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# Muscone ameliorates myocardial ischemia–reperfusion injury by promoting myocardial glycolysis

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ABSTRACT

*Objective:* The incidence of acute myocardial infarction (AMI) is increasing yearly. With the use of thrombolysis or percutaneous coronary intervention (PCI), the mortality rate of acute myocardial infarction has been significantly reduced. However, reperfusion can cause additional myocardial injury. There is still a lack of effective drugs to treat I/R injury, and it is urgent to find new therapeutic drugs.

*Methods:* In this study, network pharmacology was used to predict potential targets and biological processes involved in Muscone-mediated treatment of acute myocardial infarction. To model ischemia–reperfusion injury, a hypoxia-reoxygenation model and *in vivo* ischemia–reperfusion injury C57BL/6 mice model was constructed. Mice were treated with Muscone i.p. for 4 weeks. We detected the cardiac function on day 28.The expression levels of the apoptotic proteins Caspase-3 and Bax and the anti-apoptotic protein Bcl-2 were detected by immunoblotting after Muscone treatment of AC16 cells and *in vivo*. Additionally, the gene expression levels of the PUMA and p53 were analyzed by qRT–PCR. Molecular docking was used to evaluate the binding energy between Muscone and NLRP3-related proteins. Immunoblotting and qRT–PCR were used to assess the expression levels of NLRP3 signaling pathway-related proteins (NLRP3, ASC, and Caspase-1) and the NLRP3 gene, respectively. Moreover, the extracellular acidification rate of AC16 cells was measured using the Seahorse system to evaluate glycolysis levels after Muscone treatment. The expression of the key glycolytic enzyme PKM2 was analyzed by immunoblotting and qRT–PCR. Finally, ChIP–qPCR was performed to determine the levels of histone modifications (H3K4me3, H3K27me3, and H2AK119Ub) in the PKM2 promoter region.

*Results:* GO functional enrichment analysis revealed that muscone was involved in regulating the biological processes (BP) of AMI, which mainly included negative regulation of the apoptosis

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signaling pathway, the response to lipopolysaccharide, and blood pressure regulation. The cellular components (CC) involved in muscone-mediated regulation of AMI mainly included lipid rafts, membrane microdomains, and membrane regions. The molecular functions (MF) involved in muscone-mediated regulation of AMI mainly included oxidoreductase activity, nuclear receptor activity, and transcription factor activity. In vitro results indicated that muscone treatment could inhibit the expression levels of Bax and Caspase-3 in AC16 cells after ischemia–reperfusion while increasing the expression level of the antiapoptotic protein Bcl-2. Muscone significantly suppressed the transcription levels of p53 and PUMA in AC16 cells. Molecular docking suggested that muscone could bind well with the Cryo-EM structure of NEK7(PDB ID:6NPY). Further investigation of inflammatory pathways revealed that muscone could inhibit the expression level of NLRP3 in AC16 cells and reduce the expression levels of Caspase-1 and Caspase recruitment domain. Fluorescent quantitative PCR experiments showed that muscone significantly inhibited the transcription of NLRP3. Moreover, we found that muscone could enhance the glycolytic efficiency of AC16 cells, which may be related to the increased protein expression of PKM2 in AC16 cells. Fluorescent quantitative PCR showed that muscone could increase the transcription level of PKM2. Chromatin immunoprecipitation assays showed that muscone treatment increased the expression level of H3K4me3 in the PKM2 promoter region and inhibited the levels of H3K27me3 and H2AK119Ub in the PKM2 promoter region.
*Conclusion:* Muscone promoted myocardial glycolysis and inhibited NLRP3 pathway activation to improve myocardial ischemia–reperfusion injury.

## 1. Introduction

Acute myocardial infarction (AMI) is a major cause of morbidity and mortality in the elderly population worldwide. The occurrence of AMI is mainly attributed to coronary artery occlusion and subsequent interruption of blood flow. Currently, thrombolytic therapy and percutaneous coronary intervention (PCI) are the primary clinical treatments for AMI [1]. The widespread use of these two techniques and the early restoration of myocardial perfusion are the most effective methods for reducing infarct size and improving clinical outcomes. However, restoring ischemic myocardial blood flow may cause additional myocardial injury, which is associated with reperfusion-induced oxidative stress and is known as ischemia–reperfusion injury (I/R) [2]. Studies have shown that I/R injury can increase cardiomyocyte death and expand the area of myocardial infarction [3]. However, there is currently a lack of FDA-approved drugs to reduce reperfusion injury in clinical practice, highlighting the urgent need to identify new therapeutic drugs.

In recent years, active monomers in traditional Chinese medicine have received much attention and are expected to become new drugs for clinical treatment. Musk, a highly valued traditional Chinese medicine, has been widely used in the clinic to relieve symptoms of myocardial ischemia [4]. Musk-Bolus has a significant anti-inflammatory effect. Muscone, which is one of the main active monomers of musk, has been reported to improve ventricular remodeling after myocardial infarction [5] and exerts anti-inflammatory effects on various chronic diseases, such as intervertebral disc degeneration [6]. Our previous research showed that muscone could improve ventricular remodeling after myocardial infarction [7,8], which is related to inhibition of the oxygen-free radical-mediated proinflammatory process in macrophages [5]. However, there have been few reports on the protective effect of muscone against myocardial damage caused by ischemia–reperfusion injury, and the regulatory mechanism is not yet clear.

Similar to myocardial infarction, ischemia/reperfusion (I/R) injury can trigger local inflammation in the myocardium. Studies have shown that a moderate inflammatory response in the early stages of myocardial infarction may be beneficial for promoting myocardial repair. However, excessive and sustained chronic inflammation can worsen ventricular remodeling and lead to decreased cardiac function. These excessive proinflammatory responses may be due to an increase in oxidative stress after I/R injury, and elevated levels of reactive oxygen species (ROS) within cardiomyocytes can damage mitochondrial cristae and membranes, impairing oxidative phosphorylation within the cell [9]. Inflammasomes are multiprotein complexes within cells that are activated by various endogenous and exogenous stimuli, such as lipopolysaccharides and glycolysis. Inflammasome activation enhances the maturation and expression of proinflammatory cytokines such as interleukin-1β and interleukin-18, inducing multiorgan inflammation. Inflammasome-mediated apoptosis has been demonstrated to participate in the pathological process of myocardial infarction [10,11]. NLR family pyrin domain-containing protein 3 (NLRP3) is a key member of the inflammasome. NLRP3 recruits the caspase recruitment domain-containing apoptosis-associated speck-like protein containing a CARD (ASC) and cysteine aspartic acid-specific protease 1 (Caspase-1) proteins, thereby increasing the release of proinflammatory factors [12]. Our previous report showed that muscone inhibited the activation of local NLRP3 inflammasomes after myocardial infarction. However, it remains unclear whether muscone inhibits the activation of NLRP3 inflammasomes in myocardial cells after I/R injury and the specific regulatory mechanisms involved, which require further investigation.

Inflammasome activation after myocardial infarction is highly dependent on altered metabolic processes in cardiomyocytes. Hypoxic-ischemic conditions after myocardial infarction shift cardiomyocytes toward fatty acid utilization, leading to a change from glucose to fatty acid metabolism. Enhancing glycolysis can mitigate cardiomyocyte apoptosis and reactive oxygen species levels after I/R injury and suppress inflammation. Inhibiting glycolysis with small molecule drugs can exacerbate myocardial injury and reduce cardiac function after I/R injury [13]. This finding suggests that glycolysis protects myocardial cells against reperfusion injury after myocardial infarction, which may be related to the inhibition of inflammation [14]. However, it is currently unknown whether the

regulation of NLRP3 inflammasome activation by muscone is due to its effects on glycolysis in cardiomyocytes. Further studies are required to elucidate this mechanism.

Network pharmacology uses multidisciplinary theories such as systems biology and polypharmacology to reveal the principles and laws of interactions among organisms and drugs based on the analysis, visualization, and interpretation of biological network data, the construction and analysis of biological networks, and other related technologies and methods [15]. Molecular docking is a method for drug design based on the characteristic structure of the interactions between receptors and ligands, which involves simulating the binding state between receptors and ligands to identify the optimal binding state [16]. The aim of this study was to clarify whether muscone promotes activation of the NLRP3 pathway and its regulatory mechanisms following myocardial reperfusion injury. We predicted the potential targets and biological processes of muscone in the treatment of acute myocardial infarction using network pharmacology and validated the findings by treating the human myocardial cell line AC16 with muscone and further examining the expression of NLRP3 signaling pathway-related proteins and the level of histone modification in the NLRP3 promoter, as well as the level of glycolysis in myocardial cells.

The present study aimed to elucidate whether muscone facilitates activation of the NLRP3 signaling pathway in cardiomyocytes following reperfusion injury and its underlying regulatory mechanisms. We treated the human cardiomyocyte cell line AC16 with muscone, examined the protein expression of NLRP3 signaling pathway components and the histone modification levels of the NLRP3 promoter, and assessed the glycolytic activity of cardiomyocytes. These findings may provide new therapeutic targets and strategies for the clinical prevention and treatment of myocardial infarction.

## 2. Material and methods

### 2.1. Section on network pharmacology

#### 2.1.1. Acquisition of muscone target

The PubChem database (https://pubchem.ncbi.nlm.nih.gov/) was used to obtain the 3D structure of the Muscone compound in SDF format and its Canonical SMILES structure. The obtained structures were imported into the TargetNet database (http://targetnet.scbdd.com/calcnet/index/) to obtain predicted target proteins of Muscone [15,16]. The target proteins were filtered based on the probability score (>0) to obtain the Muscone target proteins.

#### 2.1.2. Identification of Potential Targets for Musk Ketone in Treating AMI

We identified Potential Targets for Musk Ketone in Treating AMI Utilizing the GeneCards database (https://www.genecards.org/), we performed a search with the key term “acute myocardial infarction” to retrieve relevant targets associated with AMI. Subsequently, we employed the online Venn diagram generation tool (http://www.bioinformatics.com.cn/login/) to create a Venn diagram for the targets of musk ketone as mentioned in section 1.4 and the AMI-related targets. The intersecting targets obtained represent the potential targets of musk ketone in the context of AMI.

### 2.2. Myocardial ischaemic reperfusion injury (MIRI) surgery and muscone treatment

8- week old male C57BL/6 mice were purchased from GemPharmatech (Nanjing, China) and Ischemia−reperfusion injury mice model were contructed as previous described [17].All mice were anesthetized with inhaled ether (3 min) and fixed for endotracheal intubation by the use of a small animal respirator. A longitudinal incision was made from the third to fourth ribs, exposing the heart. Then, a Prolene suture was placed around the 2 cm of the root of left anterior descending coronary artery (LAD). The suture was loosened after occlusion for 30 min, which was followed by 120-min reperfusion of LAD.Mice in sham group received the same procedure except for ligation. Muscone was administrated once a day at a dose of 2 mg/kg by gavage according to previous study [5]. All mice were bred in SPF animal conditions and housed in mouse rooms maintained at 23 °C. Our experiment was approved by Nanjing and Jiangnan Animal Experimental Ethics Committee.

### 2.3. Color Doppler echocardiography

Mice were depilated at the chest and anesthetized with 3 % sodium pentobarbital at 40 mg/kg. At the 4th week after MI, cardiac function was evaluated by the high-frequency ultrasound imaging system (Vevo 2100, Visual Sonics, Toronto, Canada) equipped with a 30-MHz transducer as described previously [18]. Left ventricular ejection fraction (LVEF) and left ventricular shortening fraction (LVFS) of each mouse were calculated as previously described [18].

### 2.4. Cell culture and treatment

The human cardiac myocyte cell line (AC16) was obtained from Shanghai Zhongqiao XinZhou Biotechnology Co., Ltd. The cells were cultured in high-glucose DMEM medium supplemented with 10 % fetal bovine serum (FBS, Invitrogen, Australia), 100 U/ml penicillin, and 100 μg/ml streptomycin at 37 °C in a humidified 5 % CO2 atmosphere. When the cells reached 80 % confluence, they were subcultured at a 1:3 ratio using trypsin (Gibco, Carlsbad, CA, USA).

### 2.5. In vitro myocardial ischemia-reperfusion cell model

In this study, a myocardial cell ischemia-reperfusion model was established using a basic culture medium without fetal bovine serum to simulate the ischemic microenvironment, and a hypoxic environment was simulated using 95 % N2 and 5 % CO2. Specifically, the AC16 cells were cultured to 80–90 % confluence, and the original culture medium was removed. The cells were then washed three times with PBS and the washing liquid was removed. Next, a basic culture medium without fetal bovine serum was added, and the cells were placed in a hypoxic apparatus (three-gas incubator) and exposed to a mixture of 95 % N2 and 5 % CO2. After 30 min, the air flow was stopped, and the culture box was sealed for 24 h. After 24 h, an equivalent volume of culture medium containing 10 % fetal bovine serum was replaced, and the cells were continued to be cultured for 1 h.

### 2.6. Enzyme-linked immunosorbent assay (ELISA)

Enzyme-linked immunosorbent assay were operated as previous described [5]. Serum samples of mice from each group were collected at the 4th week after I/R injury. Serum level of brain natriuretic peptide (BNP) and creatine kinase (CK-MB) in mice were quantified using ELISA kits (YIFEIXUE BIO TECH, China) according to the manufacturer's instructions.

### 2.7. Determination of lactate acid and ATP levels in heart tissue

Lactate Acid, and ATP Detection Kits were got from Nanjing Jiancheng Bioengineering Institute. Fresh heart tissues were collected from sham, I/R + DMSO and I/R + Muscone mice, and the levels of each metabolite were determined following the manufacturer's instructions provided with each respective kit and previous described [19].

### 2.8. Cell viability assays

CCK8 assay were performed as previous described [5]. To evaluate the protective effect of Muscone towards AC16 cells, the Enhanced Cell Counting Kit-8 (Biosharp, China) was used according to the manufacturer's instructions. AC16 were seeded in a 96-well plate at $2 \times 10^4$ cells/well and cultured to adherence. Then the culture medium was replaced by DMEM medium alone and AC16 cells were treated with muscone.Cells were then constructed *In vitro* ischemia-reperfusion cell model as previous described. Subsequently, 10 μl enhanced CCK-8 solution was added to each well and the absorbance at 450 nm was measured 2 h after incubation.

### 2.9. Lactate dehydrogenase (LDH) assay

Lactate dehydrogenase (LDH) assay was preformed as previous described [20]. LDH concentration was determined using an LDH assay kit (Beyotime Biotechnology Co., Ltd.) according to the manufacturer's instructions. The absorbance at 450 nm was determined using a microplate reader, and the OD value was recorded for further evaluation.

### 2.10. Muscone in vitro stimulation

The AC16 cells were treated with DMEM containing 25 μmol/L Muscone (Sigma-Adrich) for 24 h and then collected for further experiments. The control group was treated with DMSO.

### 2.11. Protein extraction and immunoblotting

The AC16 cells and 50 μg heart tissue were incubated with RIPA lysis buffer (Invitrogen, Carlsbad, CA, USA) to obtain the total cell or tissue lysate. The proteins were separated by SDS-PAGE and transferred onto PVDF membranes. After blocking in 5 % milk (Bio-Rad, Hercules, CA) for 1 h, rabbit monoclonal anti-NLRP3 antibody (1:1000, Abcam), anti-Caspase-1 antibody (1:1000, Abcam), anti-Caspase recruitment domain antibody (1:1000, Abcam), anti-M2-type pyruvate kinase antibody (1:500, Abcam), and anti-vitamin D receptor antibody (1:500, Santa Cruz Biotechnology) were added and incubated overnight at 4 °C. GAPDH was used as a loading control for total cell protein.

### 2.12. Chromatin immunoprecipitation assay (ChIP)

In this experiment, chromatin immunoprecipitation was performed using the SimpleChIP® Plus Sonication Chromatin IP Kit (Cell Signaling Technology) according to the manufacturer's instructions. Chromatin samples were incubated with antibodies against H3K4me3 (1:50, Cell Signaling Technology), H3K27me3 (1:50, Cell Signaling Technology), H2A-K119Ub (1:50, Cell Signaling Technology), or IgG (2 μl, Cell Signaling Technology) and then subjected to precipitation. The DNA obtained after precipitation was subjected to qPCR analysis. ChamQ SYBR qPCR Master Mix (Yeasen, China) and StepOnePlus Real-Time PCR system (Applied Biosystems, USA) were used for qPCR. Relative gene expression was calculated using the $2^{-\Delta\Delta Ct}$ method and normalized to the input internal control. The qPCR primers for ChIP assay was *PKM2*:F：TGCCCCGGAACCCATAAATC; R：GAGCGACTTTCCTCCCAGAC.

### 2.13. Real-time quantitative reverse transcription-polymerase chain reaction (Real Time-qPCR)

Total RNA was extracted from AC16 cells and heart tissuein 2.2 using the TRIzol method (Invitrogen, Carlsbad, CA, USA) and following the manufacturer's instructions and previous described [5]. HiScript III first-strand cDNA synthesis kit (Vazyme, Nanjing, China) was used to reverse transcribe 0.1 μg of RNA into complementary DNA (cDNA). qPCR experiments were performed using ChamQ SYBR qPCR Master Mix (Vazyme, Nanjing, China) reagent according to the manufacturer's instructions, and the mRNA level was normalized to GAPDH. The qPCR primer sequences for detection used in this experiment are listed in Table 1 and Table 2.

### 2.14. Extracellular acidification rate (ECAR)

AC16 cells were collected and resuspended in cell medium as previously described [21]. The cell suspension ($4 \times 10^4$ cells/plate; 100 μL) was then placed in Seahorse XF24 culture microplates in the bio-hood for 1 h, then transferred to an incubator (37 °C, 5 % CO2) for 4 h. Next, growth media (150 μL) was added gently to each well, and cells were cultured overnight. The Glycolysis Assay Extracellular Acidification Assay Kit (ab197244, company) were purchased from Abcam. For ECAR analysis, the cell suspension ($4 \times$ 104/plate) was added to 24-well plates. After baseline measurement (T0), glucose (10 mM), oligomycin (1 μM), and 2-deoxy-D-glucose (2-DG; 100 mM) were added into each well at the indicated time points.

### 2.15. Statistical analysis

All analyses were performed using the SPSS software (Version 16.0, SPSS Inc., Chicago, IL, USA). Descriptive statistics for continuous data are presented as means ± S.E.M., and group differences were compared using standard t-tests and one-way analysis of variance (ANOVA). Qualitative data are presented as percentages, and differences between groups were analyzed using the chi-square test. A two-tailed p-value of less than 0.05 was considered statistically significant in this study.

## 3. Results

### 3.1. Potential targets of muscone in the treatment of acute myocardial infarction and GO functional enrichment analysis

The structure of muscone is shown in figure (Fig. 1A). A total of 471 potential targets of muscone were obtained through intersections with 1045 disease targets of AMI, and a Venn diagram was drawn (Fig. 1B) and obtained 46 common targets between the drug and the disease. The 46 common targets were uploaded to the STRING database, and the lowest interaction score of "medium confidence (0.400)" was used as the screening condition to generate a protein interaction network diagram (Fig. 1D). Key target genes were selected based on degree centrality analysis of the interactions using Cytoscape 3.7.0 software. In the PPI network, each node represents a protein, and each edge represents the interaction relationship between proteins. Proteins located in the core position of the network may play a key role in the treatment of AMI with muscone. CytoNCA analysis showed that the top 20 core targets ranked by degree centrality value included AKT1, PTGS2, NOS3, ESR1, PTPRC, SIRT1, MAPK14, NR3C1, PPARA, HMOX1, ABCB1, AR, SLC6A4, CASP9, CYP2C19, APP, NOS2, and CNR1. Muscone is involved in regulating the biological processes (BP) of AMI, including the negative regulation of apoptotic signaling pathways, the response to lipopolysaccharides, and blood pressure regulation. The cellular components (CC) involved in regulating AMI mainly include membrane rafts, membrane microregions, and membrane regions. The molecular functions (MF) involved in regulating AMI mainly include oxidoreductase activity, nuclear receptor activity, and transcription factor activity (Fig. 1C).

### 3.2. Muscone inhibits apoptosis in myocardial cells after ischemia–reperfusion injury

To investigate whether muscone protects myocardial cells from ischemia–reperfusion injury, we constructed an ischemia–reperfusion model in AC16 cells and treated them with 25 μmol·L-1 muscone, while the control group was treated with DMSO. After modeling, we collected the cells and extracted protein and RNA. We measured the expression levels of the proapoptotic proteins Bax and Caspase-3 and the antiapoptotic protein Bcl-2 using fluorescence quantitative PCR and immunoblotting. The results showed that compared with the control group, muscone treatment inhibited the expression levels of Bax and Caspase-3 in AC16 cells after ischemia–reperfusion (Fig. 2A and B, 2C) and increased the expression level of the antiapoptotic protein Bcl-2 (Fig. 2A and D). To further verify the results, we measured the transcription levels of p53 and PUMA by fluorescence quantitative PCR and found that muscone treatment inhibited the transcription levels of p53 and PUMA in AC16 cells (Fig. 2E and F). In addtion, CCK8 assay and LDH

**Table 1**
Sequence of Primers for AC16 cells qPCR assay.

| Gene | Sequence (5′—3′) |
|---|---|
| *p53* | F : GGGACCAAATTGAGGGCTTC |
| | R : TCAACGTCACCAGTCCTCAGA |
| *PUMA* | F : GACCTCAACGCACAGTACGAG |
| | R : AGGAGTCCCATGATGAGATTGT |

**Table 2**
Sequence of Primers for qPCR assay in mice.

| Gene | Sequence (5′—3′) |
|---|---|
| *IL-1β* | F : TGCCACCTTTTGACAGTGATG |
| | R : TGATGTGCTGCTGCGAGATT |
| *IL-6* | F : CCCCAATTTCCAATGCTCTCC |
| | R : CGCACTAGGTTTGCCGAGTA |
| *TNF-α* | F : GATCGGTCCCCAAAGGGATG |
| | R : TTTGCTACGACGTGGGCTAC |
| *NLRP3* | F : ATTACCCGCCCGAGAAAGG |
| | R : TCGCAGCAAAGATCCACACAG |
| *PKM2* | F : GCCGCCTGGACATTGACTC |
| | R : CCATGAGAGAAATTCAGCCGAG |
| *GAPDH* | F : AGGTCGGTGTGAACGGATTTG |
| | R : TGTAGACCATGTAGTTGAGGTCA |

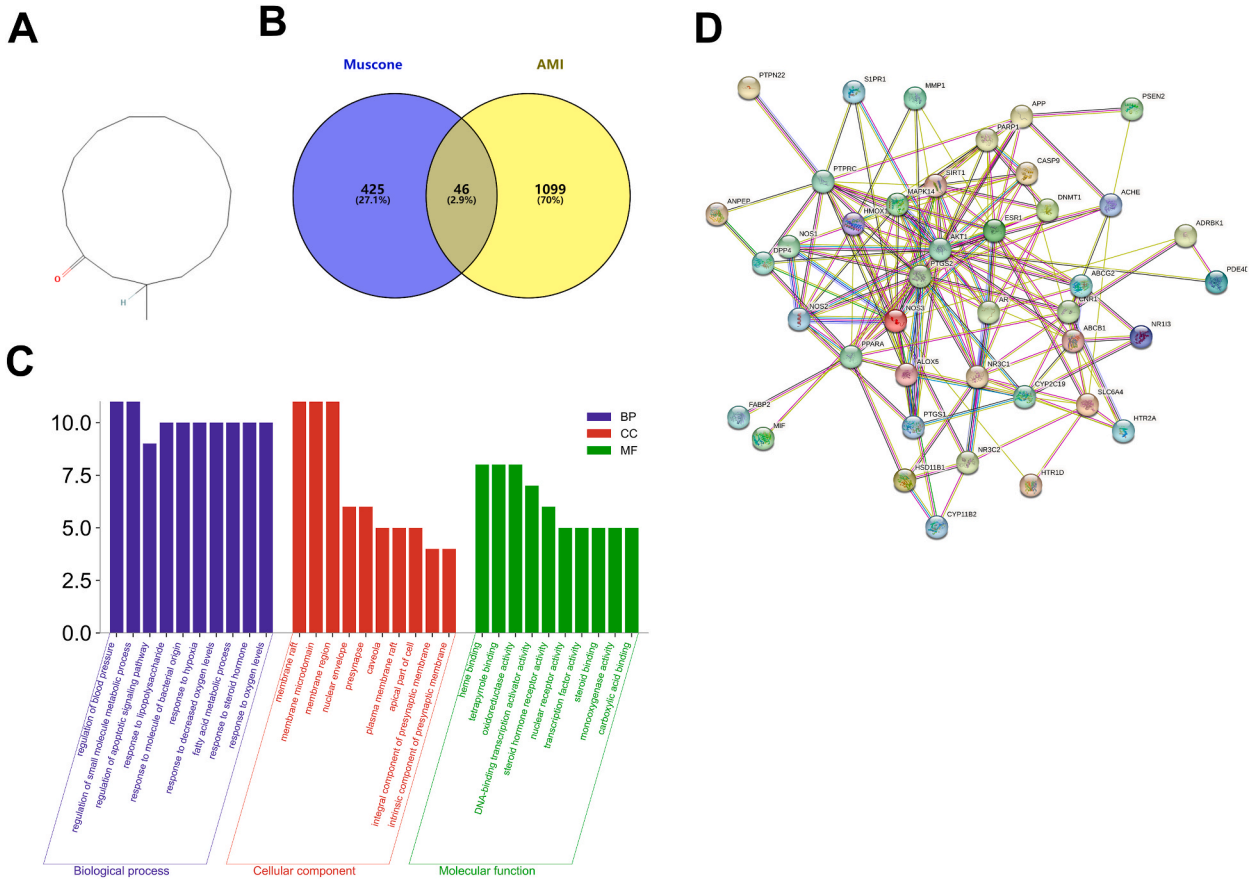


**Fig. 1.** Network pharmacology results of muscone in the treatment of AMI.
Network pharmacology results of muscone in the treatment of AMI. (A) Compound structure of muscone. (B) The compound target network based on Muscone & AMI intersection. (C) GO enrichment analysis results. Purple represents Biological process, green represents Cellular component and blue represents Molecular function. (D) Protein-Protein Interaction Networks Network nodes represent proteins; Links between proteins represent predicted functional associations:Light blue represents from curated databases; Purple represents experimentally determined; Green represents the gene neighborhood; Orange represents gene fusions; Dark blue represents gene and light green represents co-occurrence; black for textmining co-expression; Royal blue represents protein homology. The thickness of the line represents the degree of confidence in the prediction of the interaction. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

assay shwon that Muscone could promote cell viability of AC16 after ischemia–reperfusion injury (Fig. S1A), and inhibited the apoptosis levels of AC16 in vitro (Fig. S1B).These results suggest that muscone can inhibit apoptosis in myocardial cells after ischemia–reperfusion injury.

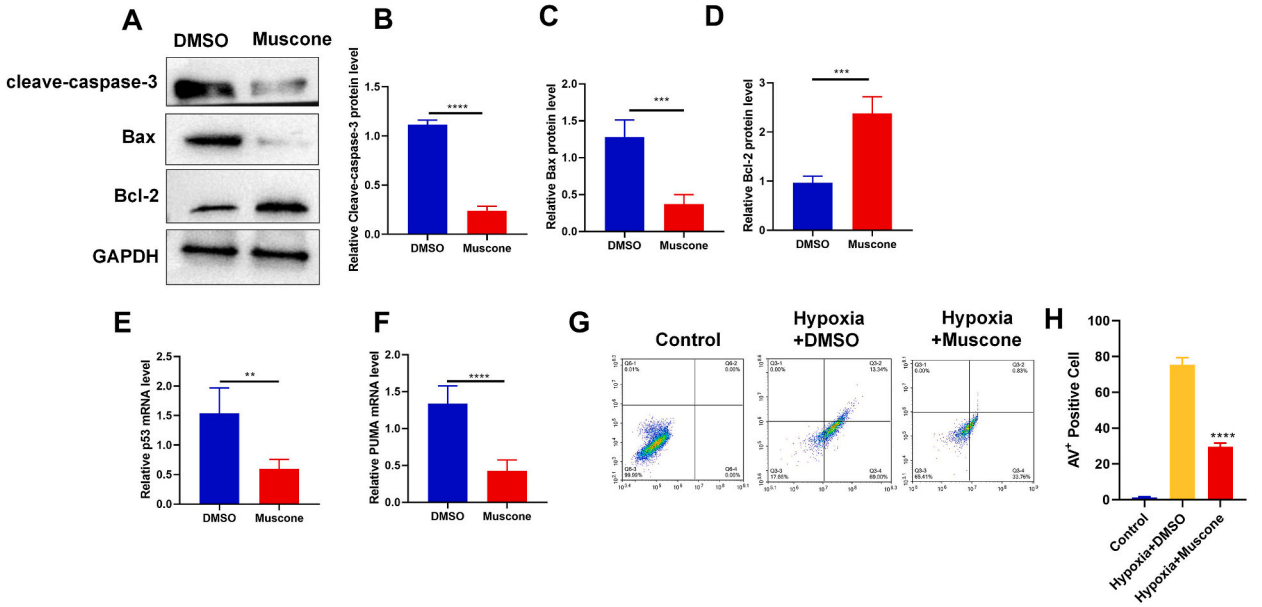


**Fig. 2.** Muscone inhibits apoptosis of myocardial cells after ischemia-reperfusion injury
Construction of an ischemia-reperfusion model using AC16 cells. Cells were treated with DMSO or muscone for 24 h and collected for subsequent experiments. (A) Immunoblotting was performed to detect the expression levels of pro-apoptotic and anti-apoptotic proteins. (B–D) Statistical graphs of Bax, Bcl-2, and Caspase-3 protein expression levels. (E–F) Fluorescence quantitative PCR was used to detect the transcription levels of p53 and PUMA in cells. *, P < 0.05; **, P < 0.01; ***, P < 0.001; ****, P < 0.0001 compared with the DMSO group.

### 3.3. *Muscone inhibits the NLRP3 pathway in myocardial cells after ischemia–reperfusion injury*

Previous studies by our group showed that muscone can inhibit activation of the NLRP3 signaling pathway in macrophages stimulated by lipopolysaccharides, but whether muscone can inhibit the expression of NLRP3 in AC16 cells is still unclear. Then, we constructed an ischemia–reperfusion model in AC16 cells and stimulated the cells with 25 μmol·L-1 muscone for 24 h. We collected the

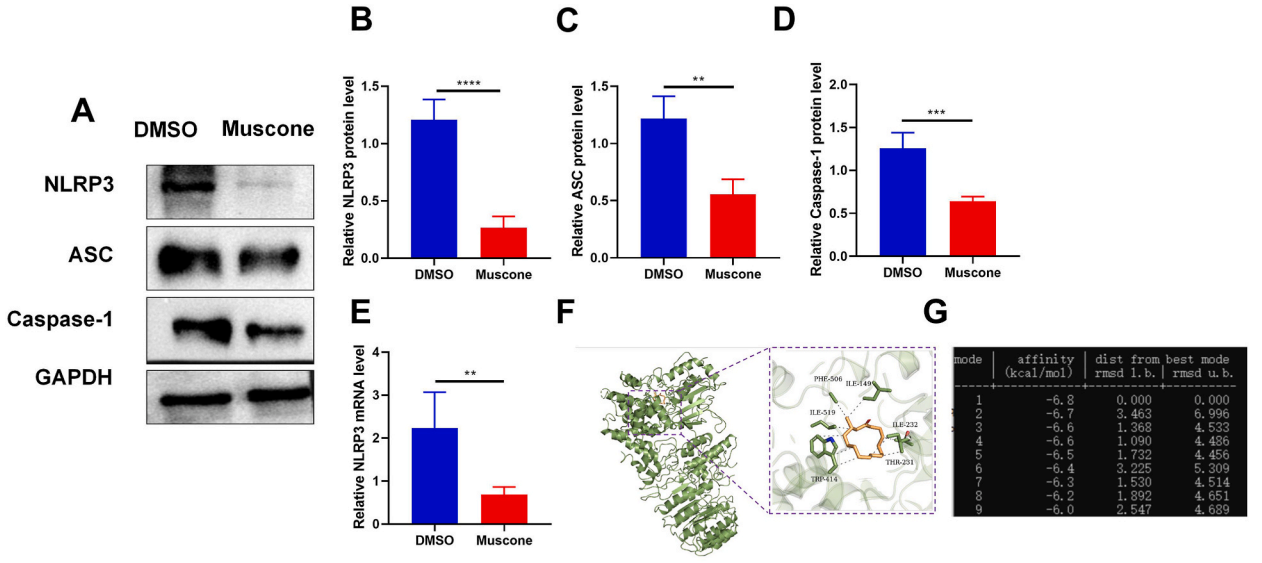


**Fig. 3.** Muscone inhibits the NLRP3 pathway in myocardial cells after ischemia-reperfusion injury
Construction of an ischemia-reperfusion model in AC16 cells, followed by treatment with DMSO or muscone for 24 h. (A) Immunoblotting was performed to detect the expression levels of NLRP3 pathway-related proteins. (B–D) Bar graphs showing the expression levels of NLRP3, ASC, and Caspase-1 proteins. (E–F) Fluorescence quantitative PCR experiments were performed to detect the transcription levels of NLRP3 in the cells. *, P < 0.05; **, P < 0.01; ***, P < 0.001; ****, P < 0.0001, compared with the DMSO group.

cells and used immunoblotting to verify the expression levels of NLRP3 pathway-related molecules (NLRP3, caspase recruitment domain, and caspase-1). Compared with that in the DMSO group, muscone treatment significantly inhibited the expression level of NLRP3 in AC16 cells (Fig. 3A and B) and reduced the expression levels of Caspase-1 (Fig. 3A and C) and the Caspase recruitment domain (Fig. 3A and D). Further fluorescent quantitative PCR analysis showed that muscone significantly inhibited the transcription of NLRP3 (Fig. 3E).To further address this issue, we used molecular docking to simulate the interaction between muscone and the cryo-EM structure of NEK7 (PDB ID: 6NPY) and evaluated the binding density by calculating the binding energy. The results revealed that muscone primarily interacted with NLRP3-associated protein through hydrophobic bonds. Cryo-EM analysis of the NLRP3-NEK7 complex indicated a strong binding affinity, with a binding energy of −6.8 kcal/mol, indicating their close interaction (Fig. 3F and G).

### *3.4. Muscone promotes glycolysis and PKM2 expression in cardiomyocytes after ischemia–reperfusion injury*

We further explored the mechanism by which muscone inhibits the NLRP3 pathway.Previous studies have shown that glycolysis plays a key regulatory role in NLRP3 activity. Therefore, we hypothesized that muscone could regulate glycolysis in cardiomyocytes. GO analysis shown that Muscone might participate in the process of cell glycolysis (Fig. 4A).We collected cells from the model group and measured the extracellular acidification rate (ECAR). The results showed that there was no significant difference between the muscone and DMSO groups during the nonglycolytic acid production phase. However, when glucose was added, the extracellular acid production rate of the muscone-treated group was significantly increased, indicating that muscone could enhance the glycolysis level of AC16 cells. Furthermore, we added oligomycin to completely inhibit oxidative phosphorylation and measured the extracellular acidification rate, which showed that the peak value of the muscone-treated group was significantly higher than that of the DMSO group (Fig. 4B), further indicating that muscone could significantly promote the glycolytic activity of AC16 cells. Based on the ECAR results, muscone can significantly promote glycolysis in AC16 cells.

To further elucidate the specific mechanism by which muscone promotes glycolysis in AC16 cells, we used immunostaining to examine the protein expression of M2-type pyruvate kinase (PKM2). The results showed that compared with DMSO treatment, muscone treatment significantly increased the protein expression of PKM2 in AC16 cells (Fig. 4C and D). Fluorescent quantitative PCR

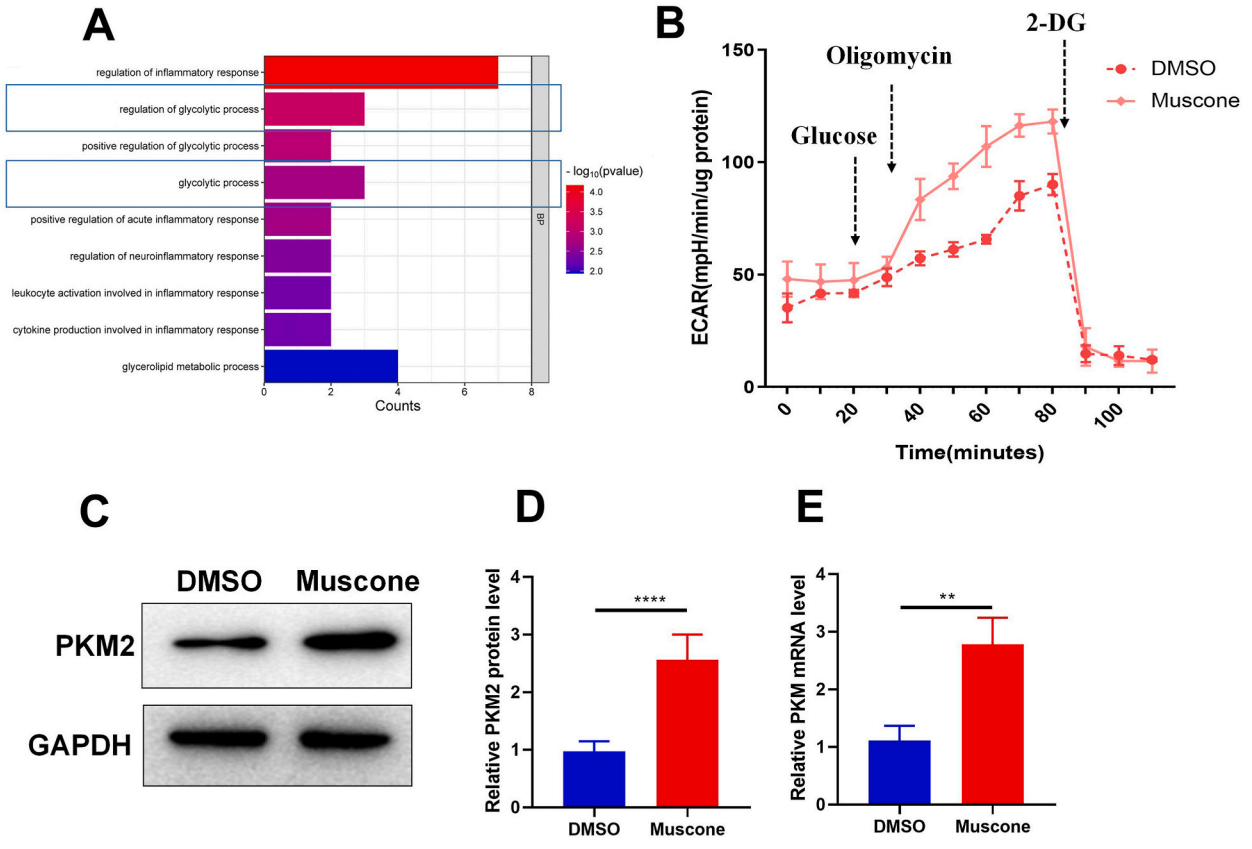


**Fig. 4.** Muscone promotes glycolysis and PKM2 expression in cardiomyocytes after ischemia-reperfusion injury
Construction of ischemia-reperfusion model in AC16 cells, treated with DMSO or muscone for 24 h and then collected for subsequent experiments. (A) Extracellular acidification rate was detected in the hippocampal system. (B–D) Statistical graph of PKM2 protein expression level. (D) Fluorescence quantitative PCR experiment was used to detect the transcription level of PKM in cells. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$; ****, $P < 0.0001$ compared with the DMSO group.

experiments also showed that muscone could increase PKM transcriptional levels (Fig. 4E).

Overall, we found that muscone could enhance glycolysis in cardiomyocytes after ischemia–reperfusion injury by promoting PKM2 expression.

### 3.5. *Muscone promotes the activation of histone modifications associated with the PKM2 promoter and reduces levels of inhibitory histone modifications in AC16 cells*

Histone modification is an important type of transcriptional regulation and an important part of epigenetic modification in cells [22]. GO analysis shown that Muscone might participate in the process of transcriptional regulation (Fig. 5A).To further clarify whether Muscone promotes the transcription of PKM2, we stimulated AC16 cells as described above and measured the levels of H3K27 trimethylation (H3K27me3), H2AK119 monoubiquitination (H2AK119Ub), and H3K4 trimethylation (H3K4me3) on the PKM2 promoter region by chromatin immunoprecipitation (ChIP). We found that Muscone treatment inhibited the levels of H3K27me3 (Fig. 5B) and H2AK119Ub (Fig. 5C) in the PKM promoter region.Furthermore, the results showed that compared with that in the DMSO group, muscone treatment increased the expression level of H3K4me3 in the PKM promoter region (Fig. 5D), and H3K4me3 modification

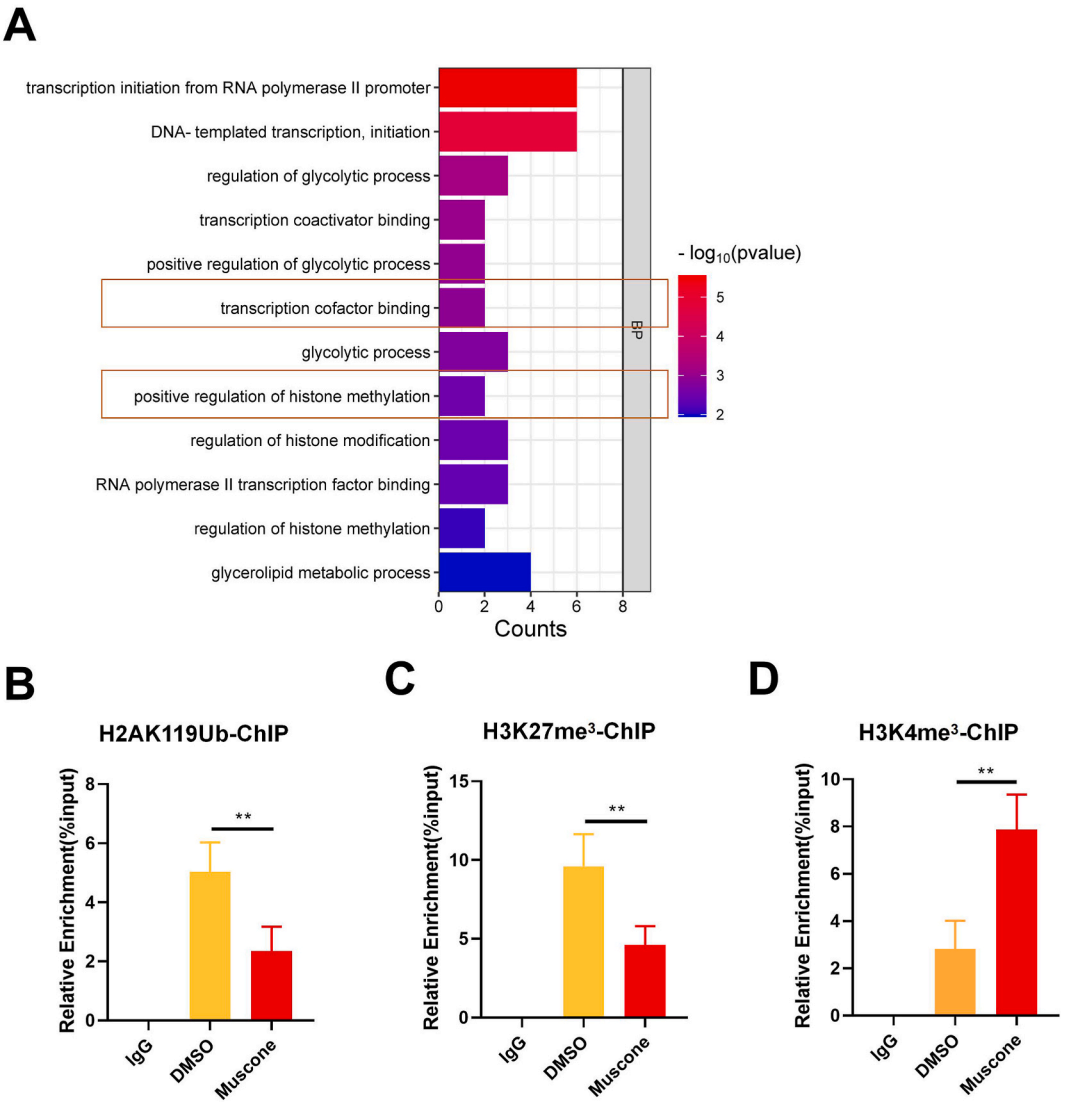


**Fig. 5.** Muscone promotes the activation of histone modifications associated with the PKM2 promoter and reduces levels of inhibitory histone modifications in AC16 cells.

Construction of an ischemia-reperfusion model using AC16 cells, treated with DMSO or muscone for 24 h prior to collecting cells for subsequent experiments. (A) ChIP assay to detect the level of H2AK119Ub in the PKM2 promoter region and its statistical graph. (B) ChIP assay to detect the level of H3K27me3 in the PKM2 promoter region and its statistical graph. (C) ChIP assay to detect the level of H3K4me3 in the PKM2 promoter region and its statistical graph. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$; ****, $P < 0.0001$ compared with the DMSO group.

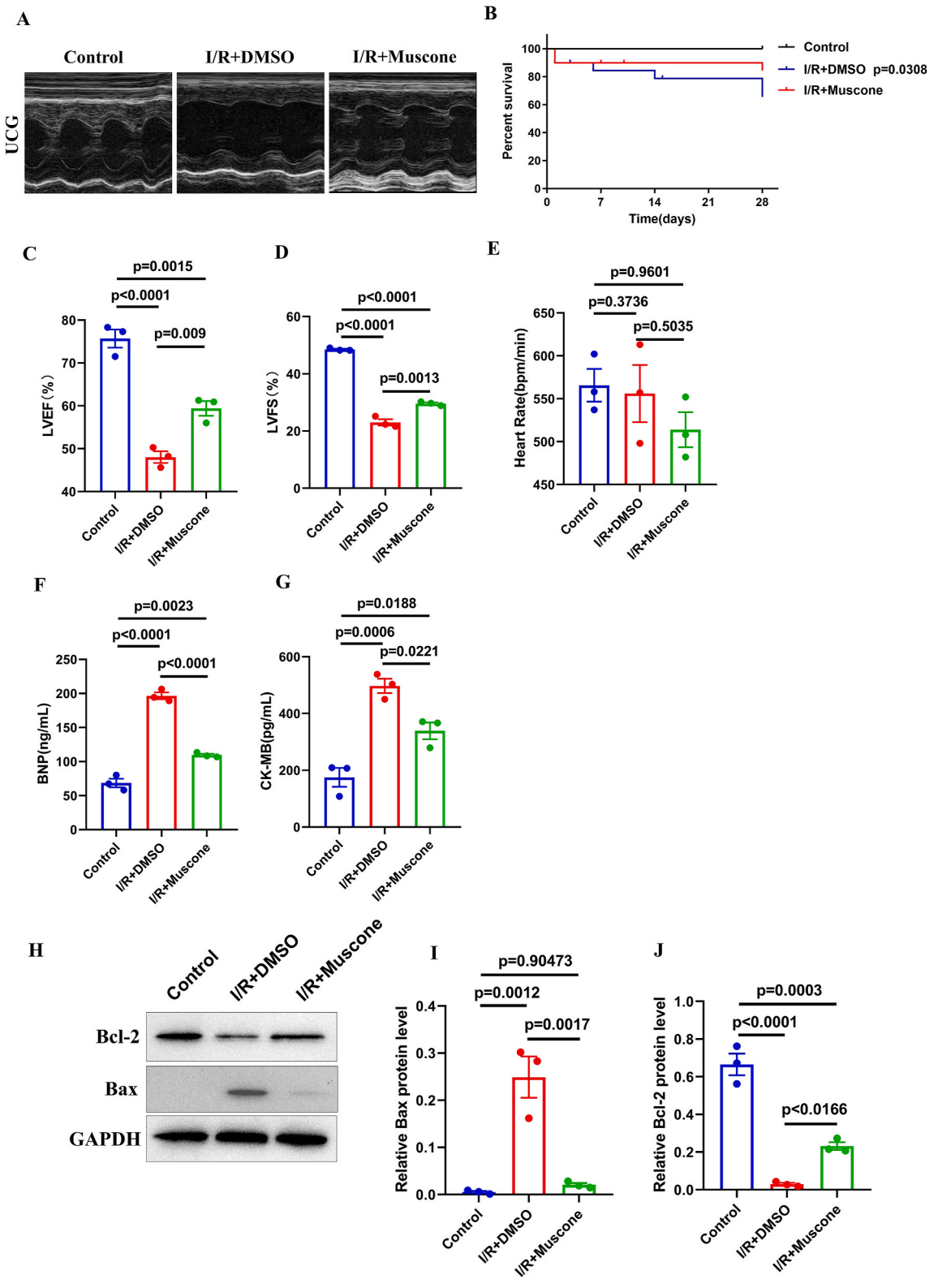


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**Fig. 6.** Muscone improves survival rate and cardiac function in I/R injury mice
Construction of an ischemia-reperfusion model in 8-week old C57BL/6 mice, treated with DMSO or muscone for 4 week and then collected heart tissues for subsequent experiments. (A) Representative M-mode echocardiograms of mice at the 4th week after MI. LVEF (C), LVFS (D) and Heart Rate(E) were measured by echocardiography. Data are expressed as mean ± SEM. (B) Survival rate of mice at the 4th week after I/R injuty. Kaplan-Meier analysis showed lower mortality in mice of I/R + muscone group compared with those in I/R group. (F–G) Serum levels of BNP and CK-MB in mice at the 4th week after I/R were measured by ELISA. Data are expressed as mean ± SEM. (H)Immunoblotting was performed to detect the expression levels of pro-apoptotic and anti-apoptotic proteins. (I–J) Statistical graphs of Bax and Bcl-2 protein expression levels.Data are expressed as mean ± SEM.

loosened chromatin and activated transcription. H3K27me3 and H2AK119Ub modification can compress chromatin and inhibit transcription. These results indicate that Muscone enhances PKM2 transcription and promotes glycolysis, by increasing the levels of activating histone modifications and reducing inhibitory histone modifications in the PKM2 promoter region.

### *3.6. Muscone improves survival rate and cardiac function in I/R injury mice*

To further clarity the protective effect of Muscone *in vivo* assay.C57BL/6 male Mice were randomly divided into sham group, I/R injury group and I/R injury + Muscone group. At the 4th week after surgery, there was no death in control group, and the survival rate in I/R + Muscone group was higher than that in I/R group (Fig. 6B). At the 4th week after I/R injury, we found Muscone treatment could increased LVEF when compared with DMSO group (Fig. 6A and C). In addition, LVFS also increased after Muscone treatment (Fig. 6D). However, the heart rate among sham group, I/R injury group and I/R injury + Muscone group had no statistical difference (Fig. 6E).Meanwhile, muscone treatment significantly reduced serum levels of BNP and CK-MB in I/R injury mice (Fig. 6F and G). To further verify the anti-apoptosis effect of Muscone in I/R injury. We detected the expression levels of Bax and Bcl-2 via Western blotting assay. The results indicated that Muscone could inhibit the expression levels of Bax (Fig. 6H and I) and increased protein level of the Bcl-2 in I/R injury (Fig. 6H and J).Taken together, these results indicated that muscone could improve survival rate and cardiac function in mice at the 4th week after cardiac I/R injury.

### *3.7. Muscone inhibites activation of NLRP3 inflammasome pathway in cardiac I/R injury mice*

Chronic inflammation plays a crucial role in post-MI ventricular remodeling.At the 4th week after I/R injury.We then detected the levels of inflammasome associated proteins (NLRP3, ASC and Caspase-1) in heart tissue via western blotting assay.The results shown that, compared with I/R + DMSO group, Muscone treatment could decrease the expression levels of NLRP3, ASC and Caspase-1 in heart tissue 4th week after I/R injury (Fig. 7A and 7B-D).Meanwhile, we also detected the levels of pro-inflammatory cytokines (IL-1β and IL-6) in heart tissue via western blotting assay.The results shown that, compared with I/R + DMSO group, Muscone treatment could decrease the expression levels of IL-1β and IL-6 in heart tissue 4th week after I/R injury (Fig. 7A and 7E-F).

To further clarify the inhibitory effect of muscone on I/R injury inflammation. Then we detected the mRNA levels of multiple pro-inflammatory cytokines (IL-1β,TNF-α and IL-6) in heart tissue via Real-Time qPCR assay.The results shown that I/R injury induced the mRNA levels of IL-1β, TNF-α and IL-6 in heart tissue, which were remarkably suppressed by Muscone (Fig. 7G).In addition, we also found that Muscone treatment could inhibite expression levels of NLRP3 *in vivo* (Fig. 7H).

### *3.8. Muscone contributes to glycolysis process in I/R injury heart tissue*

To further elucidate the specific mechanism by which muscone promotes glycolysis *in vivo*, we used immunoblotting to examine the protein expression of M2-type pyruvate kinase (PKM2). The results showed that compared with DMSO treatment, Muscone treatment significantly increased the protein expression of PKM2 in I/R injury heart tissue (Fig. 8A and C). Fluorescent quantitative PCR experiments also showed that muscone could increase PKM2 transcriptional levels (Fig. 8B).Next, we examined whether muscone administration could induced the glycolytic capacity.We found a significant increasing levels in lactic acid levels together with a significant increase in ATP content in the I/R injury heart tissues after

Muscone treatment (Fig. 8D and E).

Taken together, the above data suggests Muscone significantly promoting intracellular glycolysis in myocardial cells.

## 4. Discussion

Cardiovascular disease has always been a threat to human health. With the aging population, the incidence of myocardial infarction is gradually increasing. In recent years, with the development of percutaneous coronary intervention technology and thrombolytic drugs [23], the mortality rate of acute myocardial infarction has gradually decreased. However, studies have shown that reperfusion after myocardial infarction can aggravate myocardial damage in the infarcted area. This process is called ischemia–reperfusion injury [24]. Ischemia–reperfusion injury can significantly expand the infarcted area, cause myocardial fibrosis and ventricular remodeling, and promote the occurrence of heart failure after myocardial infarction [25,26]. However, the mechanism of ischemia–reperfusion injury after myocardial infarction is not yet clear, and there is a lack of relevant drug treatments in clinical practice. Therefore, it is urgent to conduct in-depth research on this issue.

This study aimed to explore the mechanism by which muscone promotes the inflammatory response after ischemia–reperfusion

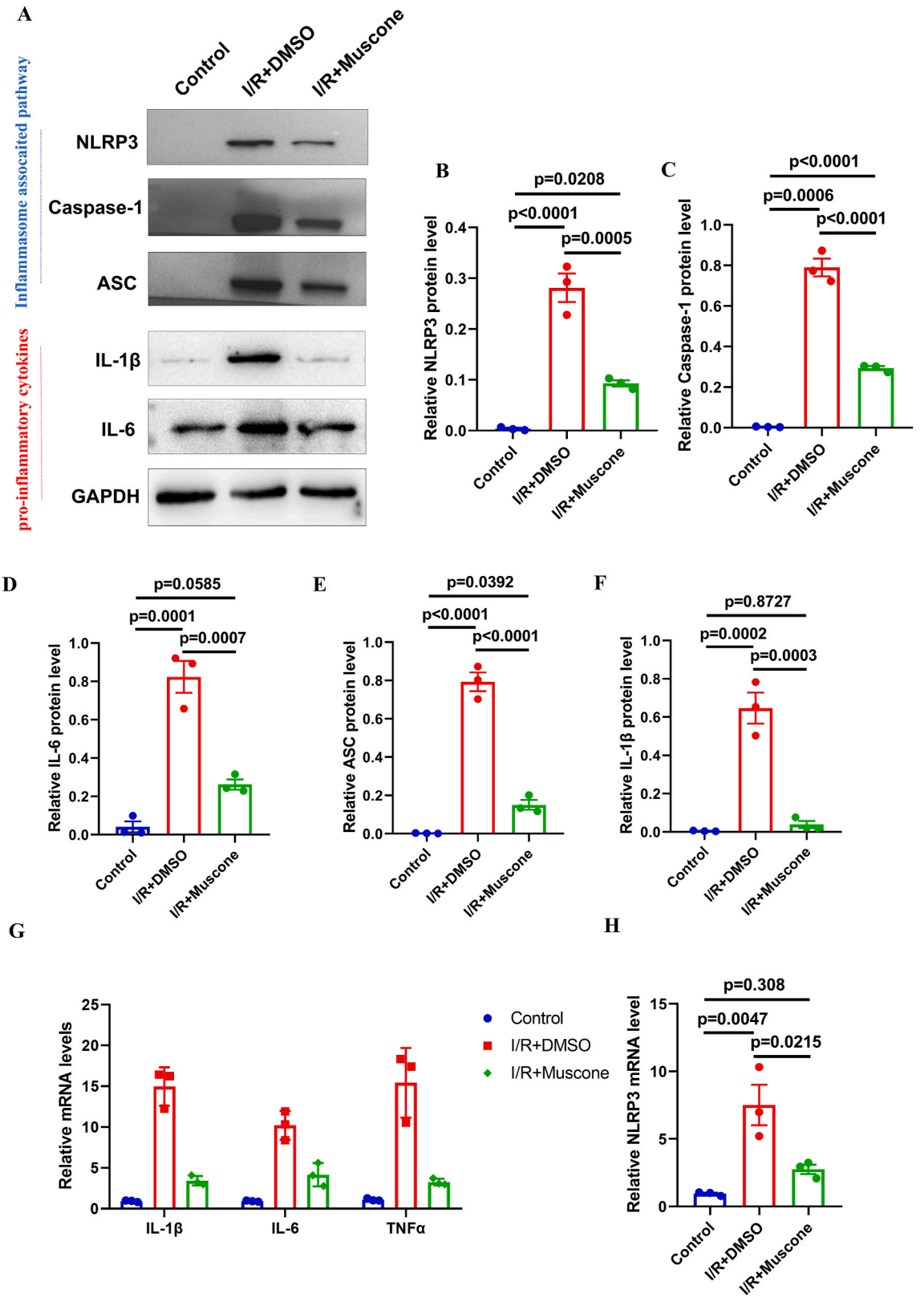


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**Fig. 7.** Muscone inhibites activation of NLRP3 inflammasome pathway in cardiac I/R injury mice
Construction of an ischemia-reperfusion model in 8-week old C57BL/6 mice, treated with DMSO or muscone for 4 week and then collected heart tissues for subsequent experiments. (A)Immunoblotting was performed to detect the expression levels of NLRP3 pathway-related proteins and pro-inflammatory cytokines (IL-1β and IL-6) in heart tissues. (B–F) Statistical graphs of NLRP3 pathway-related proteins (NLRP3, ASC and Caspase-1) and pro-inflammatory cytokines (IL-1β and IL-6) protein expression levels. Data are expressed as mean ± SEM.(G)Fluorescence quantitative PCR experiment was used to detect the transcription level of pro-inflammatory cytokines (IL-1β, IL-6 and TNF-α) in heart tissues.Data are expressed as mean ± SEM.(H)Fluorescence quantitative PCR experiments were performed to detect the transcription levels of NLRP3 in tissues.

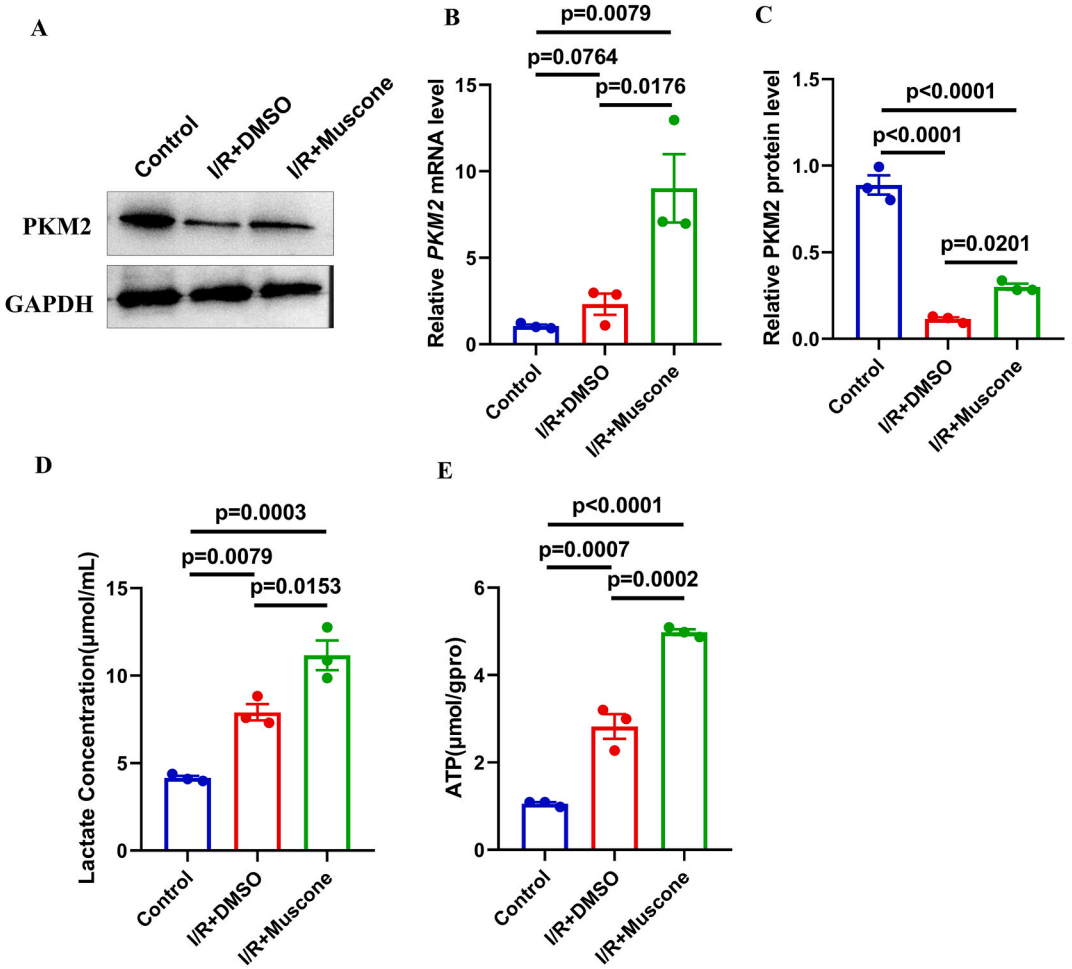


**Fig. 8.** Muscone contributes to glycolysis process in I/R injury heart tissue
Construction of an ischemia-reperfusion model in 8-week old C57BL/6 mice, treated with DMSO or muscone for 4 week and then collected heart tissues for subsequent experiments. (A)Immunoblotting was performed to detect the expression levels of PKM2 in heart tissues.(B)Fluorescence quantitative PCR experiments were performed to detect the transcription levels of PKM2 in heart tissues.(C) Statistical graphs of PKM2 protein expression levels. Data are expressed as mean ± SEM.(G) Statistical graphs of expression levels of lactate acid and ATP in I/R injury heart tissues. Data are expressed as mean ± SEM.

injury. By constructing an ischemia–reperfusion model using a human cardiac cell line (AC16) and In vivo ischemia–reperfusion injury mice model, we found that muscone could inhibit the expression levels of the proapoptotic proteins Caspase-3 and Bax and promote the level of the antiapoptotic protein Bcl-2 compared with DMSO. Also Muscone improves survival rate and cardiac function in I/R injury mice.Muscone administration could inhibit the expression levels of Bax and increased protein level of the Bcl-2 in I/R injury mice model. Transcriptional studies also showed that muscone could inhibit the transcription of p53 and PUMA, indicating that muscone could inhibit apoptosis in myocardial cells after ischemia–reperfusion injury. To further clarify the role of muscone in the inflammatory response after ischemia–reperfusion injury, we conducted an immunostaining analysis of the NLRP3 signaling pathway and found that muscone could significantly inhibit activation of the NLRP3 pathway and the transcription of NLRP3. Meadwhile, we found that Muscone inhibited activation of NLRP3 inflammasome pathway and expression levels of multiple pro-inflammatory cytokines in cardiac I/R injury mice. To further study the specific mechanism by which muscone regulates apoptosis and promotes

inflammation, we focused on the metabolic changes in myocardial cells. Previous studies have shown that the enhancement of glycolysis plays a key role in regulating reperfusion-induced myocardial injury and inflammatory responses. This finding aroused our interest, and using the Seahorse system, we found that muscone could enhance the level and efficiency of glycolysis in AC16 cells, and this process may involve muscone promoting the protein expression of PKM2 and the transcription of the PKM2 gene in AC16 cells and in heart tissue. We then found a significant increasing levels in lactic acid levels together with a significant increase in ATP content in the I/R injury heart tissues after Muscone treatment.Further analysis of the histone modifications in the PKM2 promoter region revealed that muscone treatment could increase the level of H3K4me3 in the PKM promoter region and inhibit the expression levels of H2AK119Ub and H3K27me3. These inhibitory histone modifications reduce the compactness of chromatin and increase the transcriptional openness of PKM, ultimately inhibiting PKM transcription. These results initially clarified that muscone mainly increases the level of H3K4me3 in the PKM2 promoter region, inhibits the expression levels of H2AK119Ub and H3K27me3, loosens the chromatin of the PKM2 transcription region, promotes the formation of transcription complexes, promotes the transcription of PKM2, enhances glycolysis in AC16 cells, and inhibits the inflammatory response and apoptosis after ischemia–reperfusion injury.

The inflammasome was originally discovered as a key receptor involved in host immune responses triggered by exogenous infection [27,28]. Toll-like receptors (TLRs) and nucleotide-binding leucine-rich repeat-like receptors (NLRs) are crucial in activating inflammasome [29]. TLRs and NLRs can recognize pathogen-associated molecular patterns (PAMPs) or damage-associated molecular patterns (DAMPs). The NLRP3 inflammasome is an important component of the inflammasome family and has a significant proinflammatory effect [30,31]. The NLRP3 inflammasome complex is composed of the NLRP3 protein, an apoptosis-associated speck-like protein containing a caspase recruitment domain, and the effector protein caspase-1. NLRP3 primarily depends on its N-terminal pyrin domain (PYD) to interact with the caspase recruitment domain and then forms an NLRP3-caspase recruitment domain complex, which recruits caspase-1 [27,28]. After activation, caspase-1 can convert pro-interleukin-1β or pro-interleukin-18 to their mature forms, ultimately triggering an inflammatory response [31,32]. The NLRP3 signaling pathway is significantly activated in ischemia–reperfusion injury, and Marchetti et al. reported that inhibiting NLRP3 activity could reduce the infarct size in rat myocardial ischemia–reperfusion injury [28]. Injecting NLRP3 small interfering RNA (siRNA) or intraperitoneal injection of the inflammasome inhibitor BAY-11-7028 into the myocardium reduced macrophage and neutrophil infiltration and decreased the number of myocardial cells in a mouse model of myocardial ischemia–reperfusion injury [33,34]. These results suggest that NLRP3 has a detrimental effect on ischemia–reperfusion injury. Our study showed that muscone could inhibit the activation of the NLRP3 signaling pathway and improve the response to myocardial ischemia–reperfusion injury.

Metabolic reprogramming is considered a key factor in myocardial remodeling and the inflammatory response following myocardial infarction [35]. Studies have shown that there is a shift from glucose metabolism to fatty acid metabolism in cardiomyocytes after ischemia–reperfusion injury, which can induce oxidative stress and exacerbate myocardial injury after reperfusion. Our research shows that muscone can enhance glycolysis in cardiomyocytes following ischemia–reperfusion injury, possibly by promoting the expression of M2-type pyruvate kinase. Through transcriptome analysis, we identified transcriptional regulation as a key pathway through which muscone participates in metabolic reprogramming in AC16 cells. However, other studies have shown that the activation of inflammasomes in macrophages in an inflammatory microenvironment is associated with increased glycolysis, which differs from our findings in cardiomyocytes. Zhang DM el had shown that P53-induced glycolysis and apoptosis regulator (TIGAR) ameliorates I/R-injury of brain.In addition, MCT4 could exert a cardioprotective effect after myocardial I/R injury by inducing OXPHOS/glycolysis interconversion and inhibiting oxidative stress [36].When Myocardial ischemia/reperfusion (I/R) injury occurred, increasing level of glycolysis effect could alleviate apoptosis of myocyte cells via regulating production of ATP in cells.While, over-glycolysis effect also had its side-effect.Actually, some researches published previous indicated the pro-inflammatory effect of over-glycolysis, Min Xie el shown that PKM2 inducing the over-glycolysis effect in myeloid cells, thus led to the activation of AIM2 and NLRP3 inflammasome [37].Pharmacological inhibition of the PKM2 could inhibit the activation of inflammasome pathway [37]. Glycolysis can continue to provide ATP energy for cardiomyocytes in ischemic state, which is important for cardiomyocyte survival and repair after I/R injury. However, the persistence of excessive glycolysis can promote the persistence of chronic inflammatory condition and induce ventricular remodeling after I/R injury.All these research indicated the complex relationship among metabolic remodeling,inflammation and cardiac fibrosis.

Epigenetic modifications are important for regulating gene transcription. Epigenetic regulation, including CpG island DNA methylation, histone modifications, and noncoding RNA regulation, is considered a key factor in regulating inflammasome transcription [38]. Yan Xia et al. found that RNF8-mediated H3 histone ubiquitination modification could promote glycolysis, and this process was crucial for tumorigenesis [39,40]. Under infection conditions, high deacetylation of histone H3K9/14 and high trimethylation of histone H3K4 can be observed. In this study, we found that muscone could promote H3K4me3 levels in the PKM promoter region, increase H2AK119Ub and H3K27me3 levels, reduce the level of active histone modifications, and increase the level of repressive histone modifications, leading to tighter chromatin compression in the NLRP3 promoter region and transcriptional suppression.

In summary, this study elucidates the protective role of muscone in regulating apoptosis and the inflammatory response in myocardial cells after ischemia–reperfusion injury, providing new therapeutic drugs and targets for the clinical treatment of myocardial infarction. Furthermore, molecular biology has revealed potential molecular mechanisms by which muscone exerts its protective effects and identified the key molecular role of muscone in regulating glycolysis in myocardial cells. Although this project has initially discovered an important role of muscone in regulating the epigenetic modifications of the PKM2 promoter, further clarification and research are needed on the specific mechanism of action of muscone in this precise regulation.

## Data availability

Data will be made available on request to corresponding author Wang Xiaoyan or Wang Guangyan.

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## CRediT authorship contribution statement

**Xin Gu:** Conceptualization, Data curation, Funding acquisition, Investigation, Methodology, Project administration, Software, Validation, Writing – original draft. **Neng Bao:** Conceptualization, Data curation, Investigation, Methodology, Software, Supervision. **Jing Zhang:** Conceptualization, Data curation, Investigation, Methodology, Resources. **Guangyi Huang:** Conceptualization, Data curation. **Xiaodong Zhang:** Methodology, Software, Validation, Visualization. **Zhixuan Zhang:** Conceptualization, Data curation. **Yinqiang Du:** Data curation, Resources, Software, Validation. **Haoyu Meng:** Data curation, Funding acquisition. **Jiabao Liu:** Investigation, Project administration. **Peng Wu:** Conceptualization, Validation. **Xiaoyan Wang:** Conceptualization, Data curation, Funding acquisition, Methodology, Project administration, Writing – review & editing. **Guangyan Wang:** Conceptualization, Formal analysis, Funding acquisition, Investigation, Writing – review & editing.

## Declaration of competing interest

The authors declare the following financial interests/personal relationships which may be considered as potential competing interests:

Peng Wu reports financial support was provided by Foundation for Innovative Research Groups of the National Natural Science Foundation of China. Haoyu Meng reports financial support was provided by Natural Science Foundation of Jiangsu Province. Yingqiang Du reports financial support was provided by Natural Science Foundation of Jiangsu Province. Xiaoyan Wang reports financial support was provided by Key project of Jiangsu Provincial Health Commission. Xiaoyan Wang reports financial support was provided by General project of Wuxi Science and Technology Administration. Guangyan Wang reports financial support was provided by General project of Wuxi Traditional Chinese Medicine Administration. Xiaoyan Wang reports financial support was provided by Precision Medicine Project of Wuxi Health Commission. Yingqiang Du reports financial support was provided by The Research Project of Gusu School of Nanjing Medical University. Yingqiang Du reports financial support was provided by Scientific Research Project of Gusu Health Talent Plan. Xin Gu reports financial support was provided by Wuxi Taihu Light Technology Project. The authors declare that they have no competing interests. If there are other authors, they declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

## Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.heliyon.2023.e22154.

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