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Dimethyl malonate inhibit autophagy of cardiomyocytes after Myocardial infarction via AKT /mTOR pathway

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Abstract: *Although interventional coronary reperfusion strategies have been widely and successfully applied, the incidence and mortality associated with acute myocardial infarction (AMI) remain high. Myocardial infarction continues to be the primary cause of heart failure. The present study hypothesizes that dimethyl malonate (DMM) exhibits a cardioprotective effect in cases of acute myocardial infarction (AMI), with the underlying mechanism potentially involving the inhibition of autophagy and reduction in the production of reactive oxygen species. The initial investigation delved into the impact of DMM on myocardial infarction size and cardiac function in a murine model with left anterior descending (LAD) coronary artery ligation, furthermore, it explored the potential pathway through which DMM exerts its inhibitory effect on autophagy.*

Keywords: *Dimethyl malonate; Autophagy; Cardiomyocytes; Myocardial infarction; AKT/mTOR pathway*

1. Introduction

Myocardial infarction (MI), an irreversible injury typically resulting from coronary plaque rupture, precipitates the formation and occlusion of coronary arteries. Consequently, cardiovascular diseases (CVDs), with MI being a prominent example, have emerged as the leading cause of mortality globally to date. [1] Globally, it accounts for over a third of all fatalities. The sole viable means of salvaging ischemic myocardium in acute myocardial infarction (AMI) entails prompt reperfusion strategies, encompassing drug thrombolysis and percutaneous coronary intervention (PCI). [2, 3] Nonetheless, reperfusion, while averting ischemic myocardial infarction, can also inflict distinct, additional, and irreversible damage. Despite the continuous refinement of coronary reperfusion intervention methods, the morbidity and mortality rates associated with AMI remain substantial. [4] Consequently, cardiac protection strategies, encompassing measures aimed at preventing, mitigating, and repairing damage to the myocardial tissue, are imperative.Autophagy, an evolutionarily conserved intracellular degradation process that maintains cell homeostasis by removing damaged proteins and renewing organelles, has been shown to play a wide range of physiological and pathophysiological roles in a variety of cells and tissues[5]. Poor autophagy is associated with many heart diseases, including dilated cardiomyopathy, ischemic heart disease, and heart failure.[6, 7] Our research has conclusively demonstrated that dimethyl malonate exhibits a potent inhibitory effect on excessively activated autophagy, thereby exerting a protective function in the aftermath of myocardial infarction.

Dimethyl malonate (DMM) serves as a reversible inhibitor of succinate dehydrogenase (SDH) and is an endogenously produced molecule that is readily metabolized. During reperfusion, the reintroduction of oxygen to ischemic tissue can inflict severe damage on the heart. Characterized by a surge of reactive oxygen species (ROS) within mitochondria, this damage initiates a cascade of events ultimately resulting in cardiomyocyte death due to ischemia/reperfusion (I/R) injury. Numerous studies have demonstrated the potent therapeutic effects of DMM in ischemia/reperfusion models, specifically its ability to prevent the accumulation and oxidation of succinate during I/R, consequently diminishing ROS production. Nevertheless, the precise role of DMM in modulating cellular senescence processes during cardiac injury and senescence remains elusive.This study shows that DMM can inhibit over-activated autophagy after myocardial infarction, thus playing a protective role.

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2. Materials and methods

2.1 Cell culture

H9C2 cells were procured from the Cell Bank of the Chinese Academy of Sciences, located in Shanghai, China. The cells were maintained in Gibco Dulbecco Modified Eagle Medium (DMEM), enriched with 10% fetal bovine serum and 1% penicillin/streptomycin solution, within a 37 °C incubator maintained at 5% CO2.

2.2 Myocardial infarction

Male C57BL/6 mice were randomly assigned to three groups: the SHAM group (sham-operated), the MI group (myocardial infarction group), and the MI+DMM group (receiving DMM treatment after myocardial infarction). Myocardial infarction (MI) was induced in mice by ligation of the anterior descending branch of the left coronary artery. The experimental model was established as described below: Mice were anesthetized via inhalation of 4-5% isoflurane, and subsequently underwent permanent ligation of the left anterior descending (LAD) coronary artery to induce myocardial infarction. Following this, the ribs were sutured together to close the chest wall incision and the overlying skin. Finally, the mice were placed on a heating pad until full recovery. The MI+DMM group (receiving surgical administration) received daily intraperitoneal injections of DMM at a dosage of 300 mg/kg, with the initial injection administered within 1 hour post-surgery. Heart tissues from mice in each group were harvested and subjected to analysis.

2.3 Echocardiography

One week post-myocardial infarction modeling in mice, cardiac function was assessed using a mouse cardiac ultrasound system. The specific steps were as follows: Mice were anesthetized with isoflurane gas, administered according to their body weight, and cardiac activity was induced by adjusting the isoflurane concentration. The heart rate was maintained between 450 and 500 beats per minute (bpm). After removing hair from the chest region of the mice using hair removal cream, the mice were positioned on the operating table. A small amount of ultrasonic gel was applied, and the probe was used to acquire images of the long- and short-axis sections of the heart for analysis. M-Mode ultrasound images were captured after five cardiac cycles. Using the corresponding software, left ventricular end-systolic diameter (LVIDs), left ventricular end-diastolic diameter (LVIDd), and left ventricular ejection fraction (LVEF) were measured, along with other relevant indicators.

2.4 Western Blot

Add the same amount of sample to be measured in the hole of SDS-PAGE gel, place the entire electrophoresis stand in the electrophoresis buffer system at a constant voltage of 80 V, and stop electrophoresis when the lowest marker reaches the bottom of the stand. Under low temperature conditions, the constant flow is set to 300 mA, and the membrane is blocked with 5% milk after 1.5 hours of transfer. Then, the corresponding bands are cut out according to the location of the target protein and incubated in the primary antibody solution for 16 hours. The membranes are then placed in a box containing goat anti-rabbit secondary antibody solution and incubated for 2 hours. ECL exposure solution is added to the membrane to avoid light, incubated for 10 seconds, and then exposed in the gel imager.

2.5 cck-8

The CCK-8 kit was purchased from Biyuntian (Jiangsu, China) for the detection of cell viability. The measurement procedure was carried out strictly according to the manufacturer's instructions. Cardiomyocytes were inoculated into 96-well plates at a density of 1×10^4 cells/well, and when the cell fusion was observed to reach 60%-70%, different concentrations of DMM (2.5 uM, 5 uM, 7.5 uM, 10 uM) were added to each well for 24 hours. Subsequently, 10 μL of CCK-8 solution was added to each well of the 96-well plate, following the manufacturer's instructions, and the absorbance was measured at 450 nm after incubation at 37 °C for 2 hours.

2.6 DCFH-DA reactive oxygen species detection was co-stained with Hoechst

1) In an environment protected from light, the supernatants of different treatments were removed and

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rinsed with sterile PBS buffer solution. Subsequently, the cells were diluted using DCFH-DA stock solution and added to the cell culture plate for incubation. Following incubation, they were aspirated and washed with DMEM three times before being placed under a fluorescence microscope for observation.

2) In a dark environment, the cells stained with DCFH-DA were gently washed twice with sterile DMEM. Subsequently, the Hoechst dye solution was diluted in DMEM at a 1:1000 ratio and added to the cell culture plate. The cells were then incubated in an incubator at 37 °C for 10 minutes. Following incubation, the dye was aspirated, and the cells were washed with DMEM three times before being placed under a fluorescence microscope for observation.

2.7 2,3,5-Triphenyltetrazolium Chloride (TTC) Staining

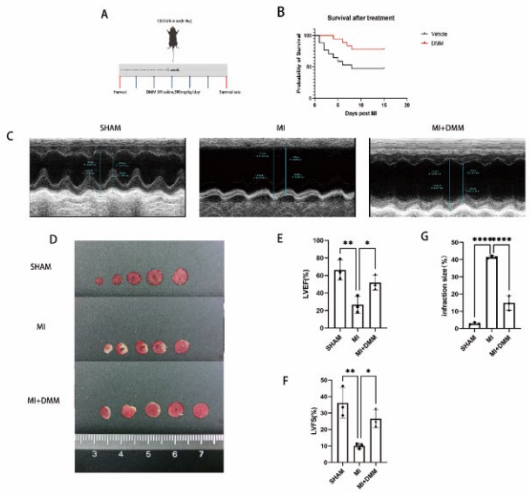
To evaluate the change in the size of the ischemic zones, 2,3,5-Triphenyltetrazolium Chloride (TTC) staining was applied. The heart tissue was cut into 1mm thick slices, and the brain tissue was gently transferred to a petri dish containing TTC dye for 30 minutes at 37 °C. TTC marks undamaged tissue, rendering the infarct area white. As previously reported, after fixation in 4% formaldehyde solution for two hours, the stained sections were scanned and photographed for further ImageJ software analysis.

2.8 Statistical analysis

When comparing two sets of measurement data, the t-test is used if the data being analyzed are normal. If the data are non-normal, a non-parametric test is employed. When comparing multiple sets of measurement data, Analysis of Variance (ANOVA) is used for data that are normally distributed, and non-parametric testing is utilized if the data are not normally distributed. A P-value less than 0.05 was considered statistically significant. Data analysis was performed using GraphPad Prism v8.0 software.

3. Results

3.1 DMM improves cardiac function after myocardial infarction in vivo



(A) Experimental Timeline for the Induction and Assessment of Myocardial Infarction in Mice. Subsequently, (B) Survival Curve Analysis of AMI Mice Demonstrating Statistical Significance ($P < 0.05$) was performed to evaluate the impact of the intervention. (C) Representative Left Ventricular Short-Axis Color Ultrasound Images of Mice from Each Group Following Myocardial Infarction were acquired to visualize cardiac function. (D) Comprehensive Observation and Tetrazolium Chloride (TTC) Staining of Hearts from SHAM, MI, and MI+DMM Groups provided insights into the structural changes. (E, F) Comparative Analysis of Left Ventricular Ejection Fraction (LVEF) and Left Ventricular Short Axis Shortening Rate (LVFS) Across Groups (n=3), Presented as Mean ± SD. Asterisks (, **, ***) Indicate Statistically Significant Differences at $P < 0.05$, $P < 0.01$, and $P < 0.001$ Respectively. Additionally, (G) Statistical Evaluation of Cardiac Tetrazolium Chloride (TTC) Staining Among SHAM, MI, and MI+DMM Groups (n=3), Reported as Mean ± SD.*

Figure 1:The effect of DMM on survival rate and cardiac function in mice with acute myocardial infarction

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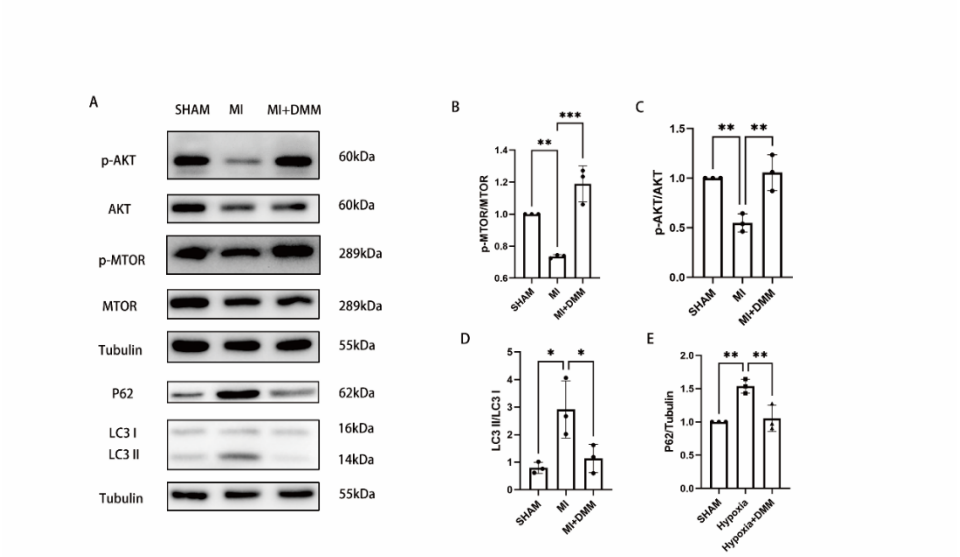
To further demonstrate the role of DMM in prognosis after acute myocardial infarction, we recorded mortality after MI in C57BL/6 mice. C57BL/6 mice of 6-8 weeks were used for the experiment. The mice were randomly divided into SHAM group, MI group, and MI+DMM group. In the MI+DMM group, DMM was injected continuously for 7 days after MI. The survival curve of MI mice was worse than that of SHAM group mice and mice treated with DMM (Figure 1A and 1B). One week after MI, echocardiography was used to assess cardiac function in mice. The results showed (Figure 1C, 1E and 1F) that compared with the SHAM group, myocardial contractility and ventricular wall motion amplitude of mice 7 days post-MI were reduced, and ventricular eject fraction (LVEF) and left ventricular short axis shortening rate (LVFS) were significantly decreased, while the LVEF and LVFS of mice treated with DMM were increased. It is suggested that DMM can improve the cardiac function of mice after myocardial infarction. TTC staining was used to evaluate myocardial infarction size in each group. The results showed that the mean infarct size of mice treated with DMM was smaller than that of mice in the MI group (Figure 1D and 1G).

3.2 In vivo, DMM effectively inhibited AKT/mTOR-mediated autophagy in mice subsequent to myocardial infarction

Western blot analysis was conducted to evaluate the in vivo protective effect of DMM on mice after acute myocardial infarction, focusing on the AKT/mTOR-mediated autophagy pathway (Figure 2A). The results revealed that, in comparison to the control group, the myocardial infarction group exhibited elevated levels of autophagy-related proteins LC3-II/I and P62, while displaying downregulated ratios of p-AKT/AKT and p-mTOR/mTOR. In the post-myocardial infarction mouse group, DMM treatment effectively reversed this trend, as evident from Figures 2B, 2C, 2D, and 2E. Collectively, these findings suggest that DMM exerts a protective effect in mice with acute myocardial infarction, potentially through the inhibition of autophagy mediated by the AKT/mTOR pathway



DMM mediates autophagy in mice post-myocardial infarction via the activation of the AKT/mTOR signaling pathway.

***(A)** Representative Western blot gel images illustrating the in vivo autophagy flux pathway proteins mediated by AKT/mTOR. **(B, C, D, E)** Histograms depict the quantification of protein levels in each group (n = 3), presented as mean ± SD. Asterisks (*, **, ***) indicate statistically significant differences at $p < 0.05$, $p < 0.01$, and $p < 0.001$, respectively.*

Figure 2:The effect of DMM on AKT/mTOR mediated autophagy pathway after myocardial infarction in mice

3.3 In vitro, DMM significantly enhanced the cellular activity of H9C2 cells following exposure to hypoxia

CCK-8 assays were conducted with H9C2 cells to determine optimal drug concentrations. The experimental results demonstrated that cell viability, following DMM pretreatment, was significantly elevated compared to the hypoxia group without DMM treatment (Figure 4A), suggesting a protective

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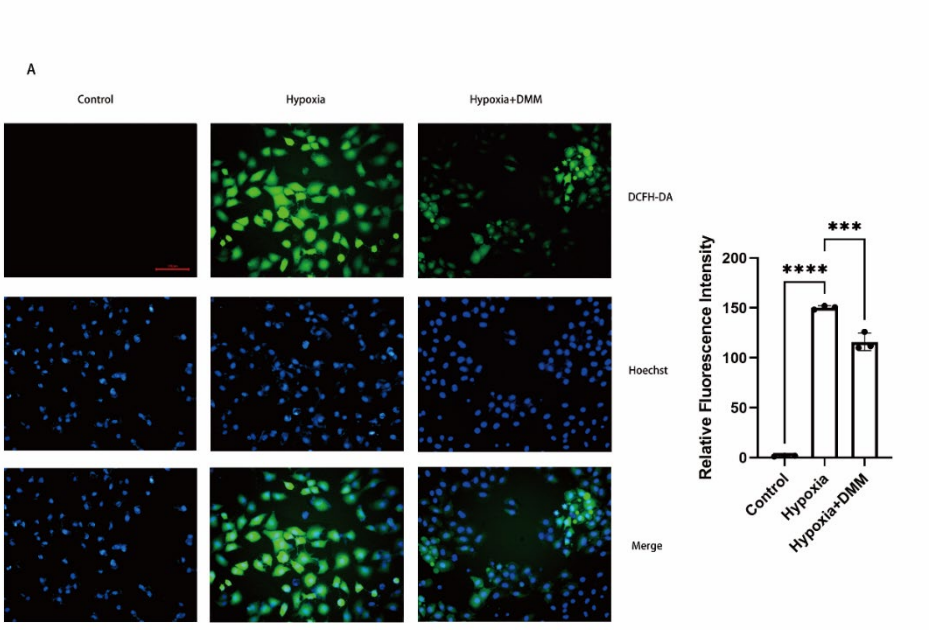
role of DMM against acute myocardial infarction in vitro. Numerous literature studies have reported that excessive ROS production can inflict severe damage on biomolecules [8]. Based on this premise, we performed DCFH-DA staining to assess reactive oxygen species (ROS) levels in H9C2 cells. The staining results (Figure 3A) revealed that in the absence of hypoxia-induced damage, the ROS content in H9C2 cells was minimal, whereas significant ROS accumulation was observed after 24 hours of hypoxia. Notably, DMM pretreatment significantly mitigated this ROS accumulation. Collectively, these findings suggest that DMM preconditioning can alleviate oxidative stress associated with acute myocardial infarction, thereby exerting a protective effect.



The histogram precisely quantifies the fluorescence intensity levels across each experimental group (n = 3), with mean ± SD values presented.
(A) Illustrative depiction of DCFH-DA reactive oxygen species staining outcomes in various groups of H9C2 cells. Asterisks (, **, ***) denote statistical significance at $p < 0.05$, $p < 0.01$, and $p < 0.001$ levels, respectively, indicating statistically significant differences among groups.*

Figure 3:The effect of DMM pretreatment on ROS levels in H9C2 cells after hypoxia

3.4 The cardioprotective effects of DMM are mediated through the downregulation of autophagy in vitro

To further elucidate the potential protective mechanism of DMM in models of acute myocardial infarction, we assessed the protective effect of DMM on hypoxia-induced H9C2 cells via western blot analysis of the AKT/mTOR-mediated autophagy pathway (Figure 4B, 4E). The results of western blot analysis indicated that LC3II/I and P62 levels were significantly elevated, whereas the ratios of p-AKT/AKT and p-mTOR/mTOR were significantly diminished in the hypoxia group in comparison to the control group. DMM pretreatment was able to reverse this trend, as evident from Figures 4C, 4D, 4F, and 4G. Based on the aforementioned findings, we hypothesize that DMM exerts a protective effect on hypoxia-induced H9C2 cells through inhibition of the AKT/mTOR-mediated autophagy pathway.

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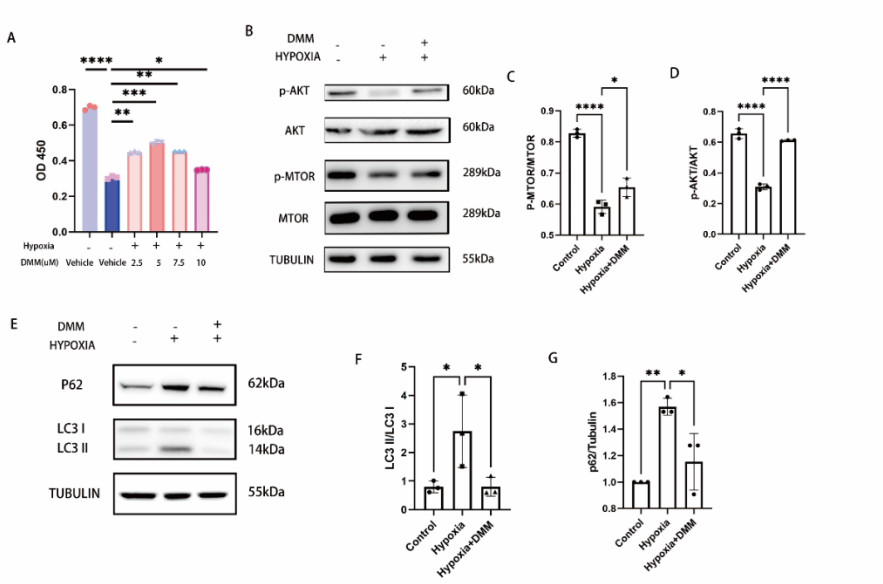

DMM mediates H9C2 autophagy via the activation of the AKT/mTOR pathway.
(A,B) Western blot gel images of in vitro AKT/mTOR-mediated autophagy flux pathway proteins.
(C,D,F,G) The histogram depicts the quantification of protein levels in each group (n = 3). Mean ± SD.
**p < 0.05, **p < 0.01, ***p < 0.001 indicate statistically significant differences.*

Figure 4:The effect of DMM on hypoxia induced autophagy in H9C2 cells

4. Discussion

Dimethyl malonate serves as a competitive inhibitor of SDH, suppressing the accumulation of succinate during ischemia and its subsequent oxidation during reperfusion, consequently mitigating mitochondrial ROS production and IR-induced damage[9]. The myocardial infarction model was established in c57bl/6 mice through ligation of the left anterior descending coronary artery (LAD). Following the successful establishment of this model, the mice were subjected to DMM injection. Cardiac ultrasound, in conjunction with TTC staining, demonstrated the protective effect of DMM on mice post-myocardial infarction. Thus, the question arises: What is the underlying mechanism responsible for the protective effects of DMM?

Autophagy is widely regarded as an adaptive cellular response to stress, safeguarding cellular homeostasis through the elimination of damaged proteins and intracellular organelles[10]. Autophagy provides protection to cells against a wide range of insults. Nevertheless, excessive or dysfunctional autophagy can be detrimental to cells, resulting in the accumulation of autophagy products, including autophagosomes, and the degradation of vital proteins and organelles essential for cell survival[11]. Our study suggests that the protective or detrimental effects of autophagy on heart tissue are contingent upon the magnitude of induction and the duration of the insult. In the context of ischemia, overactivation of autophagy can induce myocardial cell injury and expedite myocardial cell death[12]. Oxidative stress and autophagy activation are recognized as key contributors to cardiac dysfunction, which is intricately linked to cardiomyocyte death and heart injury. In this study, we have demonstrated that DMM effectively mitigates the overactivation of autophagy and diminishes the production of reactive oxygen species in the context of acute myocardial infarction.

AKT, a serine/threonine kinase, serves as a proto-oncogene crucial in regulating diverse cellular functions, encompassing metabolism, growth, proliferation, survival, transcription, and protein synthesis. As a downstream effector of AKT, mTOR is implicated in autophagy regulation[13]. In this study, our findings demonstrate that DMM effectively activates the AKT/mTOR pathway in both hypoxia-induced cell models and MI mice, evidenced by the elevated pAKT/AKT and pmTOR/mTOR ratios[14, 15]. This suggests that DMM can hinder AKT/mTOR-mediated autophagy via activates the AKT/mTOR pathway, thereby exerting a protective effect in mice with acute myocardial infarction[16].

In conclusion, our study underscores the significant potential of DMM to mitigate damage, enhance heart function, and diminish myocardial infarction size in MI mice through the inhibition of the AKT/mTOR-mediated autophagy pathway. We suggest that DMM holds promise as a potential

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therapeutic option for patients with acute myocardial infarction.

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