

Lycopene protects myocardial ischemia injury through anti-apoptosis and anti-oxidative stress

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Abstract. – **OBJECTIVE:** The aim of this research was to explore the protective effect of lycopene (Lyc) on myocardial ischemia injury through anti-apoptosis and anti-oxidative stress.

MATERIALS AND METHODS: 75 rats were divided into 5 groups: sham operation group (control group), model group, low-dose group (Lyc+2 mg/kg), medium-dose group (Lyc+4 mg/kg) and high-dose group (Lyc+6 mg/kg). The rat model of myocardial ischemia was established by a subcutaneous injection of isoproterenol (85 mg/kg) for two consecutive days. Conventional HE staining and Masson staining were performed for pathological changes. Biochemical indicators were measured by the enzyme-linked immuno sorbent assay (ELISA). Western blotting was used to measure the levels of related proteins in JNK/STAT signaling pathway.

RESULTS: Compared to control group, the levels of CK-MB, TC, and TGs were significantly increased in model group. The levels of CK-MB, TC, and TGs in each Lyc-administered group were decreased. After Lyc was administered, the SOD, CAT, GSH-Px activities and MDA content were all restored. The serum levels of IL-1 β , TNF- α and IL-6 in control group were significantly lower than in model group. When the Lyc was administered, the serum IL-1 β , TNF- α and IL-6 levels in medium-dose group and high-dose group were significantly decreased. The levels of Bax/Bcl-2, Cyt-c, and Caspase-3 in model group were significantly higher than control group. Changes of Bax/Bcl-2, Cyt-c, and Caspase-3 in medium-dose and high-dose groups after the administration of Lyc were restored significantly. The levels of p-JNK/JNK, p-STAT1 (Tyr701)/STAT1, p-STAT1 (Ser727)/STAT1, p-STAT3 (Tyr705)/STAT3 were significantly increased, while p-STAT3 (Ser727)/STAT3 was significantly decreased. When Lyc was administered, the expression levels of p-JAK/JAK, p-STAT1 (Tyr701)/STAT1, p-STAT1 (Ser727)/STAT1, p-STAT3 (Tyr705)/STAT3 protein in medium-dose group and high-dose group were significantly decreased, and the expression level of p-STAT3 (Ser727)/STAT3 protein was significantly increased.

CONCLUSIONS: Lyc could show a protective effect on oxidative stress injury and anti-cardiomyocyte apoptosis of myocardial ischemia, and its possible mechanism was to attenuate the activation of JNK/ERK signaling pathway induced by myocardial injury.

Key Words:

Lycopene, Myocardial ischemia, Apoptosis, Oxidative stress, JNK/ERK/STAT signaling pathway.

Introduction

Myocardial ischemia is a pathological condition in which the blood perfusion of the heart decreases, resulting in a decrease in oxygen supply to the heart, abnormal energy metabolism of the myocardium, and failure to support the normal function of the heart^{1,2}. Myocardial ischemia is a lack of coronary blood flow and has electrical, functional, metabolic and structural consequences for the heart. All therapeutic interventions must aim to improve the blood flow of the ischemic myocardium as quickly as possible¹. Several biochemical and metabolic changes occur in myocardial ischemia, including mitochondrial reactivation and regeneration of oxygen species, inducing oxidative stress, which can mediate myocardial injury³⁻⁵. Scholars⁶⁻⁹ have found that myocardial ischemia induces apoptosis of myocardial cells, which is involved in the formation of pathological changes. However, the mechanism of apoptosis is not clear. Lycopene (Lyc) is the main carotenoid in tomatoes and in human serum and tissues¹⁰.

Studies^{11,12} showed beneficial effects of Lyc on individuals who are antioxidant-deficient like elderly patients, or humans exposed to higher levels of oxidative stress like smokers, diabetics, hemodialysis patients and acute myocardial infarction patients. Lyc exhibits significant Re-

active Oxygen Species (ROS)-scavenging and antioxidant properties- which may prevent spermatozoa alterations caused by oxidative stress, and preserve the functionality of male reproductive cells¹³. Lyc could protect bone marrow mesenchymal stem cells from ischemia-induced apoptosis, through reducing ROS generation¹⁴. Lyc treatment (1 μ M) before reoxygenation significantly reduced cardiomyocyte death induced by hypoxia/reoxygenation, and could achieve 1 μ M concentration in circulating blood, significantly suppressed myocardial infarction, ROS production, and c-Jun N-terminal kinase (JNK) phosphorylation in the cardiac tissue of mice during *in vivo* regional myocardial ischemia-reperfusion¹⁵.

However, many aspects of Lyc *in vivo* metabolism, functions and clinical indications shall be studied. In this work, the rat model of myocardial ischemia was established by subcutaneous injection of isoproterenol (ISO), and the effect of Lyc on myocardial ischemia was evaluated by the enzyme-linked immunosorbent assay (ELISA) and Western blotting.

Materials and Methods

Ethics Statement

This investigation was approved by the Ethics Committee of the Emergency General Hospital. Animal welfare and the relevant experiment were carried out in compliance with the Guide for the Care and Use of Laboratory Animals.

Reagents and Instruments

Lycopene was purchased from Sigma-Aldrich (St. Louis, MO, USA); isoproterenol and bicinchoninic acid (BCA) protein concentration assay kit were purchased from BBI Life Sciences Corporation (Shanghai, China); Bcl-2, Bax, Caspase-3, Cyt-c, JNK, p-JNK, p-STAT1 (Tyr701), STAT1, p-STAT1 (Ser727), STAT1, p-STAT3 (Tyr705), STAT3, p-STAT3 (Ser727), STAT3 of primary antibodies, and fluorescent secondary antibodies were purchased from Cell Signaling Technology (Danvers, MA, USA). Hematoxylin and eosin (H&E) staining kit and the Masson staining kit were purchased from Beyotime Biotechnology Co. Ltd. (Shanghai, China). The instruments included refrigerated centrifuge Allegra X-30R (Beckman, Brea, CA, USA); iMark microplate reader (Bio-Rad, Hercules, CA, USA); CX41 fluorescence microscope (Olympus, Tokyo, Japan), RM22335 paraffin slicer (Leica, Nussloch, Ger-

many) and NanoDrop 2000C UV spectrophotometers (Thermo Scientific, Waltham, MA, USA).

Establishment of Animal Model, Grouping and Administration

Seventy-five Specific-Pathogen-Free (SPF) male Sprague-Dawley (SD) rats, weighing 275 ± 25 g, were purchased from the Shanghai Experimental Animal Center of the Chinese Academy of Sciences. Each cage was kept at room temperature (25°C), constant humidity (50% \pm 5%). The house was ventilated and dry, and kept quiet, with a 12-h cycle. Experimental rats began the experiment after one week of adaptation. Rats were randomly divided into 5 groups: sham operation group (control group), model group, low-dose group (Lyc+2 mg/kg), medium-dose group (Lyc+4 mg/kg) and high-dose group (Lyc+6 mg/kg). The rat model of myocardial ischemia was established by a subcutaneous injection of isoproterenol (85 mg/kg) for two consecutive days. After 7 d of continuous administration, the protective effect of Lyc on the myocardial injury was observed.

Pathological Changes in Myocardial Tissue

Rats were anesthetized by an intraperitoneal injection of sodium pentobarbital (40-60 mg/kg) and perfused with 4% of paraformaldehyde (PFA), and then, the heart tissue was removed. After 4% of PFA was fixed for 24 h, it was subjected to dehydration treatment and was embedded, and sliced up to a thickness of about 4 μ m. Conventional HE staining and Masson staining were performed, and after mounting, changes in the myocardial histopathological morphology were observed under a light microscope.

Biochemical Indicators Measured by ELISA

Experimental rats were anesthetized by an intraperitoneal injection of sodium pentobarbital (40-60 mg/kg). Then, rats were sacrificed by decapitation and the heart tissue was quickly taken.

The tissue was fully ground in an ice bath with sterile saline, and centrifuged at 4°C, 3500 rpm/min for 10 min. The supernatant was taken, and then, the superoxide dismutase (SOD), glutathione peroxidase (GSH-Px), catalase (CAT) activities and the malondialdehyde (MDA) content in the myocardial homogenate were measured. The levels of IL-1 β , TNF- α and IL-6 were measured and the level changes of myocardial creatine kinase isoenzymes (CK-MB), transcobalamin (TC) and triacylglycerols (TGs) in serum were also me-

asured. All operations were performed according to the kit instructions.

Western Blotting for Related Proteins in JNK/ STAT Signaling Pathway

The appropriate amount of myocardial tissue was weighed. After ground by liquid nitrogen, the tissue protein lysate was added in a ratio of 10:1, and then was added a broad spectrum of phosphatase inhibitor (1:100) and preparations of phenylmethylsulfonyl fluoride (PMSF) (1:100) on the ice bath. After standing for 2 h, the mixture was continuously shaken, and then the supernatant was centrifuged (4°C, 3500 rpm, 10 min). The protein concentration was measured by the BCA method. The corresponding sample volume was calculated, and the corresponding buffer was added, then heat denatured for 10 min. The sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) was performed, the protein was transferred to the polyvinylidene difluoride (PVDF) membrane after electrophoresis, and blocked with 5% of fetal bovine serum (BSA) for 2 h. The primary antibodies were washed and incubated overnight at 4°C, and the membrane was washed with Tris-Buffered Saline and Tween containing 0.2-0.4% Tween-20 (TBST) for 5 min×3 times. Then, a fluorescent secondary antibody was added for exposure, and the relative gray value of the target protein was analyzed using Image Studio software.

Statistical Analysis

Data were analyzed using SPSS 19.0 (SPSS Inc., IBM Corp. Released 2010. IBM SPSS Statistics for Windows, Armonk, NY, USA). The experimental data were expressed as $\bar{x} \pm s$, and the differences between groups were compared by one-way analysis of variance (ANOVA), and Duncan methods as post-hoc test. A significant difference was considered at $p < 0.05$.

Results

Pathological Changes of HE Staining and Masson Staining

In control group, the contraction bands of the myocardium were clear, the myocardial cells were arranged neatly and densely; the nucleus structure was clear, the extracellular matrix was poor, and a few fibroblasts were seen. There were no evident cell necrosis and inflammatory cell infiltrations, and there were no patent abnormalities in the epi-

cardium and endocardium. In model group, the myocardial tissue was disordered, the cell morphology was ambiguous, and the large nucleus was dissolved, reduced, and disappeared; it was patent that a large number of focal myocardial fibers were severely degenerated and necrotic, and a large number of inflammatory cells infiltrated. When Lyc was administered, the degree of myocardial fibrosis and necrosis and inflammatory cell infiltration were significantly reduced in each administration group. The high-dose improvement effect was the most evident, and the pathological features such as myocardial cell degeneration, necrosis and inflammatory cell infiltration were significantly improved, and close back to normal.

In control group, the myocardial cells were arranged neatly, the cardiomyocyte interstitial had a small amount of collagen fibers, and there was no fibro-proliferation. The collagen tissue distribution was rare. The myocardial tissue of model group was almost completely replaced by a large collagen tissue, and the number of myocardial cells was markedly reduced and disordered; myocardial fibrosis was severe, a large number of myocardial cells collagen fibers proliferated significantly. When Lyc was administered, each drug-administered group showed significant improvement symptoms compared to model group, myocardial cells were aligned and the degree of fibrosis was significantly improved. Among them, the high-dose group had the most evident improvement (Figure 1).

Effect of Lyc on Key Enzyme Activities in Myocardial Injury

Compared to control group, the levels of CK-MB, TC, and TGs in model group were significantly increased ($p < 0.01$). When Lyc was administered, the levels of CK-MB, TC, and TGs in the serum of rats in each drug-administered group were decreased. The levels of CK-MB, TC, and TGs in the medium-dose group and high-dose group were significantly lower than those in model group, indicating that Lyc could regulate the level changes of CK-MB, TC, and TGs in serum, and then could protect against the myocardial damage (Figure 2).

Effect of Lyc on the Activities of SOD, CAT, GSH-Px and MDA Content in Serum of Experimental Rats

Results showed that the activities of SOD (Figure 3A), CAT (Figure 3B) and GSH-Px (Figure 3C) in model group were significantly lower than that in

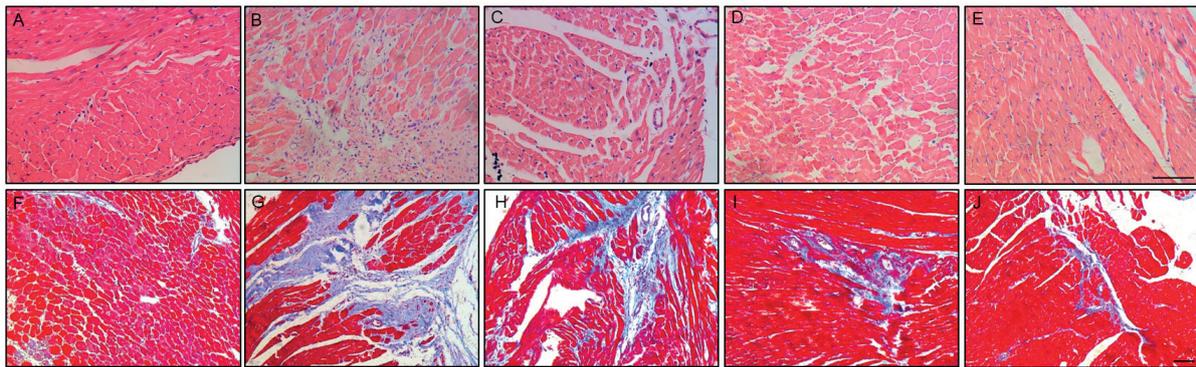


Figure 1. Pathological changes of HE staining and Masson staining. *A-E*, HE staining results in control group, model group, low-dose group (Lyc+2 mg/kg), medium-dose group (Lyc+4 mg/kg) and high-dose group (Lyc+6 mg/kg), scale bar=200 μ m. *F-J*, Masson staining results in control group, model group, low-dose group (Lyc+2 mg/kg), medium-dose group (Lyc+4 mg/kg) and high-dose group (Lyc+6 mg/kg), scale bar=100 μ m.

control group, and the content of MDA (Figure 3D) was significantly increased, indicating that rats in model group were in a state of peroxidative stress. When Lyc was administered, the body's SOD, CAT, GSH-Px activities and MDA content were all restored, and both medium-dose group and high-dose group were significantly restored, indicating that Lyc had a protective effect on oxidative stress injury of myocardial ischemia induced by ISO in rats.

Effect of Lyc on the Levels of Inflammatory Factors (IL-1 β , TNF- α , and IL-6) in Serum of Experimental Rats

In this study, serum IL-1 β , TNF- α , and IL-6, were measured. Results showed that the serum levels of IL-1 β (Figure 4A), TNF- α (Figure 4B) and IL-6 (Figure 4C) were significantly increased in model group compared to control group. When the Lyc was administered, the serum IL-1 β , TNF- α and IL-6 levels in medium-dose group and high-dose group were significantly decreased,

indicating that Lyc had a role as an anti-inflammatory factor.

Effect of Lyc on Apoptotic Signaling Pathway

As shown in Figure 5, the levels of Bax/Bcl-2, Cyt-c and Caspase-3 in model group were significantly elevated, when compared to control group, indicating that myocardial ischemia resulted in a significant increase in pro-apoptotic-related proteins in the rat myocardial tissue. Changes of Bax/Bcl-2, Cyt-c and Caspase-3 in medium-dose and high-dose groups after the administration of Lyc were restored significantly, indicating that Lyc had an anti-cardiomyocyte apoptosis caused by myocardial ischemia, and then played a protective role.

Effect of Lyc on JNK/ERK/STAT Signaling Pathway

Compared to blank group, the expression levels of JNK, STAT1 and STAT3 proteins in

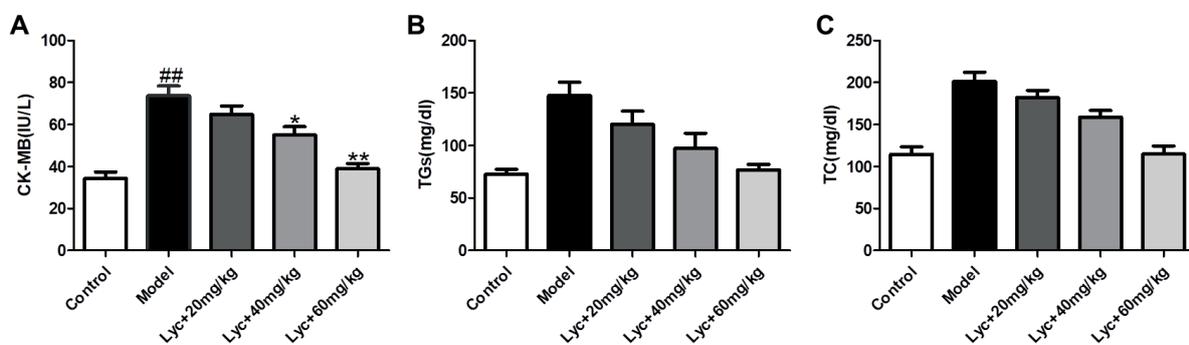


Figure 2. Effect of Lyc on levels of CK-MB (A), TGs (B), and TC (C) in serum of experimental rats.

model group did not change significantly, while the levels of p-JNK/JNK, p-STAT1 (Tyr701)/STAT1, p-STAT1 (Ser727)/STAT1, p-STAT3 (Tyr705)/STAT3 were significantly increased, while p-STAT3 (Ser727)/STAT3 was significantly decreased. When Lyc was administered, the expression levels of p-JAK/JAK, p-STAT1 (Tyr701)/STAT1, p-STAT1 (Ser727)/STAT1, p-STAT3 (Tyr705)/STAT3 protein in medium-dose group and high-dose group were significantly decreased, and the expression level of p-STAT3 (Ser727)/STAT3 protein was significantly increased, indicating that Lyc could attenuate the activation of JNK/ERK signaling pathway induced by myocardial injury (Figure 6).

Discussion

Ischemic heart disease or myocardial ischemia is a disease characterized by the reduced blood supply to the myocardium, usually due to coronary artery disease¹⁶. In this study, the rat model

of myocardial ischemia was established by a subcutaneous injection of isoproterenol. Lyc, as the main carotenoid in tomatoes, is used to treat myocardial ischemia in rats. Lyc can regulate changes in key enzymes and inflammatory factors in the myocardial injury, protect the oxidative stress injury induced by myocardial ischemia, and play a role in the apoptotic signaling pathway and in the JNK/ERK signaling pathway, which indicates that Lyc has a dose-dependent protective effect on myocardial ischemia.

The myocardium contains abundant diagnostic markers of myocardial infarction, such as CK-MB, TC, and TGs, which are released into the extracellular fluid once metabolism is impaired^{17,18}. In arsenic-induced myocardial injury rats, quercetin could significantly restore the elevated levels of cardiac markers (LDH, CK-MB, AST, ALT, and ALP), altered lipid metabolism (total cholesterol, triglyceride, LDL, HDL, and VLDL)¹⁹. The lipid profile showed that Lyc decreased TC, LDL-C, VLDL-C and triglycerides compared to control²⁰. CK-MB, TC and TG levels in patients with myo-

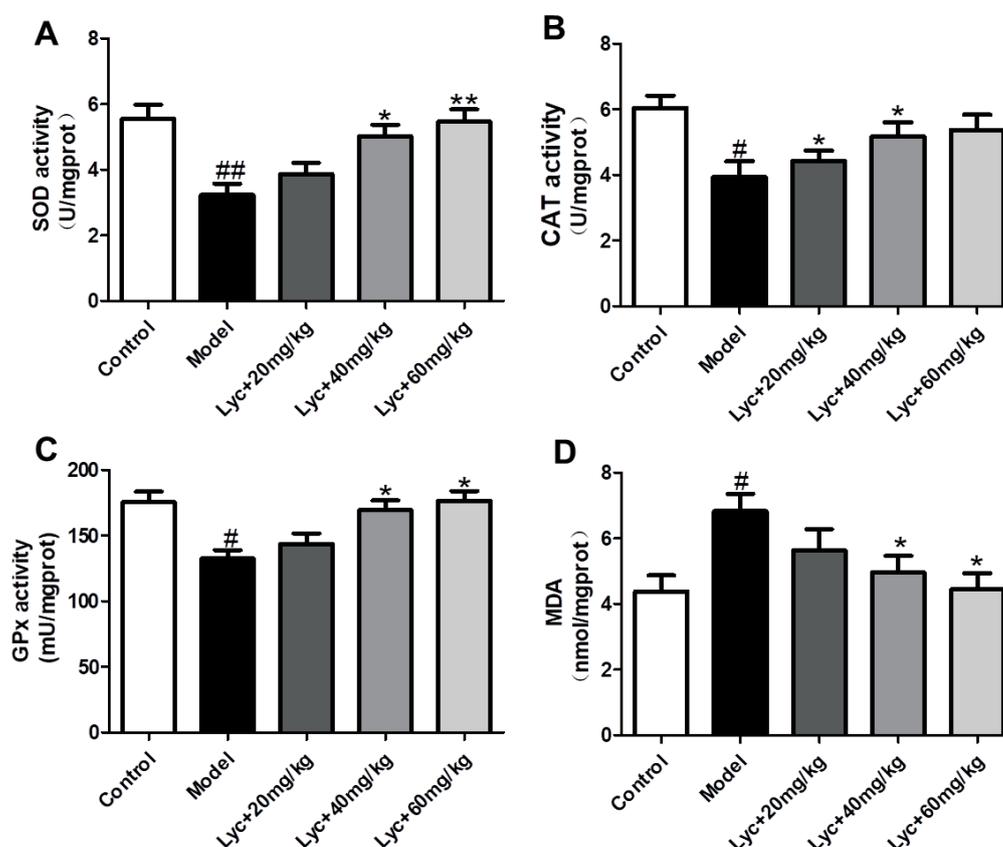


Figure 3. Effect of Lyc on activities of SOD (A), CAT (B), GSH-Px (C) and MDA (D) in serum of experimental rats.

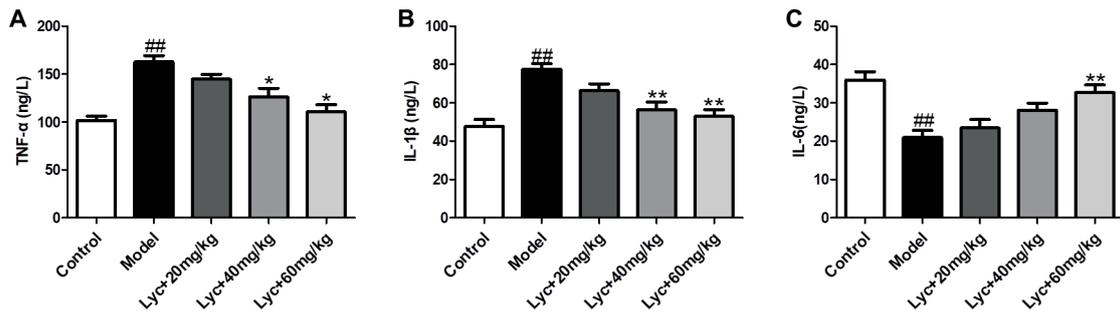


Figure 4. Effect of Lyc on levels of IL-1 β (A), TNF- α (B), and IL-6 (C) in serum of experimental rats.

cardial ischemia-reperfusion injury were significantly higher than those in patients without myocardial ischemia-reperfusion injury²¹. The above results are consistent with our results. The levels of CK-MB, TC and TG in model group were significantly increased. Lyc could significantly restore the levels of CK-MB, TC, and TGs in the serum of rats in drug-treated groups. It indicated that Lyc could regulate the levels of CK-MB, TC and TG in the serum and prevent myocardial injury.

Oxidative and reductive stress are dual dynamic phases experienced by the cells undergoing

adaptation towards endogenous or exogenous *noxious stimulus*²². Changes of SOD, CAT, GSH-Px activity and MDA content are important markers of oxidative stress *in vivo*. The volatile oil of *Angelica sinensis* was used in pituitrin-induced acute myocardial ischemic injury in mice. The activities of SOD, GSH-Px and CAT increased, while the content of MDA decreased²³. Previous studies have found that Lyc has remarkable ROS scavenging and antioxidant properties, which can prevent sperm changes caused by oxidative stress and preserve the function of male germ

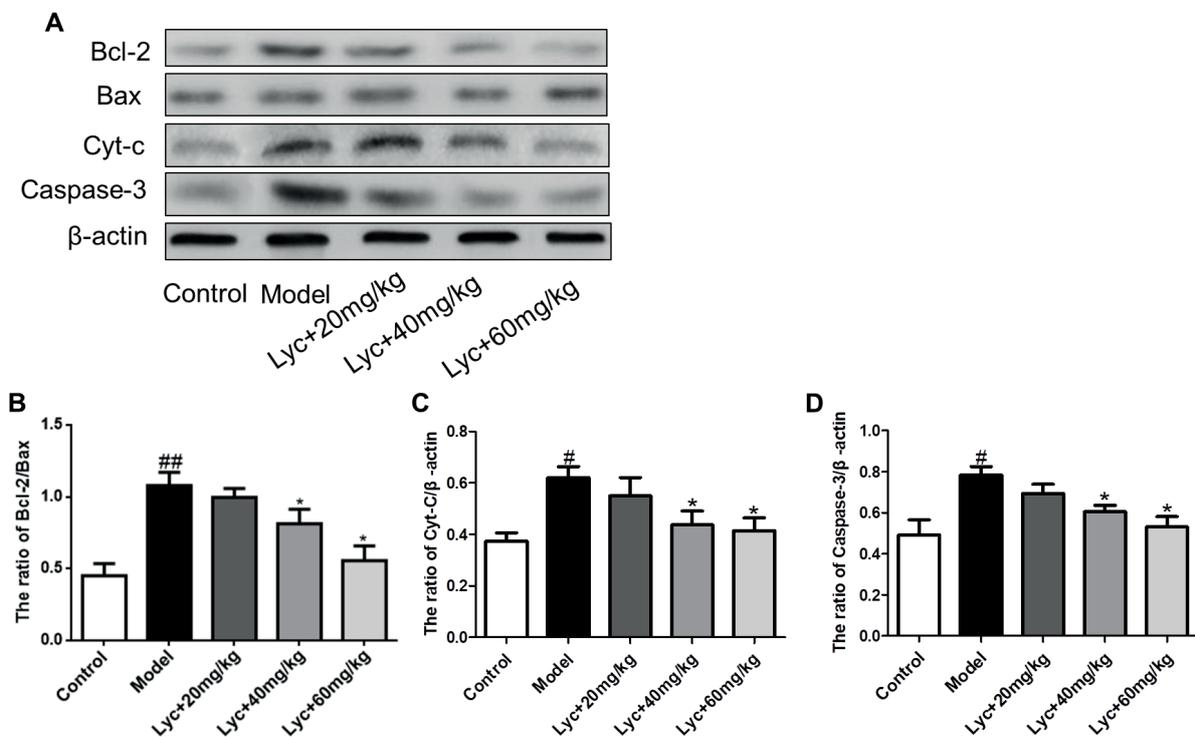


Figure 5. Effect of Lyc on levels of Bax/Bcl-2 (A-B), Cyt-c (C-D), and Caspase-3 (E-F) in experimental rat myocardial tissue.

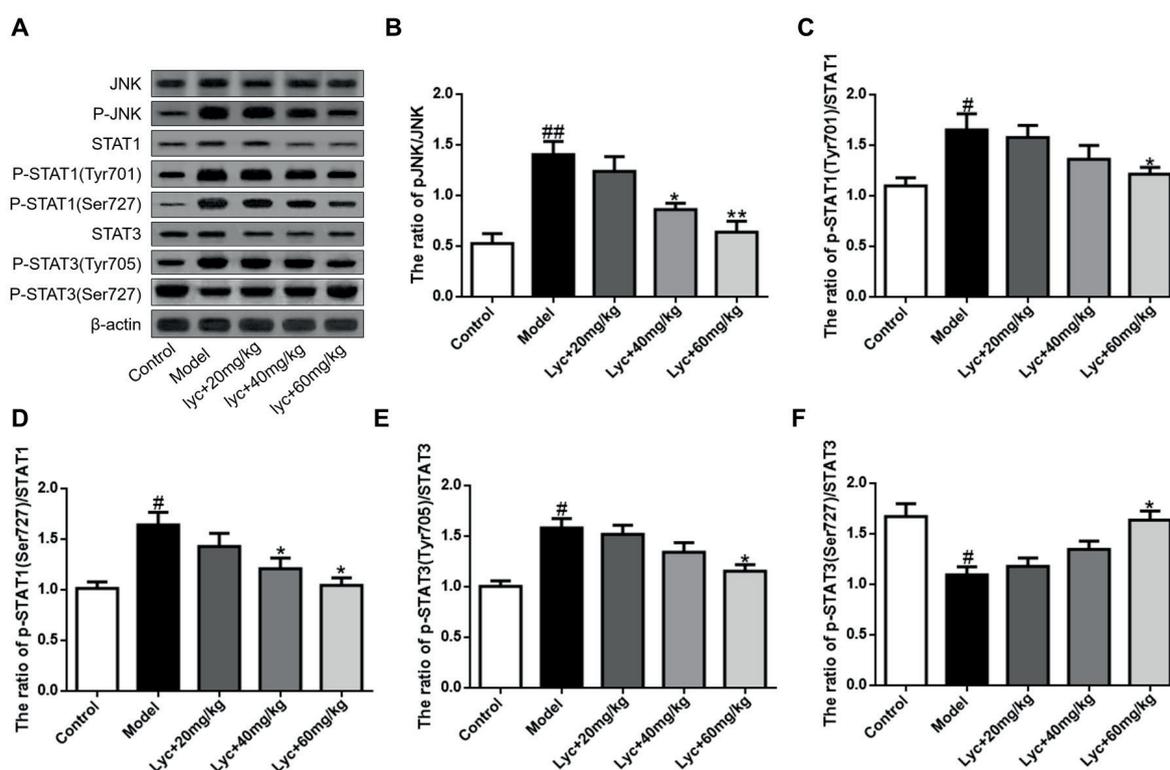


Figure 6. Effect of Lyc on levels of p-JNK/JNK, ERK1/2/p-ERK1/2, p-STAT1(Tyr701)/STAT1, p-STAT1(Ser727)/STAT1, p-STAT3 (Tyr705)/STAT3, p-STAT3 (Ser727)/STAT3 in experimental rat myocardium.

cells²⁴. Lyc was a potent neuroprotectant against apoptosis, oxidative stress and mitochondrial dysfunction, and could be administered to prevent neuronal injury or death²⁵. Lyc exhibited significant ROS-scavenging and antioxidant properties (SOD, CAT, GPx and GSH) which may prevent spermatozoa alterations caused by oxidative stress, and preserve the functionality of male reproductive cells²⁶. In this study, the activities of SOD, CAT and GSH-Px in model group were significantly lower than that in control group, and the content of MDA was significantly increased, indicating that rats in model group were in a state of peroxidative stress. The SOD, CAT, GSH-Px activities and MDA content were all restored after Lyc was administered, indicating that Lyc had a protective effect on oxidative stress injury of myocardial ischemia induced by ISO.

Cytokines and chemokines mediate the immune response of many immune and non-immune cells after myocardial ischemia. PPAR gamma agonists have protective effects on cerebral ischemia-reperfusion injury in rats. The mechanism is related to the decrease of IL-1beta, IL-6 and

TNF-alpha in brain tissue²⁷. Cardiac cytokines (IL-6, IL-1β and TNFα) peaked at 302 h reperfusion, but IL-1β and TNFα levels were unaffected by IL-6 deficiency²⁸. Lyc inhibited LPS-induced production of NO and IL-6 with decreased mRNAs of inducible nitric oxide synthase and IL-6 but had no effect on TNF-α²⁹. In cells treated with Lyc and LPS, the expression of TNF-alpha, IL-1beta, IL-6, iNOS and COX-2 decreased significantly in a dose-dependent manner³⁰. All these results were similar to our study. When the Lyc was administered, the serum IL-1β, TNF-α and IL-6 levels in medium-dose group and high-dose group were significantly decreased in a dose-dependent manner, indicating that Lyc could be served as an anti-inflammatory factor.

Studies have shown that JNK/STAT is activated in the pathogenesis of many heart diseases. JNK/STAT signaling pathway is an important signaling pathway leading to apoptosis and participates in the inflammatory response of myocardial injury³¹⁻³³. The ERK signaling pathway is mainly related to pathological processes such as growth and apoptosis of cardiomyocytes³⁴. Activation of

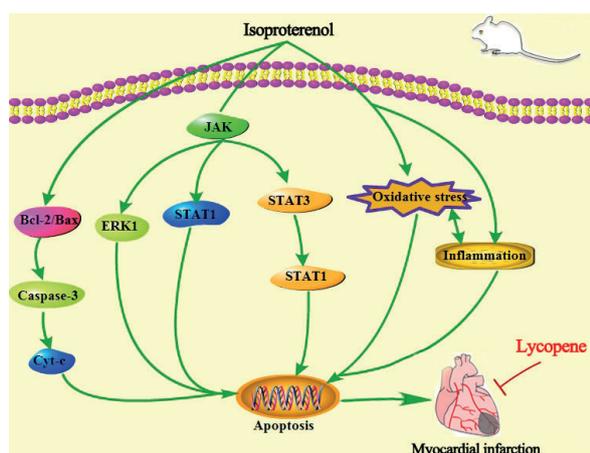


Figure 7. Graphic description of this study.

ERK1/2 can reduce apoptosis induced by myocardial injury. Lyc prevents coal burning fluorosis-induced spermatogenic cell apoptosis through the suppression of oxidative stress-mediated JNK and ERK signaling pathway, which could be an alternative therapeutic strategy for the treatment of fluorosis. Lyc protects SAP in rats by inhibiting the expression of injury-related molecular patterns and antioxidant properties, which may be related to its anti-inflammatory properties, thus maintaining cell homeostasis and preventing phosphorylation of the JNK pathway³⁵. The changes of Cyt-c and Caspase-3 were significantly restored after Lyc administration, suggesting that Lyc has an anti-apoptotic effect and protective effect on myocardial ischemia. The effects of Lyc on the levels of p-JNK/JNK, p-STAT1 (Tyr701)/STAT1, p-STAT1 (Ser727)/STAT1, p-STAT3 (Tyr705)/STAT3 and p-STAT3 (Ser727)/STAT3 indicate that Lyc can attenuate the activation of the JNK/ERK signaling pathway induced by myocardial injury.

Conclusions

The rat model of myocardial ischemia was established by subcutaneous injection of isoproterenol (85 mg/kg) for two consecutive days. We showed that Lyc could prevent myocardial injury and protect the oxidative stress injury of myocardial ischemia. As an anti-inflammatory factor, Lyc could cut off myocardial apoptosis. The possible mechanism of Lyc is to attenuate the activation of the JNK/ERK signaling pathway induced

by myocardial injury. However, our study was conducted in animal models with limited sample size and clinical significance. Further studies are needed to confirm the protective effect of Lyc in patients with clinical myocardial ischemia.

Conflict of Interest

The Authors declare that they have no conflict of interest.

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