

Lycopene protects against myocardial ischemia-reperfusion injury by inhibiting mitochondrial permeability transition pore opening

This article was published in the following Dove Press journal:
Drug Design, Development and Therapy

Xuying Li^{1,*}
Pengyu Jia^{1,*}
Zijun Huang¹
Shuang Liu¹
Jiaxin Miao¹
Yuxuan Guo¹
Nan Wu²
Dalin Jia¹

¹Department of Cardiology, ²The Central Laboratory, The First Affiliated Hospital of China Medical University, Shenyang, Liaoning 110001, People's Republic of China

*These authors contributed equally to this work

Background: Mitochondria permeability transition pore (MPTP) is an important therapeutic target for myocardial ischemia-reperfusion injury (MIRI). Lycopene (LP) is a potent antioxidant extracted from the mature fruits of plants and has been reported to protect against MIRI; however, its mechanism of action has yet to be completely elucidated. The present study aimed to investigate the role of MPTP in the cardioprotection of LP.

Methods: H9c2 cells were pretreated with LP for 12 hrs and were subjected to 12-hr hypoxia/1-hr re-oxygenation, and cell viability was measured by a Cell Counting Kit-8 (CCK-8) assay. Male rats were subsequently intraperitoneally injected with LP for 5 consecutive days. At 24 hrs following the final injection, the rat hearts were isolated and subjected to 30-min ischemia/120-min reperfusion using Langendorff apparatus. The myocardial infarct size was measured by a TTC stain. Opening of the MPTP was induced by CaCl₂ and measured by colorimetry. The change in mitochondrial transmembrane potential ($\Delta\Psi_m$) was observed under a fluorescence microscope. Apoptosis was measured by flow cytometry and a TUNEL stain, and the expression of apoptosis-related proteins was detected by Western blotting.

Results: LP pretreatment significantly increased cell viability, reduced myocardial infarct size and decreased the apoptosis rate. In addition, opening and the decrease of $\Delta\Psi_m$ were attenuated by LP and the expressions of cytochrome *c*, APAF-1, cleaved caspase-9 and cleaved caspase-3 were also decreased by LP. However, these beneficial effects on MIRI were abrogated by the MPTP opener (atractyloside). Furthermore, LP treatment markedly increased Bcl-2 expression, decreased Bax expression and the Bax/Bcl-2 ratio.

Conclusion: The results of the present study demonstrated that LP protects against MIRI by inhibiting MPTP opening, partly through the modulation of Bax and Bcl-2.

Keywords: myocardial ischemia reperfusion injury, lycopene, mitochondrial permeability transition pore, apoptosis

Correspondence: Dalin Jia
Department of Cardiology, The First Affiliated Hospital of China Medical University, 155 North of Nanjing Street, Shenyang, Liaoning 110001, People's Republic of China
Tel +86 024 8328 2728
Email jdl2001@126.com

Nan Wu
The Central Laboratory, The First Affiliated Hospital of China Medical University, 155 North of Nanjing Street, Shenyang, Liaoning 110001, People's Republic of China
Tel +86 024 8328 2728
Email imwunan@163.com

Introduction

Acute myocardial infarction (AMI) is the leading cause of morbidity and mortality worldwide.¹ Reperfusion, the restoration of blood flow, has been considered to be the most effective treatment for ischemic heart disease, in particular, AMI.¹ However, reperfusion has also been labeled as a “double-edged sword” as reperfusion itself may lead to aggravation of myocardial injury, termed myocardial ischemia-reperfusion injury (MIRI).² With the wide application of reperfusion therapy,

such as drug thrombolysis, percutaneous coronary intervention (PCI) and coronary artery bypass grafting (CABG), the identification of the most effective mechanism of MIRI prevention has become imperative.^{3–5}

Lycopene (LP) is a well-established potent antioxidant extracted from the mature fruits of plants.^{6,7} The range of biological effects of LP include the suppression of oxidative stress,⁸ tumor formation⁹ and inflammation.¹⁰ Lycopene has been demonstrated to serve a role in protection against myocardial ischemia/reperfusion injury in neonatal rats.¹¹

Mitochondrial permeability transition pore (MPTP) is a non-specific channel located in the inner mitochondrial membrane.¹² MPTP remains closed during ischemia; however, rapidly opens following the commencement of reperfusion.¹³ Opening of the MPTP is considered to be one of the important mechanisms of MIRI.¹² In addition, the inhibition of MPTP opening by cyclosporine A (CsA) may attenuate MIRI.¹⁴ Therefore, MPTP is considered to be an important therapeutic target for the prevention of MIRI. However, the effect of LP on MPTP in MIRI remains unknown. Therefore, the present study hypothesized that LP would attenuate MIRI by inhibiting the opening of MPTP.

The aim of the present study was to investigate the effects of LP on MIRI in an H9c2 cell model and an isolated rat heart model, and to further explore the role of MPTP in the cardioprotection of LP. The present study demonstrated that LP could inhibit MPTP opening, partly via modulation of Bax and Bcl-2, thereby protecting against MIRI.

Materials and methods

Drug

Lycopene and medicinal corn oil were purchased from Beijing Solarbio Biological Technology Co., Ltd. (Lycopene CAS: 502-65-8, purity: $\geq 98\%$; medicinal corn oil CAS: 8001-30-7, purity: $\geq 98\%$). Atractyloside (ATR) was purchased from Nantong Feiyu Biological Technology Co. Ltd. (CAS: 102130-43-8, purity: $\geq 98\%$). 2,3,5-triphenyl-tetrazolium chloride (TTC) was purchased from Sigma-Aldrich (CAS: 298-96-4, Merck KGaA). Lycopene and ATR powder used in our animal experiment were soluted in 2 mL medicinal corn oil and normal saline in preparation for intraperitoneal injection, respectively. Lycopene and ATR powder used in the cell experiments were dissolved in dimethyl sulfoxide (DMSO) (CAS: 67-68-5, Sigma-Aldrich, Merck KGaA).

Cell culture

H9c2 cells (Shanghai Institute for Biological Sciences, China) were cultured in Low Glucose Dulbecco's Modified Eagle's Medium (DMEM) (Gibco; Thermo Fisher Scientific, Inc., Waltham, MA, USA) containing 10% fetal bovine serum (FBS) (TBD, Tianjin, China) and 1% penicillin/streptomycin (Hyclone; GE Healthcare Life Sciences, Logan, UT, USA) under humid conditions in incubators with 5% CO₂ at 37°C.

Cell preparation

H9c2 cells were subjected to hypoxia/re-oxygenation (H/R) to mimic the MIRI model in vitro. Specifically, they were pre-starved using DMEM medium without fetal bovine serum for 12 hrs when the H9c2 cells reached 70% confluence in the aforementioned culture dishes. The culture medium was then removed and replaced with Earle's medium.¹⁵ The H9c2 cells underwent hypoxic induction via culturing in tri-gas incubator with 90% N₂, 5% CO₂ and 5% O₂ in a mixed-gas perfusion hypoxia chamber, and were incubated for an additional 12 hrs at 37°C.¹⁶ Following hypoxic incubation, Earle's medium was removed and the H9c2 cells were restored to 95% air and 5% CO₂ at 37°C with 10% medium for re-oxygenation for an additional 1 hr. The H/R model of H9c2 cells was prepared.

Cell experimental protocol

The experiment consisted of two phases. Firstly, H9c2 cells were randomly divided into 8 groups at the first phase as follows: (i) control group (Control): H9c2 cells were incubated in normoxic conditions at 37°C in a cell culture incubator for 13 hrs; (ii) hypoxia/re-oxygenation group (H/R): H9c2 cells were subjected to hypoxia for 12 hrs followed by re-oxygenation for 1 hr; (iii) solvent group (Vehicle): H9c2 cells were pretreated with 0.1% DMSO for 12 hrs and underwent HR group procedures; (iv)–(viii) 2.5 μ M LP pretreatment group (LP 2.5 μ M), 5 μ M LP pretreatment group (LP 5 μ M), 10 μ M LP pretreatment group (LP 10 μ M), 20 μ M LP pretreatment group (LP 20 μ M), 40 μ M LP pretreatment group (LP 40 μ M): H9c2 cells were pretreated with LP at the dose of 2.5 μ M, 5 μ M, 10 μ M, 20 μ M and 40 μ M for 12 hrs and underwent HR group procedures.

In the second phase (according to the result of first phase), 10 μ M LP was selected to explore the mechanism further. Therefore, H9c2 cells were further divided into 4 groups as follows: (i) Control group; (ii) HR group; (iii)

LP 10 μ M group; (iv) LP 10 μ M combined with ATR pretreatment group (LP 10 μ M + ATR): H9c2 cells were pretreated with 10 μ M LP and 20 μ M ATR for 12 hrs and underwent HR group procedures.

Animals

A total of 110 healthy male Wistar rats of grade SPF, weighing 280 ± 10 g, were purchased from Changsheng Biotechnology Co., Ltd. (Benxi, Liaoning). The experimental rats were kept at 25°C and were fed on water and normal rat food. All rats were treated and procedures abided to the Guide for the Care and Use of Laboratory Animals (NIH, USA). The animal experiments were approved by the institutional Ethics Committee of China Medical University.

Establishment of MIRI model in isolated rat hearts

Heart preparation was performed according to the protocol outlined in a previous study.¹⁷ Pentobarbital sodium (100 mg/kg) was administered intravenously to anesthetize the rats. Meanwhile, heparin (1,500 IU/kg) was injected intravenously to block coronary artery thrombosis. Following opening of the thoracic cavity, the heart was swiftly removed and immediately immersed in ice-cooled heparinized Krebs-Henseleit (K-H) solution.¹⁸ The isolated heart was then mounted on Langendorff apparatus from the root of the aorta and subjected to perfusion with K-H solution saturated with 95% O₂+5% CO₂ at a constant temperature of 37°C. All isolated hearts were continually perfused with K-H solution for 10 mins of stabilization prior to the commencement of ischemia. All isolated rat hearts were subjected to 30 mins of global ischemia, followed by 120 mins of reperfusion to generate the MIRI model.¹⁹

Animal experimental protocol

Similar to the cell experimental protocol, the animal experimental protocol consisted of two phases. In the first phase, 30 rats were divided into the following 5 groups with 6 rats per group: (i) ischemia-reperfusion group (IR): As described above; (ii) solvent group (Vehicle): The rats were pretreated with 2 mL medicinal corn oil by intraperitoneal injection once per day for 5 days, and underwent IR procedures; (iii–v) 10 mg/kg LP pretreatment group (LP 10 mg/kg), 20 mg/kg LP pretreatment group (LP 20 mg/kg), 40 mg/kg LP pretreatment group (LP 40 mg/kg): The rats

were pretreated with 10, 20, 40 mg/kg LP by intraperitoneal injection once per day, for a total of 5 days, and underwent IR group procedures.²⁰

In the second phase (according to the results in the first phase), 40 mg/kg LP was selected as the most appropriate dose. A total of 80 rats were divided into the following 4 groups with 20 rats per group in the second phase: (i) IR group; (ii) Vehicle group; (iii) LP 40 mg/kg group; (iv) 40 mg/kg LP in combination with ATR pretreatment group (LP 40 mg/kg + ATR): 5 mg/kg ATR was injected intraperitoneally 30 mins prior to extraction of the heart, and the remainder of the procedure was as described for the LP 40 mg/kg group.

Measurement of H9c2 cells viability

H9c2 cells viability was determined by the Cell Counting Kit-8 (CCK-8) and the Cell proliferation toxicity test kit (Biosharp, Beijing, China) according to the manufacturer's protocols at 450 nm by an ultramicro microporous plate spectrophotometer (Biotek, USA).²¹

Measurement of infarct size

Following reperfusion, the hearts were removed and frozen at -20°C for 1 hr; the frozen hearts were then rapidly cut into 1–2 mm sections and incubated in 1% TTC at 37°C for 20 mins. The heart slices were photographed using a digital camera and the infarcted area was analyzed by Image J software (National Institutes of Health, Bethesda, MD, USA). The results were expressed as a percentage of infarct size by the division of the calculated total infarct size with the total heart volume.

Change of the mitochondrial transmembrane potential ($\Delta\Psi_m$)

The change of $\Delta\Psi_m$ was detected by using Mitochondrial Membrane Potential Assay Kit with JC-1 (Beyotime Institute of Biotechnology, Shanghai, China) according to the manufacturer's protocol. To be specific, JC-1 is an ideal fluorescent probe widely used for the detection of $\Delta\Psi_m$. When $\Delta\Psi_m$ is normal and the state of MPTP was closed, JC-1 accumulates in the matrix of mitochondria and forms J-aggregates that produce red fluorescence. However, when $\Delta\Psi_m$ is decreased once the MPTP opened, JC-1 cannot aggregate in the matrix of the mitochondria and maintains a monomer that produces green fluorescence. The fluorescence was detected under a fluorescence microscope and photographed.

Sensitivity of MPTP to calcium

The mitochondria were isolated from heart tissues using Tissue Mitochondria Isolation kit (Beyotime Institute of Biotechnology) according to the manufacturer's protocol. The reaction of MPTP to calcium was determined using Purified Mitochondrial Membrane Pore Channel Colorimetric Assay kit (GENMED, Shanghai, China) according to the manufacturer's protocol.²² Specifically, MPTP opening was induced by CaCl_2 . The value of optical density (OD) was read at 520 nm from 0 to 10 mins by an ultramicro microporous plate spectrophotometer (Biotek, USA). The decrease in OD reflected the extent of MPTP opening.

Measurement of apoptosis

The determination of H9c2 cells apoptosis was based on the Annexin V-Fluorescein isothiocyanate (FITC)/Propidium Iodide (PI) Kit (KeyGEN BioTECH, Jiangsu, China) according to the manufacturer's protocol, using a flow cytometer (BD Biosciences, Franklin Lakes, NJ, USA). Myocardial apoptosis was detected by a Terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) assay with the In Situ Cell Death Detection kit (Roche Diagnostics) according to the manufacturer's protocol.

Western blotting

H9c2 cells and left ventricle tissues were homogenized with radioimmunoprecipitation buffer (Beyotime Institute of Biotechnology, Shanghai, China) and protease inhibitor phenylmethanesulfonyl fluoride (PMSF) (Beyotime Institute of Biotechnology) on ice for 20 mins. Proteins were extracted from lysates following centrifugation. The concentration of protein was measured using Enhanced BCA Protein Assay kit (Beyotime Institute of Biotechnology). For Western blotting analysis, 50 μg protein was denatured with heat and loaded onto 10% SDS polyacrylamide gels and then transferred to PVDF membranes. The membrane was blocked with 5% skim milk for 1 hr and incubated overnight at 4°C with primary antibodies (Abcam, Cambridge, UK), including monoclonal anti-cytochrome *c* (1:1,000), anti-apoptotic protease activating factor-1 (APAF-1) (1:1,000), anti-cleaved caspase-9 (1:1,000), anti-cleaved caspase-3 (1:1,000), anti-B cell lymphoma-2 (Bcl-2) (1:1,000), anti-Bax (1:1,000) and anti- β -actin (1:2,000). The membranes were then incubated with horseradish peroxidase-conjugated goat anti-rabbit or rabbit anti-mouse immunoglobulin G (1:2,000; Santa Cruz Biotechnology, Inc., Dallas, TX, USA) at 37°C for 2 hrs. The detection of protein bands

was performed using an enhanced chemiluminescence (ECL) for Western blotting kit (Santa Cruz Biotechnology, Inc.) according to the manufacturer's protocol. The levels of phosphorylated proteins were normalized to their corresponding total protein levels. Relative densitometry was calculated using Image J2x analysis software (NIH).

Measurement of survival rate

According to the manufacturer's protocol of Trypan blue staining cell survival (Beyotime Institute of Biotechnology), the H9c2 cells were stained, observed and photographed using a biological microscope (Nikon Corporation, Tokyo, Japan). The cell viability was quantified as percentage of unstained cells over total cells.

Measurement of lactate dehydrogenase (LDH) activity

LDH activity of the H9c2 cells was determined by LDH assay kit (Nanjing Jiancheng Bioengineering Institute, Nanjing, China) according to the manufacturer's protocols at 450 nm by ultramicro microporous plate spectrophotometer (Biotek, USA).

Statistical analysis

All data were expressed as mean $\pm$ standard deviation and statistically analyzed using software SPSS 17.0 version (SPSS, Inc., Chicago, IL, USA). The differences between groups were evaluated using one-way analysis of variance (ANOVA); if a significant difference was revealed, multiple comparison analysis was performed using Fisher's Least Significant Difference (LSD) test. $P < 0.05$ was considered to indicate a statistically significant difference.

Results

LP prevents MIRI in vitro and ex vivo

The result of the CCK-8 assay revealed that pretreatment with 2.5, 5, 10 and 20 μM LP significantly increased cell viability. As a dose of 10 μM LP achieved a higher cell viability when compared with 2.5, 5 and 20 μM LP (Figure 1A), 10 μM LP was the selected dose for the subsequent in vitro studies.

The results of the TTC stain revealed that the infarct size in the LP treatment group (10, 20 and 40 mg/kg) was lower than the IR group. In addition, the LP 40 mg/kg group had a reduced percentage of myocardial infarct size, when compared with the 10 and 20 mg/kg groups (Figure 1B). Therefore, a dose of 40 mg/kg LP was selected for further study ex vivo.

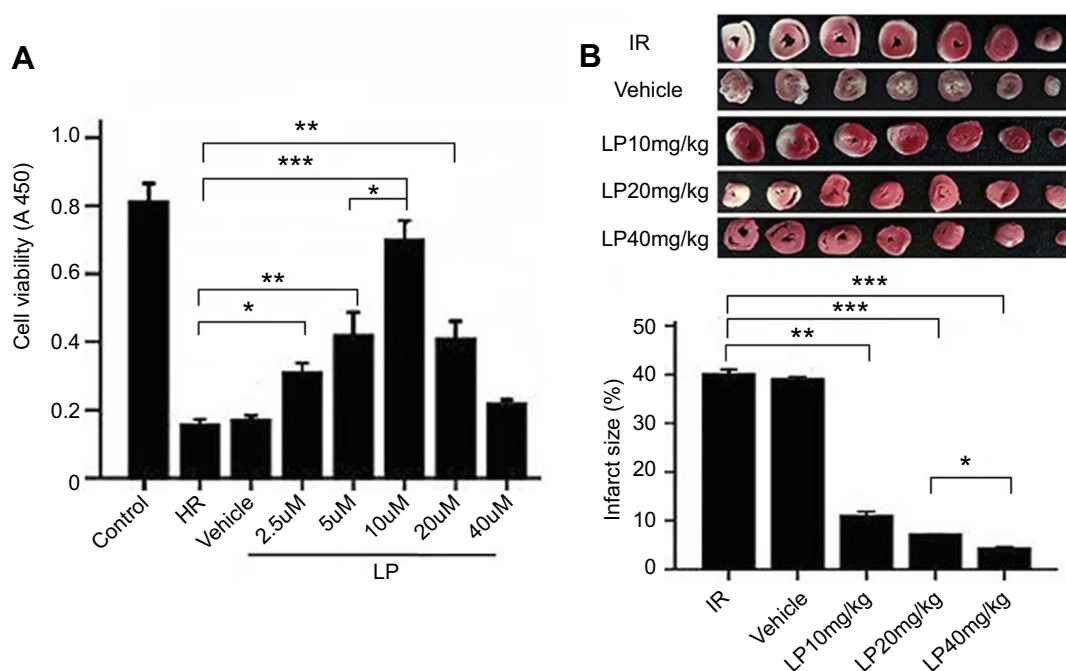


Figure 1 Protective effect of lycopene (LP) on myocardial ischemia-reperfusion injury. **(A)** H9c2 cells were pretreated with different concentrations of LP for 12 hrs, which was followed by 12 hr-hypoxia/1-hr reoxygenation (H/R). Cell viability was detected by the CCK-8 assay. Data were expressed as mean±standard SEM, n=3. * $P<0.05$, ** $P<0.01$, *** $P<0.001$. **(B)** The rats were injected with different doses of LP for 5 consecutive days, at 24 hrs following the final injection, the hearts were isolated and underwent 30 mins ischemia/120 mins reperfusion (IR). The myocardial infarct size was assessed by TTC staining. The non-infarction areas were red or pink stained, whereas the infarction areas were white or gray. Data were expressed as mean±standard SEM; n=6 per group; * $P<0.05$, ** $P<0.01$, *** $P<0.001$.

Effect of LP on the opening of MPTP

MPTP opening is primarily induced by calcium²³ and the sensitivity of MPTP to calcium can be considered to be an indicator of MPTP opening.^{22,24} The isolated mitochondria in the LP 40 mg/kg group were more resistant to stimulation by Ca^{2+} compared with the IR group (Figure 2A and B). $\Delta\Psi_m$ will be dramatically decreased, once MPTP opens. Therefore, the change of $\Delta\Psi_m$ reflects the extent of MPTP opening. The results demonstrated that the LP treatment group retained a higher level of $\Delta\Psi_m$ than the HR group (Figure 2C). Taken together, the aforementioned results suggest that LP inhibited the opening of the MPTP.

Effect of LP on the apoptosis

Cell apoptosis was determined by flow cytometry and the TUNEL assay. The flow cytometry results revealed that the apoptosis rate in the LP treatment group was lower than that in the HR group (Figure 3A); however, the apoptosis rate returned to its increased levels following treatment with ATR. The results of the TUNEL assay exhibited a similar trend as the flow cytometric analysis (Figure 3B). Therefore, the above results suggest that LP could inhibit cell apoptosis through the MPTP.

Regulation of LP on mitochondrial apoptotic pathway

MPTP opening usually leads to the release of cytochrome *c*, subsequently leading to the activation of caspases. Western blot analysis performed by the present study revealed that in the LP treatment group, the expression of cytochrome *c* was significantly decreased, as were the expressions of APAF-1, cleaved caspase-9 and cleaved caspase-3 in cardiomyocytes (Figure 4A) and the myocardium (Figure 4B). However, following treatment with ATR, these protein expressions were returned to their elevated levels. Taken together, these results suggest that LP could repress the mitochondrial apoptotic pathway through MPTP.

Regulation of LP on Bax and Bcl-2

The Bcl-2 family of proteins are key regulators of MPTP opening.²⁵ The present study measured the changes in the expressions of Bax and Bcl-2 by Western blotting, and the results demonstrated that treatment with LP significantly increased the expression of Bcl-2, whereas the expression of Bcl-2 was decreased. In addition, the ratio of Bax in relation to Bcl-2 was significantly decreased following treatment with LP (Figure 5A and B).

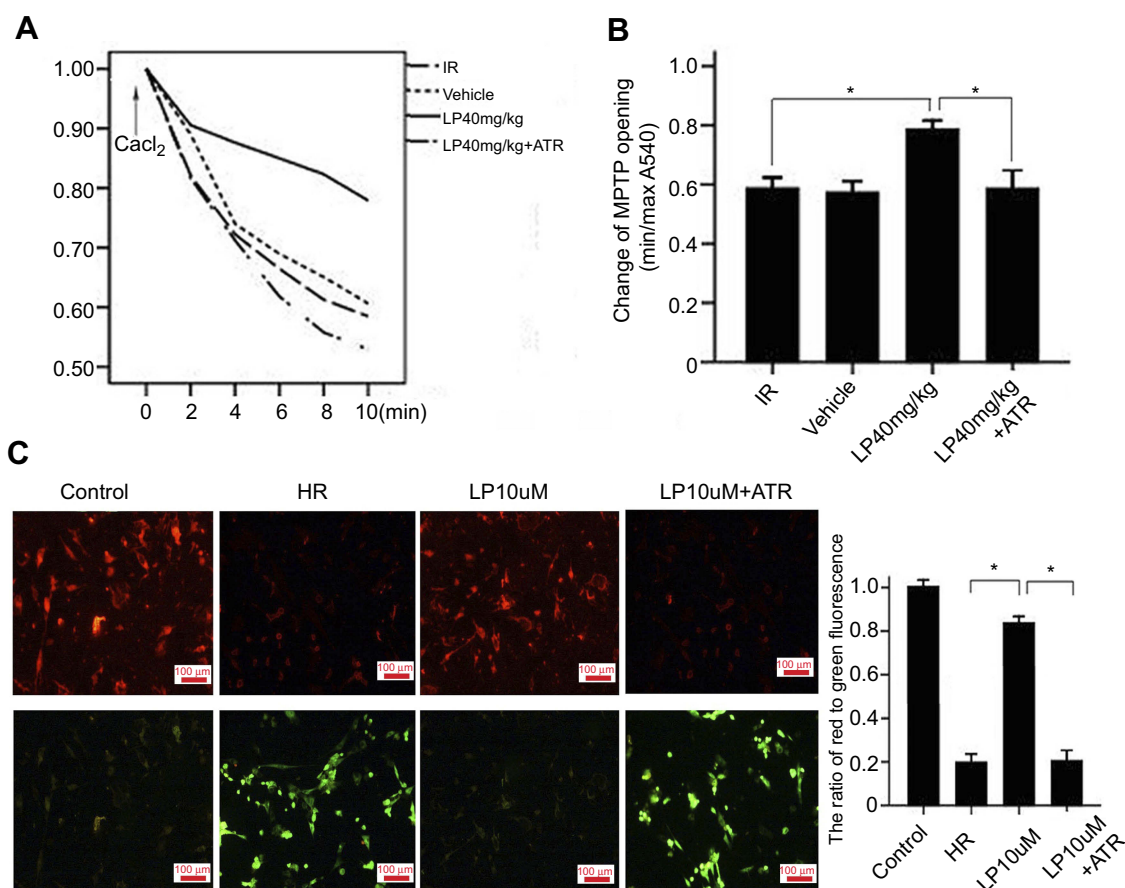


Figure 2 Effect of lycopene (LP) on mitochondrial permeability transition pore (MPTP) opening. The rats were injected with 40 mg/kg/d LP for 5 consecutive days, at 24 hrs following the final injection, the hearts were isolated and underwent 30 mins ischemia/120 mins reperfusion (IR). A total of 5 mg/kg atractyloside (ATR), an MPTP opener, was injected intraperitoneally at 30 mins prior to extraction of the heart. **(A)** MPTP opening was induced by CaCl_2 . **(B)** Statistical analysis for MPTP opening. Minimum optical density (min OD) represents the OD value recorded at the onset of the experiment (0 min); maximum optical density (max OD) represents the OD value recorded at the end of the experiment (10 mins). Min/max OD is negatively associated with the extent of MPTP opening. Data were expressed as mean $\pm$ standard SEM; $n=6$ per group; $*P<0.05$. **(C)** H9c2 cells were pretreated with 10 μM LP/20 μM ATR for 12 hrs, followed by 12 hr-hypoxia/1h-reoxygenation (H/R). The change of mitochondrial membrane potential was observed under a fluorescence microscope. The level of mitochondrial membrane potential was quantified by the ratio of red-to-green fluorescence intensity. Magnification $\times 400$, scale bar: 100 μm . Data were expressed as mean $\pm$ standard SEM. $n=3$ per group; $*P<0.05$.

ATR abrogated the cardioprotection of LP

To further validate the role of MPTP in the protection of MIRI, the influence of an MPTP opener (ATR) on the cardioprotection of LP was investigated. As illustrated in Figure 6, the increase in cell viability, the reduction in LDH release and myocardial infarct size by LP treatment were also abolished following treatment with ATR. These results confirmed that LP protects against MIRI by inhibiting MPTP opening.

Discussion

In the present study, pretreatment with the pharmacological agent LP at a dose of 2.5, 5, 10 or 20 μM was revealed to increase the survival rate of H9c2 cells, with a dose 10 μM LP achieving the highest cell viability. However, no effect was observed at a dose of 40 μM . Meanwhile, LP at a dosage of 10, 20 or 40 mg/kg was revealed to reduce the myocardial

infarction area and apoptosis to the myocardium in rats, which suggested that LP could protect against MIRI. Therefore, it was inferred that the dose-related effect of LP occurred between 5 and 20 μM ; this is consistent with the results of a previous study by Gao Y et al,²⁶ who used H9c2 cells to perform in vivo hypoxia for 4 hrs and re-oxygenation for 4 hrs. In the present study, the H9c2 cells were pretreated with LP at a concentration of 10 μM prior to hypoxia. The results demonstrated that LP attenuated ERS and apoptosis induced by H/R in H9c2 cells via inhibiting the expression of CHOP, p-JNK and caspase-12 cellular apoptosis pathways. However, in the present study, pretreatment with LP at doses of 2.5, 5, 10 and 20 μM could successfully attenuate the decrease in cell viability. The reason for this disparity may include distinctions between the duration of procedures and dosing methods.

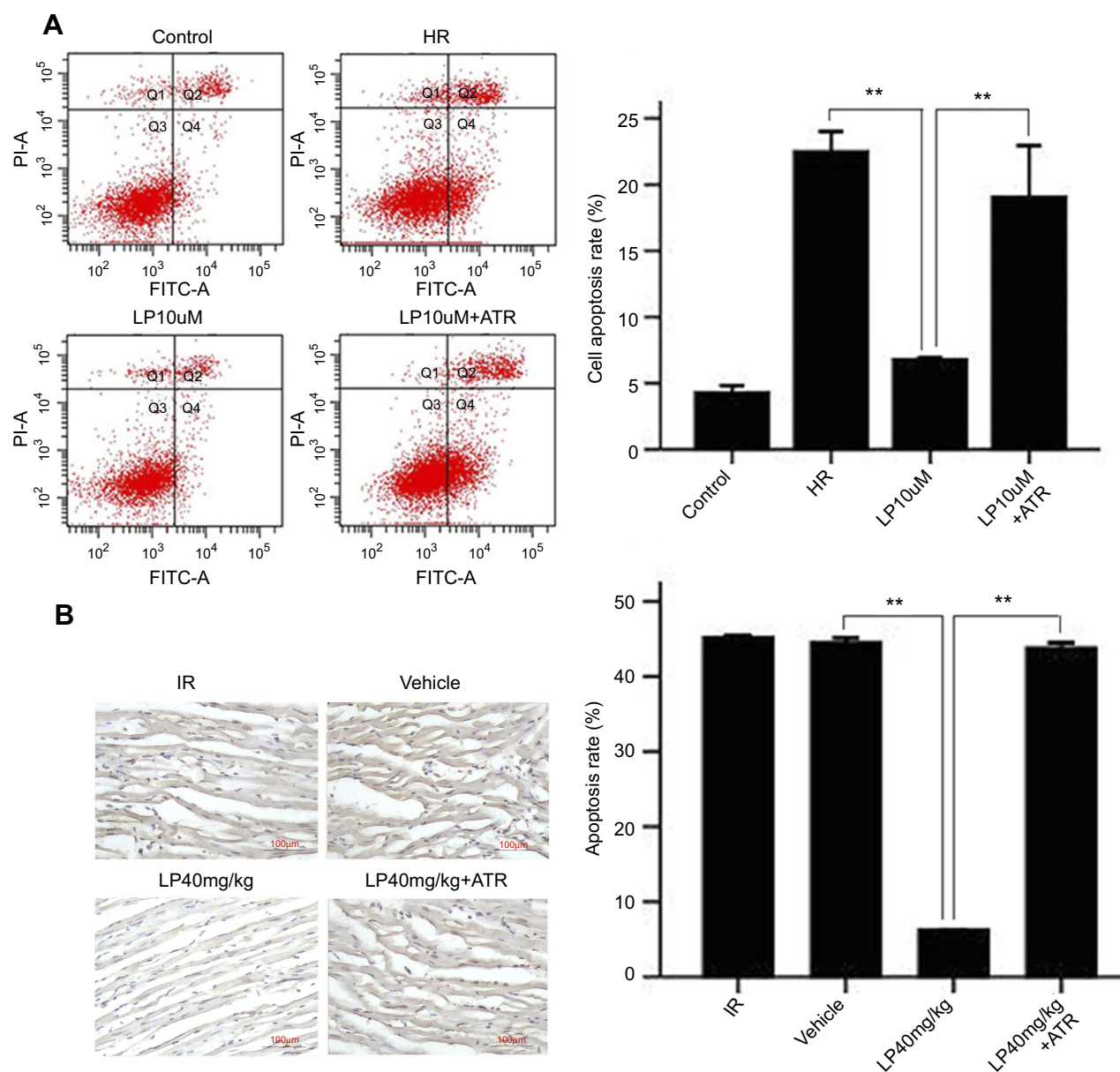


Figure 3 Effect of lycopene (LP) on apoptosis. **(A)** H9c2 cells were pretreated with 10 μ M LP/20 μ M atractyloside (ATR) for 12 hrs, followed by 12-hr hypoxia/1-hr reoxygenation (H/R). Apoptotic cells were detected with flow cytometry by AnnexinV-FITC and PI counterstaining. The viable cells were denoted with a Q3, late and early apoptotic cells were denoted with a Q2 and a Q4, respectively, and necrotic cells were denoted with a Q1. Data were expressed as mean $\pm$ standard SEM, n=3, ** P <0.01. **(B)** The rats were injected with 40 mg/kg/d LP for 5 consecutive days, at 24 hrs following the final injection, the hearts were isolated and underwent 30-min ischemia/120-min reperfusion (IR). A total of 5 mg/kg atractyloside (ATR), an MPTP opener, was injected intraperitoneally at 30 mins prior to extraction of the heart. Morphological characteristics of cardiac tissue apoptosis were determined by staining with the TUNEL assay. Apoptotic cardiomyocyte nuclei appeared with brown staining whereas negative nuclear appeared blue. Photomicrographs were taken at x400 magnification. Data were expressed as mean $\pm$ standard SEM, n=6 per group, ** P <0.01.

To the best of our knowledge, the present study is the first to demonstrate the effect of LP on the inhibition of MPTP in MIRI. Over-opening of MPTP has been reported to cause irreversible damage to the heart.¹² According to previous reports, the core components of MPTP are Voltage-Dependent Anion Channel (VDAC), Adenine Nucleotide Transporter (ANT) and cyclophilin-D.^{27,28} Over-opening of MPTP can be induced by insufficient

intracellular ATP resynthesis, reactive oxygen species-induced oxidative stress, Ca^{2+} and phosphate overloading.^{29,30} Several studies have previously demonstrated that MIRI is closely associated with the opening of the MPTP.³¹ Certain pharmacological preconditioning treatments such as Irisin, melatonin and carnosis acid have been reported to be helpful in alleviating MIRI via inhibition of MPTP opening.^{16,32} In the present study, it

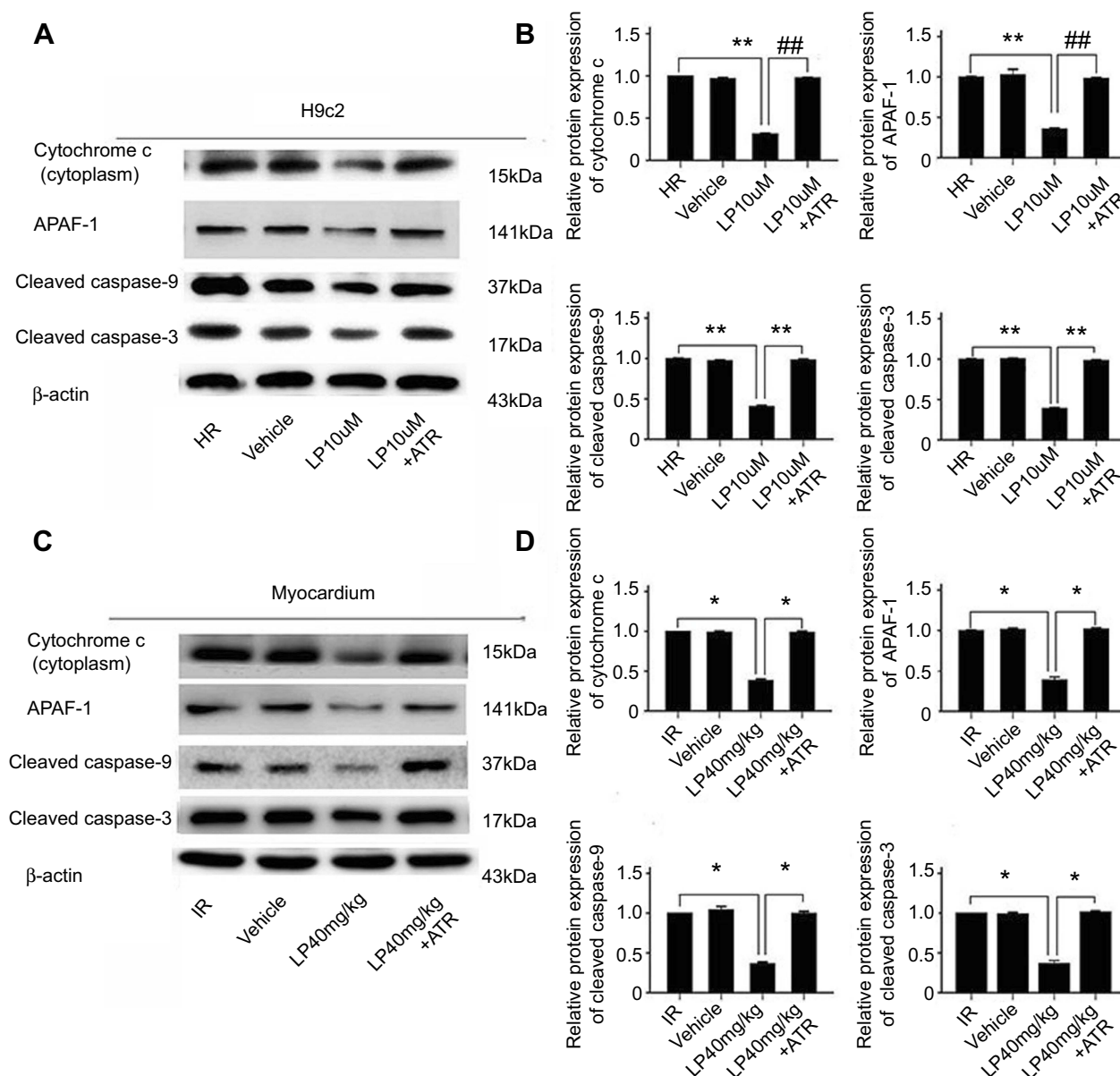


Figure 4 Effect of lycopene (LP) on mitochondrial downstream apoptotic pathway. **(A)** H9c2 cells were pretreated with 10 μ M LP/20 μ M atractyloside (ATR)/0.1% DMSO (Vehicle) for 12 hrs, followed by 12-hr hypoxia/1-hr reoxygenation (H/R). The protein expressions of cytochrome c, APAF-1, cleaved caspase-9 and cleaved caspase-3 were measured by Western blotting. Data were expressed as mean $\pm$ standard SEM, $n=3$, ** $P<0.01$. **(B)** The rats were injected with 40 mg/kg/d LP or 2 mL medicinal corn oil (Vehicle) for 5 consecutive days, 24 hrs after last injection, the hearts were isolated and undergone 30-min ischemia/120-min reperfusion (IR). 5 mg/kg atractyloside (ATR), an MPTP opener, was injected intraperitoneally 30 mins prior to extraction of the heart. The protein expressions of cytochrome c, APAF-1, cleaved caspase-9 and cleaved caspase-3 were measured by Western blot analysis. Data were expressed as mean $\pm$ standard SEM, $n=3$, * $P<0.05$, ** $P<0.01$.

was demonstrated that LP inhibited MPTP opening during the process of MIRI. First, Ca^{2+} overload induced MPTP opening, and compared with the IR group, it was observed that isolated mitochondria of rat hearts pretreated with LP were more resistant to Ca^{2+} stimulation, which indicated that LP could inhibit the opening of the MPTP. In addition, MPTP opening could result in a decrease in $\Delta\Psi_m$. Thus, the prevention of $\Delta\Psi_m$ by treatment with LP also provided evidence to support the hypothesis that LP could inhibit

the opening of the MPTP. However, the decrease/increase of $\Delta\Psi_m$ not only resulted from MPTP opening/closure but also from decreased/increased efficacy of the electron transport chain. Therefore, this is considered as an indirect evidence. Second, it was revealed that LP pretreatment reduced the expression of cytochrome c, APAF-1, cleaved caspase-9 and cleaved caspase-3, and decreased the rate of cell apoptosis. This was similar to the results of a study by Yang W et al³³ which was investigated using rat brains.

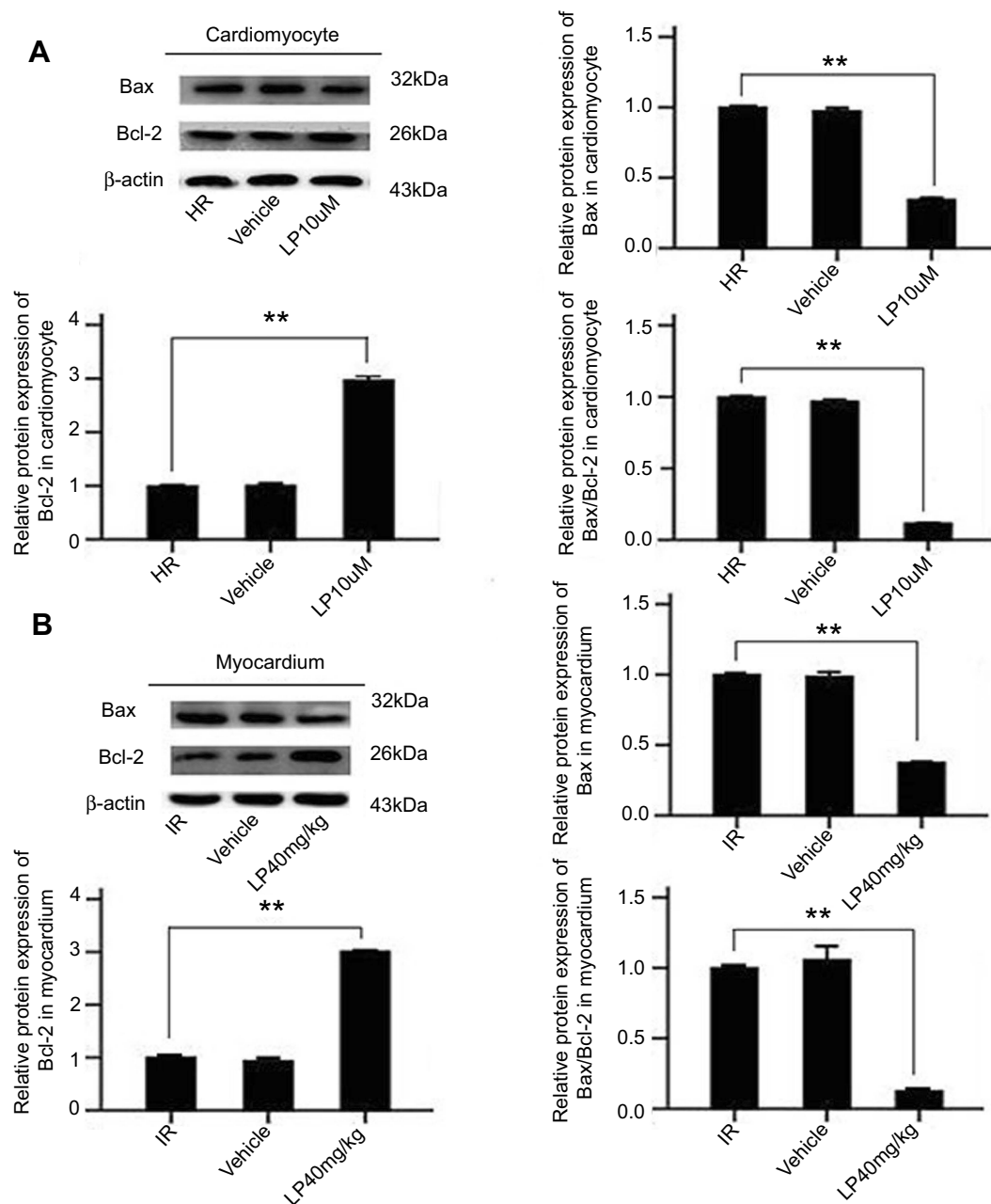


Figure 5 Effects of lycopene (LP) on the expression of Bax and Bcl-2. **(A)** H9c2 cells were pretreated with 10 μ M LP/0.1% DMSO (Vehicle) for 12 hrs, followed by 12-hr hypoxia/1-hr reoxygenation (H/R). The protein expressions of Bax and Bcl-2 were measured by Western blot analysis. Data were expressed as mean $\pm$ standard SEM, n=3, ** P <0.01. **(B)** The rats were injected with 40 mg/kg/d LP or 2 mL medicinal corn oil (Vehicle) for 5 consecutive days, 24 hrs following the last injection, the hearts were isolated and underwent 30-min ischemia/120-min reperfusion (IR). The protein expressions of Bax and Bcl-2 were measured by Western blot analysis. Data were expressed as mean $\pm$ standard SEM, n=3, ** P <0.01.

Nevertheless, all of the protective effects of LP were notably abolished by pretreatment with ATR, which supports the hypothesis that LP exerts a protective effect against MIRI by inhibiting MPTP opening.

MPTP opening has been observed to be regulated by Bax and Bcl-2.³⁴ Bax can promote MPTP opening through directly binding to ANT or VDAC, two major components of MPTP. While Bcl-2 can suppress MPTP opening through

interfering the interaction between Bax and ANT/VDAC by competing with ANT for the Bax binding site; therefore, the Bax/Bcl-2 balance is critical for maintaining MPTP opening.^{35,36} In the present study, LP pretreatment was observed to increase the expression of Bcl-2, meanwhile decrease the expression of Bax, and the Bax/Bcl-2 ratio. Therefore, we hypothesize that LP may inhibit ischemia/reperfusion-induced MPTP opening via regulating the

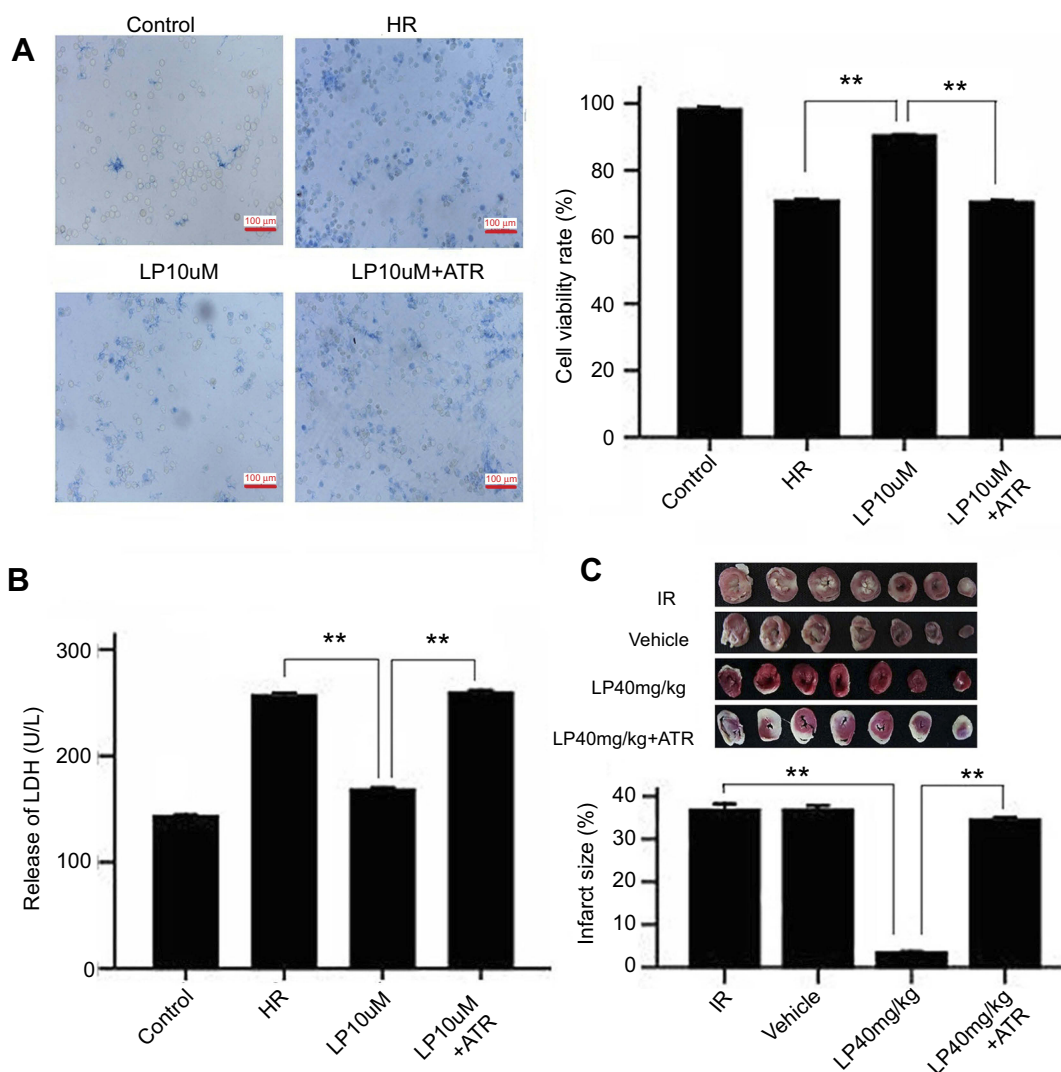


Figure 6 Cardioprotection of lycopene (LP) was abrogated by atractyloside (ATR). H9c2 cells were pretreated with 10 μ M LP/20 μ M ATR for 12 hrs, followed by 12-hr hypoxia/1-h reoxygenation (H/R). **(A)** H9c2 cell viability was assessed by trypan blue staining. Trypan staining can dye dead cells blue, and surviving cells were colored and transparent. Data were expressed as mean $\pm$ standard SEM, $n=3$, $**P<0.01$. **(B)** LDH activity in culture medium. Data were expressed as mean $\pm$ standard SEM, $n=3$, $**P<0.01$. **(C)** The rats were injected with 40 mg/kg/d LP for 5 consecutive days, at 24 hrs following the injection, the hearts were isolated and underwent 30-min ischemia/120-min reperfusion (IR). A total of 5 mg/kg atractyloside (ATR), an MPTP opener, was injected intraperitoneally at 30 mins prior to extraction of the heart. The myocardial infarct size was assessed by TTC staining. The non-infarction areas were stained red or pink, whereas the infarction areas were white or gray. Data were expressed as mean $\pm$ standard SEM, $n=6$ per group, $**P<0.01$.

expression of Bcl-2 and Bax (Figure 7). However, MPTP opening is also regulated by other proteins. For example, inactivation of glycogen synthase kinase 3 β (GSK-3 β) by its phosphorylation at Ser9 has been reported to elevate the threshold for MPTP opening, thereby suppressing the extent of MPTP opening.³⁶ Therefore, whether LP has an effect on GSK-3 β should be investigated in future studies.

There are limitations to the present study. First, the isolated rat heart model used was deprived of neural and humoral regulation, and therefore could not completely mimic pathophysiological changes which occur during MIRI. Therefore, the in vivo cardioprotective effects of LP

should be examined in future studies. Second, it was only demonstrated that LP inhibited MPTP opening via Bcl-2, and other upstream pathways of MPTP, such as PI3K/Akt, GSK-3 β and janus kinase/signal transducer and activator of transcription pathways were not confirmed in the present study.

Conclusion

The present study demonstrated that LP could inhibit MPTP opening, partly via modulation of Bax and Bcl-2. However, the detailed underlying mechanisms of action warrant further investigation in future studies.

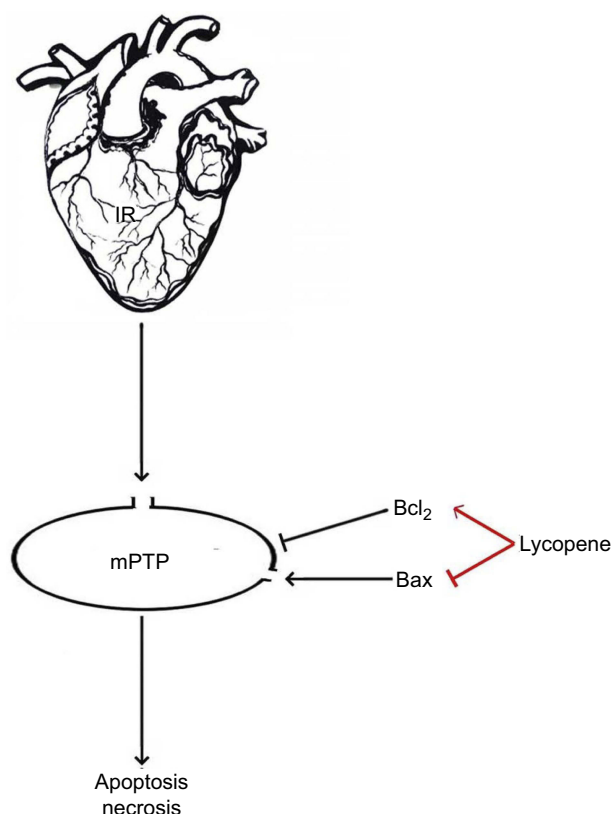


Figure 7 Schematic presentation of the inhibition of lycopene on mitochondrial permeability transition pore (mPTP) opening.

Acknowledgments

This study was supported by National Natural Science Foundation of China (81670320 and 81800232) and Natural Science Foundation of Liaoning Province (No.201602826).

Disclosure

The authors report no conflicts of interest in this work.

References

- Bajaj A, Sethi A, Rathor P, Suppogu N, Sethi A. Acute complications of myocardial infarction in the current era: diagnosis and management. *J Investig Med*. 2015;63(7):844–855. doi:10.1097/JIM.0000000000000232
- Han D, Wang YJ, Chen J, et al. Activation of melatonin receptor 2 but not melatonin receptor 1 mediates melatonin-conferred cardio-protection against myocardial ischemia/reperfusion injury. *J Pineal Res*. 2019;22:e12571. doi:10.1111/jpi.12571
- Hafez P, Chowdhury SR, Jose S, et al. Development of an in vitro cardiac ischemic model using primary human cardiomyocytes. *Cardiovasc Eng Technol*. 2018;9(3):529–538. doi:10.1007/s13239-018-0368-8
- Morciano G, Bonora M, Campo G, et al. Mechanistic role of mPTP in ischemia-reperfusion injury. *Adv Exp Med Biol*. 2017;982:169–189. doi:10.1007/978-3-319-55330-6_9
- Kazmi DH, Kapoor A, Sinha A, et al. Role of metabolic manipulator trimetazidine in limiting percutaneous coronary intervention-induced myocardial injury. *Indian Heart*. 2018;3:S365–S371. doi:10.1016/j.ihj.2018.10.415
- Tian Y, Xiao Y, Wang B, Sun C, Tang K, Sun F. Vitamin E and lycopene reduce coal burning fluorosis-induced spermatogenic cell apoptosis via oxidative stress-mediated JNK and ERK signaling pathways. *Biosci Rep*. 2018;38(4):BSR20171003. doi:10.1042/BSR20171003
- Llansola-Portoles MJ, Redekas K, Streckaitė S, et al. Lycopene crystallites exhibit singlet exciton fission in tomatoes. *Phys Chem Chem Phys*. 2018;20(13):8640–8646. doi:10.1039/c7cp08460a
- Zhang XF, Huang FH, Zhang GL, et al. Novel biomolecule lycopene-reduced graphene oxide-silver nanoparticle enhances apoptotic potential of trichostatin A in human ovarian cancer cells (SKOV3). *Int J Nanomedicine*. 2017;13(12):7551–7575. doi:10.2147/IJN.S144161
- Jain A, Sharma G, Ghoshal G, et al. Lycopene loaded whey protein isolate nanoparticles: an innovative endeavor for enhanced bioavailability of lycopene and anti-cancer activity. *Int J Pharm*. 2018;546(1–2):97–105. doi:10.1016/j.ijpharm.2018.04.061
- Gul Baykalir B, Aksit D, Dogru MS, et al. Lycopene ameliorates experimental colitis in rats via reducing apoptosis and oxidative stress. *Int J Vitam Nutr Res*. 2016;86(1–2):27–35. doi:10.1024/0300-9831/a000280
- Petyaev IM. Lycopene deficiency in ageing and cardiovascular disease. *Oxid Med Cell Longev*. 2016;2016:3218605. doi:10.1155/2016/3218605
- Jia P, Liu C, Wu N, Jia D, Sun Y. Agomelatine protects against myocardial ischemia reperfusion injury by inhibiting mitochondrial permeability transition pore opening. *Am J Transl Res*. 2018;10(5):1310–1323.
- Liu S, Wu N, Miao J, et al. Protective effect of morin on myocardial ischemia-reperfusion injury in rats. *Int J Mol Med*. 2018;42(3):1379–1390. doi:10.3892/ijmm.2018.3743
- Lee YJ, Lee C. Porcine deltacoronavirus induces caspase-dependent apoptosis through activation of the cytochrome c-mediated intrinsic mitochondrial pathway. *Virus Res*. 2018;15(253):112–123. doi:10.1016/j.virusres.2018.06.008
- He F, Wu Q, Xu B, et al. Suppression of Stim1 reduced intracellular calcium concentration and attenuated hypoxia/reoxygenation induced apoptosis in H9C2 cells. *Biosci Rep*. 2017;37(6):BSR20171249. doi:10.1042/BSR20171249
- Liu P, Dong J. Protective effects of carnosis acid against mitochondria-mediated injury in H9c2 cardiomyocytes induced by hypoxia/reoxygenation. *Exp Ther Med*. 2017;14(6):5629–5634. doi:10.3892/etm.2017.5243
- Xiao J, Ke ZP, Shi Y, Zeng Q, Cao Z. The cardioprotective effect of thymoquinone on ischemia-reperfusion injury in isolated rat heart via regulation of apoptosis and autophagy. *J Cell Biochem*. 2018;119(9):7212–7217. doi:10.1002/jcb.26878
- Wu N, Zhang X, Jia P, Jia D. Hypercholesterolemia abrogates the protective effect of ischemic postconditioning by induction of apoptosis and impairment of activation of reperfusion injury salvage kinase pathway. *Biochem Biophys Res Commun*. 2015;458(1):148–153. doi:10.1016/j.bbrc.2015.01.084
- Gonca E, Kurt Ç. Cardioprotective effect of Thymoquinone: A constituent of *Nigella sativa* L., against myocardial ischemia/reperfusion injury and ventricular arrhythmias in anesthetized rats. *Pak J Pharm Sci*. 2015;28(4):1267–1273.
- Zhao Y, Xin Z, Li N, et al. Nano-liposomes of lycopene reduces ischemic brain damage in rodents by regulating iron metabolism. *Free Radic Biol Med*. 2018;20(124):1–11. doi:10.1016/j.freeradbiomed.2018.05.082
- Xu J, Hu H, Chen B, et al. Lycopene protects against hypoxia/reoxygenation injury by alleviating ER stress induced apoptosis in neonatal mouse cardiomyocytes. *PLoS One*. 2015;10(8):e0136443. doi:10.1371/journal.pone.0136443

22. Maulik A, Davidson SM, Piotrowska I, Walker M, Yellon DM. Ischaemic preconditioning protects cardiomyocytes from anthracycline-induced toxicity via the PI3K pathway. *Cardiovasc Drugs Ther.* **2018**;32(3): 245–253.
23. Nesci S, Ventrella V, Trombetti F, Pirini M, Pagliarini A. Preferential nitrite inhibition of the mitochondrial F1FO-ATPase activities when activated by Ca(2+) in replacement of the natural cofactor Mg(2+). *Biochim Biophys Acta.* **2016**;1860(2):345–353. doi:10.1016/j.bbagen.2015.11.004
24. Xu D, Duan H, Zhang Z, et al. The novel tetramethylpyrazine bis-nitrone (TN-2) protects against MPTP/MPP+-induced neurotoxicity via inhibition of mitochondrial-dependent apoptosis. *J Neuroimmune Pharmacol.* **2014**;9(2):245–258. doi:10.1007/s11481-013-9514-0
25. Ryu SY, Peixoto PM, Teijido O, Dejean LM, Kinnally KW. Role of mitochondrial channels in cell death. *Biofactors.* **2010**;36(4):255–263. doi:10.1002/biof.101
26. Gao Y, Jia P, Shu W, Jia D. The protective effect of lycopene on hypoxia/reoxygenation-induced endoplasmic reticulum stress in H9C2 cardiomyocytes. *Eur J Pharmacol.* **2016**;5(774):71–79. doi:10.1016/j.ejphar.2016.02.005
27. Siemen D, Ziemer M. What is the nature of the mitochondrial permeability transition pore and what is it not? *IUBMB Life.* **2013**;65(3):255–262. doi:10.1002/iub.1130
28. Kwong JQ, Molkentin JD. Physiological and pathological roles of the mitochondrial permeability transition pore in the heart. *Cell Metab.* **2015**;21(2):206–214. doi:10.1016/j.cmet.2014.12.001
29. Zhang C, Yuan X, Hu Z, et al. Valproic acid protects primary dopamine neurons from MPP (+)-Induced neurotoxicity: involvement of GSK3 β phosphorylation by Akt and ERK through the mitochondrial intrinsic apoptotic pathway. *Biomed Res Int.* **2017**;2017:8124501.
30. Sun MF, Zhu YL, Zhou ZL, et al. Neuroprotective effects of fecal microbiota transplantation on MPTP-induced Parkinson's disease mice: gut microbiota, glial reaction and TLR4/TNF- α signaling pathway. *Brain Behav Immun.* **2018**;70:48–60. doi:10.1016/j.bbi.2018.02.005
31. Wang Y, Cui R, Xiao Y, Fang J, Xu Q. Correction: effect of carotene and lycopene on the risk of prostate cancer: a systematic review and dose-response meta-analysis of observational studies. *PLoS One.* **2015**;10(10):e0140415. doi:10.1371/journal.pone.0140415
32. Zhou H, Zhang Y, Hu S, et al. Melatonin protects cardiac microvasculature against ischemia/reperfusion injury via suppression of mitochondrial fission-VDAC1-HK2-mPTP-mitophagy axis. *J Pineal Res.* **2017**;63:1. doi:10.1111/jpi.12413
33. Buyuklu M, Kandemir FM, Ozkaraca M, et al. Beneficial effects of lycopene against contrast medium-induced oxidative stress, inflammation, autophagy, and apoptosis in rat kidney. *Hum Exp Toxicol.* **2015**;34(5):487–496. doi:10.1177/0960327114542964
34. Rekha KR, Selvakumar GP. Gene expression regulation of Bcl2, Bax and cytochrome-C by geraniol on chronic MPTP/probenecid induced C57BL/6 mice model of Parkinson's disease. *Chem Biol Interact.* **2014**;25(217):57–66. doi:10.1016/j.cbi.2014.04.010
35. Keskin-Aktan A, Akbulut KG, Yazici-Mutlu Ç, Sonugur G, Ocal M, Akbulut H. The effects of melatonin and curcumin on the expression of SIRT2, Bcl-2 and Bax in the hippocampus of adult rats. *Brain Res Bull.* **2018**;137:306–310. doi:10.1016/j.brainresbull.2018.01.006
36. Ruefli-Brasse A, Reed JC. Therapeutics targeting Bcl-2 in hematological malignancies. *Biochem J.* **2017**;474(21):3643–3657. doi:10.1042/BCJ20170080

Drug Design, Development and Therapy

Dovepress

Publish your work in this journal

Drug Design, Development and Therapy is an international, peer-reviewed open-access journal that spans the spectrum of drug design and development through to clinical applications. Clinical outcomes, patient safety, and programs for the development and effective, safe, and sustained use of medicines are a feature of the journal, which has also

been accepted for indexing on PubMed Central. The manuscript management system is completely online and includes a very quick and fair peer-review system, which is all easy to use. Visit <http://www.dovepress.com/testimonials.php> to read real quotes from published authors.

Submit your manuscript here: <https://www.dovepress.com/drug-design-development-and-therapy-journal>