffin, and cut into 4μm sections. Tissue sections were then prepared and stained with Hematoxylin and Eosin (H&E) to evaluate histopathological changes. The sections were examined and documented through microscopic photography.

ROS staining of myocardial tissue: Heart tissue was embedded in Tissue Tek OCT compound and sectioned continuously to a thickness of 10 μm. The frozen sections were treated with 10 μmol/L DHE (3560, Solarbio, China) and incubated in a humid darkroom at 37°C for 30 minutes. The generation of ROS was signified by the presence of red fluorescence, as observed through a fluorescence microscope.

TTC staining to calculate myocardial infarct size and mass: Blood was collected from the abdominal aorta of anesthetized SD rats. Subsequently, the thoracic cavity was opened and the heart was removed from the left auricle. The heart was then rinsed with ice-cold PBS buffer. After drying the surface moisture of the heart tissue, the heart tissue was preserved. 24 hours post-perfusion, the size of the myocardial infarction was evaluated through staining with 2,3,5-triphenyltetrazolium chloride (TTC) (T8170, Solarbio, China). The procedure involved euthanizing rats and excising their hearts. The left ventricle was then frozen at -80°C for 10 minutes, followed by sectioning transversely into slices 5 μm thick. These slices were incubated at 37°C for 8-10 minutes in 1% TTC dissolved in PBS. Subsequently, the slices were immersed in 4% formaldehyde for 12 hours and photographed.

Images captured by the digital camera were analyzed using Image-Pro Plus V6.0 software to measure the myocardial infarct size statistically. The tissue in the infarcted size is separated and the mass of the infarcted size and the whole heart is weighed separately to calculate the percentage of the infarcted size to whole heart mass.

RESULTS

Myocardial apoptosis: Specify the TUNEL kit used for apoptotic cell detection. DAPI is a nuclear staining that is mainly displayed in blue under a fluorescence microscope. Normal cells emit blue fluorescence, while myocardial apoptotic cells emit green fluorescence. The nucleus of apoptotic cardiomyocytes shows varying degrees of green I staining. Compared with the Sham group, the apoptotic cardiomyocytes in MIRI rats were higher, reaching a statistically significant level; The number of apoptotic cardiomyocytes in the TMZ group was significantly lower than that in MIRI rats; The number of apoptotic cardiomyocytes in the TMZ+DZ group was lower than that in the TMZ group; The number of apoptotic cardiomyocytes in the TMZ+5-D group was higher than that in the TMZ group (Fig. 1). This indicates that TMZ inhibits myocardial cell apoptosis caused by ischemia-reperfusion injury, and this process is related to the mitoKATP signaling pathway.

Content of inflammatory factors IL-6 and TNF-α in myocardial tissues: Inflammation in rats was evaluated by measuring serum levels of IL-6 and TNF-α. Compared with the Sham group, the contents of IL-6 and TNF-α in the myocardial tissues of rats in the MIRI group were significantly increased ($P<0.05$) (Fig. 2). Compared with the MIRI group, the contents of IL-6 and TNF-α in myocardial tissues of rats in the TMZ group decreased ($P<0.05$), and the contents of IL-6 and TNF-α in myocardial tissues of rats in the TMZ+DZ group decreased compared with those in the TMZ group; compared with the TMZ group, the contents of IL-6 and TNF-α in the myocardial tissues of rats in the TMZ+5-HD group increased.

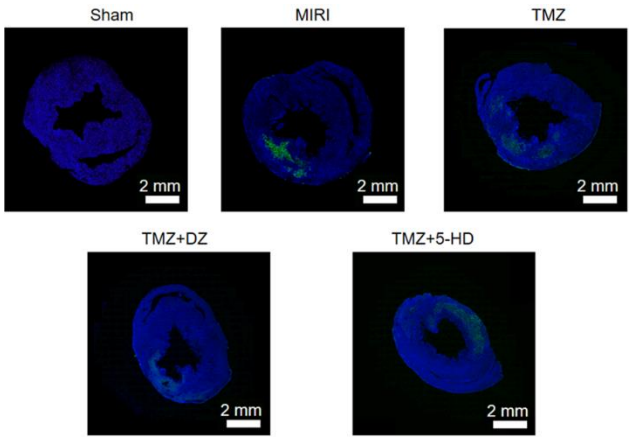

Fig. 1: TMZ monotherapy or combination therapy of TMZ and DZ improved cardiomyocyte apoptosis in MIRI rats. Representative fluorescent images of cardiac slices from the Sham, MIRI, TMZ treated MIRI (TMZ), TMZ+DZ treated MIRI (TMZ+DZ), and TMZ+5-HD treated MIRI (TMZ+5-HD) groups. Scale bar, 2 mm.

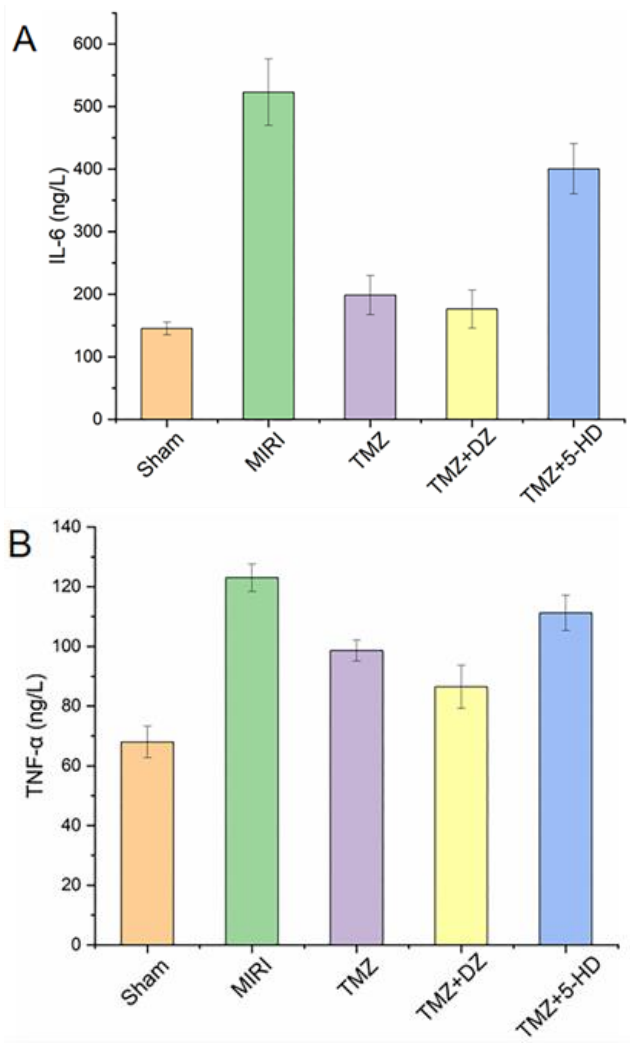

Fig. 2: TMZ monotherapy or combination therapy of TMZ and DZ reduced serum IL-6 and TNF-α levels in MIRI rats. Serum IL-6 and TNF-α levels in the five groups were measured at the end of the experiment. Data are presented as mean ± SD.

Content of serum cardiac enzymes in rats in each group: Compared with the Sham group, the serum levels of CK, CK-MB and LDH in the MIRI group were significantly higher ($P<0.05$). Compared with the MIRI group, the serum levels of CK, CK-MB, and LDH were significantly lower in the TMZ group ($P<0.05$) and the serum levels of CK, CK-MB, and LDH were significantly lower in the TMZ+DZ group compared with the TMZ group ($P<0.05$). The serum levels of CK, CK-MB, and LDH were increased in the TMZ+5-HD group ($P<0.05$) (Table 1).

Table 1: Changes of serum myocardial enzymes CK, CK-MB, and LDH in rats of each group.

Group	CK(U/L)	CK-MB(U/L)	LDH(U/L)
Sham	7.2±0.5	5.7±0.8	356.2±26.3
MIRI	13.1±0.3[a]	16.2±1.5[a]	934.1±76.9[a]
TMZ	9.4±1.1[b]	10.8±1.4[b]	490.0±52.1[b]
TMZ+DZ	7.9±0.1[c]	7.3±2.4[c]14.3±1.7[d]	400.1±32.6[c]
TMZ+5-HD	11.8±1.6[d]		728.7±39.5[d]

Values are presented as mean ± SD. [a]$P<0.05$ vs Sham group. [b]$P<0.05$ vs MIRI group. [c]$P<0.05$ vs TMZ group. [d]$P<0.05$ vs TMZ+DZ.

Myocardial infarct size after ischemia-reperfusion injury: To assess the viability of rat cardiomyocytes, TTC staining was conducted. After TTC staining, the ischemic area was grayish white the normal area was brick red, and the ratio of myocardial infarct size in the five groups of rats was statistically different compared to each other ($P<0.05$). As shown in Fig. 3, myocardial infarct size and infarct mass were increased in the MIRI group compared with the Sham group; myocardial infarct size and infarct mass were decreased in the TMZ group compared with the MIRI group; myocardial infarct size and infarct mass were decreased in the TMZ+DZ group compared with the TMZ group; myocardial infarct sizes and infarct mass were increased in the TMZ+5-HD group compared with the TMZ group (Fig. 3 and Table 2).

Table 2: The effect of trimetazidine on the proportion of infarcted mass and infarcted size (%).

Group	Number of mice	Infarct mass/whole heart mass (%)	Infarct size/whole heart area (%)
Sham	10	0.42±0.37	0.28±0.15
MIRI	10	40.85±2.45	41.25±6.58
TMZ	10	31.18±5.74	31.53±7.37
TMZ+DZ	10	25.64±4.78	25.74±6.39
TMZ+5-HD	10	36.35±6.21	36.89±7.61

Values are presented as mean ± SD.

Histopathology of rat myocardium: According to the results of H&E staining under a light microscope, the myocardial fibers in the Sham group were neatly aligned, the transverse lines were visible, the cell structure was intact, the morphology was normal, there was no inflammatory infiltration, the nuclei were in normal position, and there was no vacuolar degeneration. In rats from the MIRI group, there was a notable decrease in cardiomyocytes, accompanied by a disorganized arrangement of muscle fibers and widespread infiltration by inflammatory cells. Compared with the TMZ group, the myocardial tissue in the TMZ+DZ group had normal morphology and less damage, a slightly disorganized myocardial fiber arrangement, a few fiber breaks, a small amount of inflammatory cell infiltration, and a small amount of cell edema and necrosis. Compared with the TMZ group, the TMZ+5-HD group showed heavier myocardial cell edema and an increased number of neutrophil infiltration (Fig. 4).

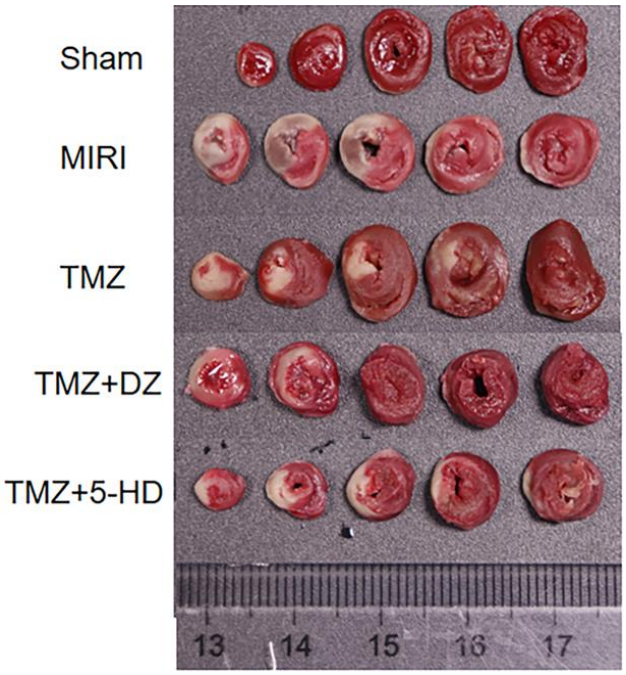

Fig. 3: Effect of TMZ monotherapy or combination therapy of TMZ and DZ or 5-HD on myocardial infarction size in MIRI rats. Representative images of the heart sections of the Sham, MIRI, TMZ- treated MIRI (TMZ), TMZ+DZ- treated MIRI (TMZ+DZ) and TMZ+5-HD treated MIRI (TMZ+5-HD) groups.

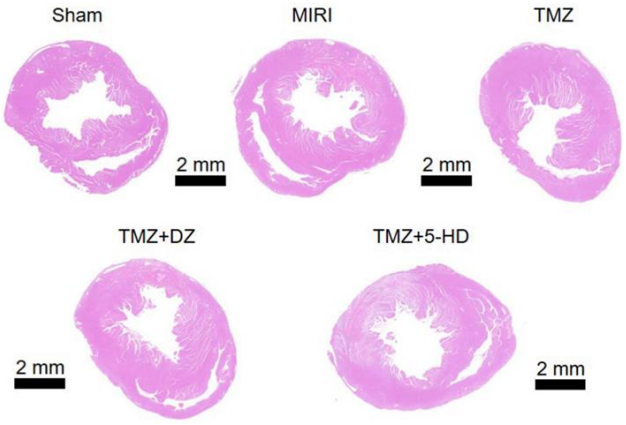

Fig. 4: Staining of myocardial tissue using hematoxylin and eosin. Hematoxylin-Eosin (H & E) staining of heart sections Sham, MIRI, TMZ-treated MIRI (TMZ), TMZ+DZ- treated MIRI (TMZ+DZ) and TMZ+5-HD treated MIRI (TMZ+5-HD) groups. Scale bar, 2 mm.

Myocardial tissue ROS detection: We also investigated the effect of TMZ on ROS in the myocardial tissue of rats, and the results showed that the amount of ROS in myocardial tissue was higher in the MIRI group compared with the Sham group; the amount of ROS in myocardial tissue was significantly lower in the TMZ group compared with the MIRI group; the amount of ROS in myocardial tissue was also somewhat lower in the TMZ+DZ group compared with the TMZ group; however, the amount of ROS in the TMZ+5 -HD group rats had a similar amount of ROS in myocardial tissues as the MIRI group, and the amount of ROS remained high (Fig. 5).

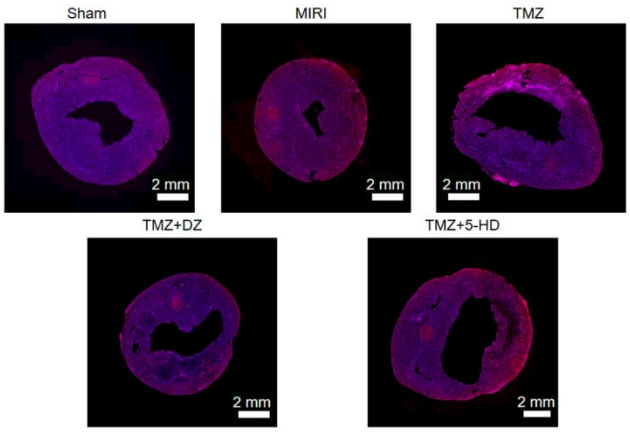

Fig. 5: TMZ monotherapy or TMZ combined with DZ reduced ROS levels in cardiomyocytes of MIRI rats. Representative fluorescent images of ROS assay in Sham, MIRI, TMZ treated MIRI (TMZ), TMZ+DZ treated MIRI (TMZ+DZ), and TMZ+5-HD treated MIRI (TMZ+5-HD) groups. Scale bar, 2 mm.

Note: The content of ROS in myocardial tissue of the MIRI group was higher than that of the Sham group. Compared with the MIRI group, ROS content in the myocardial tissue of the TMZ group was significantly decreased. Compared with the TMZ group, ROS content in myocardial tissue of the TMZ+DZ group was also decreased. The ROS content in the myocardial tissue of the TMZ+ 5-HD group was similar to that of the MIRI group, and the ROS content was high.

DISCUSSION

Ischemic heart disease has been a major cause of death worldwide. The most effective treatment for acute myocardial infarction is to restore perfusion as soon as possible, but the process of reperfusion can also lead to ischemia-reperfusion injury. Continuous myocardial ischemia-reperfusion induces cardiomyocyte death and coronary microvascular injury. Therefore, preventing and reducing MIRI is particularly important (Gerd, 2020; Gerd, 2015). Current clinical medication for myocardial ischemia includes percutaneous coronary intervention (PCI), coronary artery bypass grafting (CABG) operations, and thrombolytic technology, which is utilized to address ischemic heart disease and limit infarct size to salvage the ischemic myocardium 2. These interventions aim to provide timely reperfusion to alleviate the effects of MIRI (Zhuoran *et al.*, 2022). In this study, we found that treatment with trimethyldiazine significantly reduced myocardial infarction area after I/R, while reducing cardiomyocyte inflammation, ROS levels, and cardiomyocyte apoptosis. Interestingly, the combination of TMZ and diazoxide was more effective than TMZ monotherapy in treating MIRI rats.

TMZ is a free fatty acid oxidation inhibitor that can convert heart and muscle metabolism into glucose utilization. Because of its mechanism of action, TMZ has been found to have a cardioprotective effect on patients with angina pectoris, diabetes, left ventricular (LV) dysfunction, and patients undergoing revascularization surgery, without related side effects. In addition, the lack of interference with heart rate, arterial pressure, and most common comorbidities makes TMZ an attractive choice for patients and clinicians (Mario *et al.*, 2019). Diazepam is a benzodiazepine drug used to treat various central nervous system diseases. Research has found that diazepam has a cardioprotective effect on stress-induced cardiac dysfunction. Diazepam can improve the serum levels of creatine kinase, creatine kinase MB, LDH, High sensitivity C-reactive protein, and troponin I in heart disease model rats, and reduce the serum levels of sodium, potassium, calcium and magnesium in diseased animals (Fahad *et al.*, 2020). Hence, the emergence of mitoKATP openers and blockers has provided a more focused and scientific approach to studying MIRIThe emergence of mitoKATP openers and blockers has made the study of MIRI more directional and scientific, providing new targets for the study of anti-myocardial ischemic drugs (Duan and Kasper, 2011). Experiments have shown that mitoKATP not only resists MIRI by reducing calcium overload and apoptosis experiments have shown that mitoKATP not only resists MIRI by reducing calcium overload and apoptosis but also reduces the damage caused by oxygen radical release and protects the mitochondrial respiratory chain (Kvietys and Granger, 2012). The oxygen radicals generated during MIRI cause a cascade of free radicals, which decreases membrane lipid fluidity and increases permeability, leading to mitochondrial swelling, cellular metabolic disorders, and damage. In this study, by constructing an MIRI model, we found that trimetazidine has anti-hypoxia and reoxygenation damage, and this effect can be partially blocked by 5-HD.

Domestic and international experiments have shown that diazoxide and nicorandil, as specific openers of mitoKATP channels, have a significant protective effect on the heart, which can be attenuated by selective inhibitors of mitoKATP (Seidlmayer *et al.*, 2019). Thus, the protective effect of trimetazidine on MIRI in rats may mimic the protective mechanism of its opener on the heart, which may be related to the maintenance of proper mitochondrial volume, inhibition of mitochondrial calcium overload during reperfusion, and alteration of cellular ROS production (Pell *et al*., 2018). The present experimental study, based on the demonstration of the protective effect of trimetazidine on MIRI in rats, only preliminarily explored whether this effect is related to mitoKATP channels, and further experimental studies are needed to explore and verify the specific protective mechanism.

This study has several limitations. While we observed that the combination of Trimetazidine with Diazepam (TMZ+DZ) exhibited superior therapeutic effects compared to other combinations, future research could investigate additional drug combinations. This could include interactions with other cardiovascular drugs or agents known to counteract oxidative stress, inflammation, or cellular apoptosis in MIRI. Additionally, while this study highlighted the involvement of the mitoKATP signaling pathway, further molecular insights are necessary. This could entail comprehensive genomic, proteomic and metabolomic studies to delineate and quantify the broader cellular and molecular alterations associated with the use of Trimetazidine and its combinations. Lastly, translating these findings from rat models to clinical trials represents a critical next step. Clinical studies should evaluate the efficacy and safety of

Trimetazidine and its combinations in companion animals with MIRI, comparing these outcomes to standard care. Pursuing these research directions not only builds upon the current findings but also opens new pathways for enhancing our understanding and treatment of myocardial ischemia-reperfusion injury.

Conclusions: In conclusion, our study provides compelling evidence that Trimetazidine, especially when combined with DZ, significantly attenuates myocardial ischemia-reperfusion injury in rats through a mechanism involving the mitoKATP signaling pathway. These findings offer valuable insights into the therapeutic potential of TMZ+DZ in the treatment of MIRI, highlighting the importance of targeting mitochondrial pathways to optimize myocardial energy metabolism and reduce oxidative stress for myocardial protection. Further research is warranted to explore the clinical applications of these findings in the treatment of MIRI.

Data availability statement: Data for this study is available from the corresponding author upon reasonable request.

Funding: This work was self-funded.

Conflicts of interest: The authors declare that there is no conflict of interest regarding the publication of this paper.

Authors contributions: Xiaoqi Wang, Guohai Su, and Keqing Hu participated in the design of this study, and Zhongyang Xu and Xiaobin Guo performed the statistical analysis. Yan Zhuang carried out the study and collected background information. Xiaoqi Wang drafted the manuscript. All authors read and approved the final manuscript.

Ethics statement: The study was approved by the Ethics Committee of Central Hospital Affiliated to Shandong First Medical University (No.20230130-002).

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