

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

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Abstract. Hydroxytyrosol (HT), a phenolic compound extracted from olive oil, is reported to protect against myocardial ischemia reperfusion injury (MIRI), but its mechanism has not been fully elucidated. The mitochondria permeability transition pore (MPTP) is an important therapeutic target for MIRI. The present study aimed to investigate the role of MPTP in the cardioprotection of HT. Isolated rat hearts were mounted on a Langendorff apparatus and subjected to 30 min of ischemia followed by 120 min of reperfusion to mimic a MIRI model. Isolated hearts were pretreated with different doses of HT (10, 100 and 1,000 μM) for 10 min prior to ischemia. Myocardial infarct size was detected using TTC staining. Changes in myocardial cell structure were observed using hematoxylin and eosin staining. MPTP opening was detected spectrophotometrically. Myocardial cell apoptosis was observed with terminal deoxynucleotidyl-transferase-mediated dUTP nick end labeling assays. The expression of apoptosis-associated proteins was measured by western blot analysis. The data revealed that HT (100 and 1,000 μM) treatment significantly alleviated pathological damage in ischemic myocardium and reduced myocardial infarct size compared with the untreated control. However, no significant difference was observed in the 10 μM HT treatment group compared with the untreated control. It was further revealed that HT decreased the B cell lymphoma-2 (Bcl-2)-like protein 4 (Bax)/Bcl-2 ratio, suppressed MPTP opening and

subsequently decreased the expression of cytochrome *c*, cleaved caspase-9 and -3, thereby inhibiting apoptosis. Additionally, the beneficial effects of HT on MIRI were reversed by atractyloside, which induces MPTP opening. In conclusion, the present study demonstrated that HT inhibited MPTP opening, partially via modulation of Bax and Bcl-2, thereby protecting against MIRI and thereby providing a pharmacological basis for future research and treatment of MIRI.

Introduction

Acute myocardial infarction (AMI) is the leading cause of morbidity and mortality worldwide (1). Reperfusion, the restoration of blood flow, has been considered the most effective treatment of ischemic heart disease, in particular AMI (2). However, reperfusion has further been referred to as a double-edged sword, as reperfusion may cause aggravation of myocardial injury, termed myocardial ischemia reperfusion injury (MIRI) (3). With the wide application of reperfusion therapy, including drug thrombolysis, percutaneous coronary intervention and coronary artery bypass grafting, the elucidation of the mechanism of MIRI prevention has become imperative (4-7).

The mitochondrial permeability transition pore (MPTP) is a non-specific channel located in the inner mitochondrial membrane (8). MPTP remains closed during ischemia, but rapidly opens following the commencement of reperfusion (9). MPTP opening is considered an important mechanism of MIRI (10). Additionally, the inhibition of MPTP opening by cyclosporine A attenuates MIRI (11). Therefore, MPTP is considered an important therapeutic target for the prevention of MIRI.

Hydroxytyrosol (HT), known as 3,4-dihydroxyphenyl ethanol, is a phenolic compound extracted from Mediterranean virgin olive oil (12). Biological effects of HT include the suppression of oxidative stress, inflammation and tumor formation, and protection of the cardiovascular system and neurological function (13-17). HT serves a role in the protection against liver ischemia/reperfusion injury in mice (18,19). Pei *et al* (20) revealed that HT affects MIRI via the phosphatidylinositol-4,5-bisphosphate 3 kinase (PI3K)/protein kinase B

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(Akt) signaling pathway. However, the effect of HT on MPTP in MIRI remains unknown. Therefore, the present study suggested that HT attenuated MIRI by inhibiting MPTP opening.

The aim of the present study was to investigate the effects of HT on MIRI in an isolated rat heart model and to further explore the role of MPTP in the cardioprotection of HT.

Materials and methods

Experimental animals and reagents. A total of 100 healthy male Wistar rats (age, 4 weeks; weight, 250±20 g) were purchased from Changsheng Biotechnology Co., Ltd. (Beijing, China). Rats were housed in environmentally controlled conditions (20–25°C, 5–65% relative humidity, with a 12-h light/dark cycle) with a common 1 week acclimatization period. All rats had access to fresh food and water ad libitum. The procedures for handling and caring for animals adhered to the guidelines in compliance with the Guide for the Care and Use of Laboratory Animals (21). The experimental protocol was approved by the Institutional Ethics Committee of China Medical University (Shenyang, China).

HT was purchased from Dalian Meilun Biology Technology Co., Ltd. (Dalian, China). Atractyloside (ATR) and 2,3,5-triphenyltetrazolium chloride (TTC) were purchased from Sigma-Aldrich (Merck KGaA, Darmstadt, Germany).

Establishing MIRI in isolated rat hearts. Pentobarbital sodium (100 mg/kg) was administered intravenously to anesthetize the rats. Heparin (1,500 IU/kg) was injected intravenously to prevent intracoronary clot formation. Following opening of the thoracic cavity, hearts were swiftly removed and immediately immersed in ice-cooled heparinized Krebs-Henseleit (K-H) buffer (0.15 mol/l NaCl, 0.006 mol/l KCl, 0.002 mol/l CaCl₂, 0.002 mol/l NaHCO₃) and saturated with 100% oxygen (22). Isolated hearts were transferred to a Langendorff perfusion system (Taimeng Science and Technology Ltd., Chengdu, China) to perform heart perfusion with K-H solution, saturated with 95% O₂+5% CO₂ at 37°C. A water-filled latex balloon was inserted into the left ventricle through the left atrium and connected to a pressure transducer for pressure measurement. All isolated hearts were continually perfused with K-H solution for 10 min stabilization, prior to the commencement of ischemia. All isolated rat hearts were subjected to 30 min global ischemia, followed by 120 min of reperfusion to generate the MIRI model.

Experimental protocol. The experimental protocol consisted of two phases. In the first phase, 40 rats were divided into the following 4 groups with 10/group: i) Ischemia reperfusion group (IR): As described above; ii) 10 μM HT treatment group (HT 10 μM): Isolated hearts were perfused with 10 μM HT for 10 min and K-H solution for 5 min prior to induction of ischemia; iii) 100 μM HT treatment group (HT 100 μM): Isolated hearts were perfused with 100 μM HT for 10 min and K-H solution for 5 min prior to induction of ischemia; iv) 1,000 μM HT treatment group (HT 1,000 μM): Isolated hearts were perfused with 1,000 μM HT for 10 min and K-H solution for 5 min prior to induction of ischemia.

According to the results of the first phase, 100 μM HT was chosen for the second phase. A total of 60 rats were divided into the following 3 groups with 20/group: i) IR group; ii) HT

100 μM group: The heart rate was stabilized and the heart was perfused with HT for 10 min, then with K-H solution for 5 min, ischemia/reperfusion was performed as in IR group. iii) 100 μM HT in combination with ATR treatment group (HT 100 μM+ATR): 5 mg/kg ATR was injected intraperitoneally 30 min prior to extraction of the heart, the remainder of the procedure was as described for the HT 100 μM group.

Cardiac function monitoring. Change in cardiac function was evaluated monitoring heart rate (HR) and coronary flow (CF). HR and CF were measured prior to ischemia, and 30 and 60 min post initiation of reperfusion.

Hematoxylin and eosin (HE) staining. Following reperfusion, hearts were harvested. Heart tissues were fixed in 4% paraformaldehyde for 24–72 h at room temperature and washed with flowing water for 4 h. Heart tissue samples were subsequently dehydrated in an ascending ethanol series (70% ethanol for 2 h, 80% ethanol overnight, 90% ethanol for 2 h, 100% ethanol I for 1 h and 100% ethanol II for 1 h) at room temperature. The tissue samples were embedded in paraffin embedded and the paraffin-embedded tissue samples were cut into 5-μm-thick sections. The tissue sections were subsequently deparaffinized in xylene I for 15 min and xylene II for 15 min at room temperature and rehydrated in a descending ethanol series (100% ethanol I for 5 min, 100% ethanol II for 5 min, 95% ethanol for 2 min, 85% ethanol for 2 min, 75% ethanol for 2 min) and distilled water for 2 min at room temperature. Deparaffinized section were incubated with hematoxylin solution for 5 min, 1% hydrochloric acid alcohol for 3 sec and eosin solution for 3 min at room temperature. Pathological changes were observed under a light microscope (Olympus BX51; Olympus Corporation, Tokyo, Japan; magnification, x400).

Measurement of myocardial infarct size. Following reperfusion, the hearts were removed, frozen at -20°C for 1 h and sliced into 1–2 mm thick sections. The sections were incubated in a 1% TTC solution for 20 min at 37°C. Tissue sections were washed with 1X PBS and fixed in 4% paraformaldehyde overnight at room temperature. Images of the stained slices were captured using a digital camera and analyzed using Image J2X analysis software (National Institutes of Health, Bethesda, MD, USA). The severity of the myocardial infarction was indicated by the ratio of the infarct area to the total area.

Apoptosis. Myocardial apoptosis was detected by terminal deoxynucleotidyl-transferase-mediated dUTP nick end labeling (TUNEL) assay using the In Situ Cell Death Detection kit (cat. no. 11684817910; Roche Diagnostics, Indianapolis, IN, USA), as previously described (23). Apoptotic cells were observed under a light microscope (magnification, x400) in three randomly selected fields. Image-Pro Plus (version 6.0; Media Cybernetics, Inc., Rockville, MD, USA) was used for cell counting.

MPTP sensitivity to Ca²⁺. Mitochondria were isolated from heart tissues using the Tissue Mitochondria Isolation kit (Beyotime Institute of Biotechnology, Shanghai, China), according to the manufacturer's protocol. The sensitivity of MPTP to Ca²⁺ was determined using the Purified

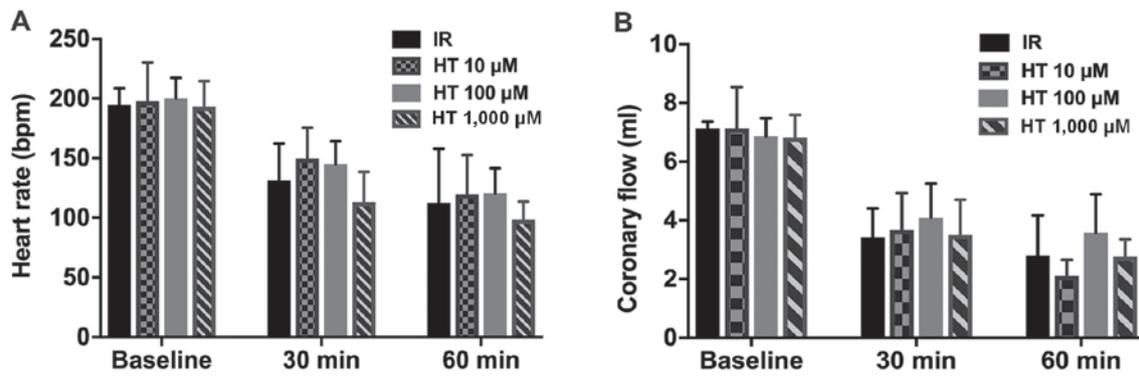


Figure 1. HT treatment does not alter HR and CF. (A) HR and (B) CF determined in animals treated with HT at 0, 10, 100 and 1,000 μM at various times following IR (n=6/group). IR, ischemia reperfusion; HT, hydroxytyrosol; HR, heart rate; CF, coronary flow.

Mitochondrial Membrane Pore Channel Colorimetric Assay kit (cat. no. GMS10095; Shanghai Genmed Pharmaceutical Technology Co., Ltd., Shanghai, China), according to the manufacturer's protocol. The larger the min/max ratio, the lower the sensitivity of MPTP opening to Ca^{2+} , conversely, the smaller the min/max ratio, the higher the sensitivity of MPTP opening to Ca^{2+} .

Western blot analysis. The left ventricle tissues were homogenized with radioimmunoprecipitation buffer (Beyotime Institute of Biotechnology) and protease inhibitor phenylmethanesulfonyl fluoride (Beyotime Institute of Biotechnology) on ice for 20 min. Proteins were extracted from lysates following centrifugation at 13,000 \times g for 15 min at 4°C. Total protein was quantified using the Enhanced BCA Protein Assay kit (Beyotime Institute of Biotechnology). Subsequently, 50 μg protein was denatured at 100°C for 10 min, and separated via SDS-PAGE on a 10% gel. The separated proteins were transferred onto polyvinylidene difluoride membranes and blocked with 5% skimmed milk for 1 h at room temperature. The membranes were incubated with primary antibodies against B-cell lymphoma 2 (Bcl-2)-associated X protein (Bax; 1:1,000; cat. no. WL01637), Bcl-2 (1:1,000; cat. no. WL01556; both Shenyang Wan Biotechnology Co., Ltd., Shenyang, China), cytochrome *c* (1:1,000; cat. no. ab90529), apoptotic protease activating factor-1 (APAF-1; 1:1,000; cat. no. ab2001), cleaved caspase-3 (1:1,000; cat. no. ab2302; all Abcam, Cambridge, UK), cleaved caspase-9 (1:1,000; cat. no. 40503-1; Signalway Antibody LLC, College Park, MD, USA) and β -actin (1:1,000; cat. no. TA-09; OriGene Technologies, Inc., Beijing, China) overnight at 4°C. Following primary incubation, the membranes were incubated with horseradish peroxidase-labeled goat anti-rabbit immunoglobulin G (1:4,000; cat. no. E030120-01) or goat anti-mouse immunoglobulin G (1:4,000; cat. no. E030110-01; both EarthOx Life Sciences, Millbrae, CA, USA) at 37°C for 2 h. Protein bands were visualized using BeyoECL Star (Beyotime Institute of Biotechnology), according to the manufacturer's protocol. Levels of phosphorylated proteins were normalized to the corresponding total protein levels. Relative densitometry was calculated using ImageJ (version 1.50e; National Institutes of Health).

Statistical analysis. Data are expressed as the mean \pm standard deviation from three independent experiments. All statistical

analysis was performed using SPSS 17.0 (SPSS, Inc., Chicago, IL, USA). Differences between groups were evaluated using one-way analysis of variance followed by Fisher's least significant difference tests. $P < 0.05$ was considered to indicate a statistically significant difference.

Results

Effects of HT on HR and CF. As presented in Fig. 1, no statistical significance was detected for HR or CF in rats of the various experimental groups at various times ($P > 0.05$).

Effects of HT on myocardial injury. HE staining revealed that HT (100 and 1,000 μM) pretreatment markedly alleviated pathological damage in the ischemic myocardium; however, pretreatment with 10 μM HT did not reveal any alleviation of pathological damage in the ischemic myocardium (Fig. 2A). Results of the TTC assay revealed the infarct size, which is presented as white tissue sections compared with red normal tissue sections (Fig. 2B). The myocardial infarct size of the 100 and 1,000 μM HT groups was significantly decreased compared with the IR group (30.9 ± 2.9 and 32.3 ± 3.2 vs. $54.3 \pm 3.0\%$; $P < 0.05$; Fig. 2C). However, pretreatment with 10 μM HT revealed no significant change in the myocardial infarct size compared with the IR group ($P > 0.05$; Fig. 2C). The protective effect on MIRI was not improved when increasing HT from 100 to 1,000 μM . Therefore, 100 μM HT was selected for second-stage experiments.

ATR reverses protective effects of HT. To elucidate the role of HT in cardioprotection during MIRI, the effects of ATR, a MPTP opener, were investigated. The results revealed that myocardial injury was worsened in the HT 100 μM + ATR group compared with the HT 100 μM group (Fig. 3A). Myocardial infarct size was significantly increased in the HT 100 μM + ATR group compared with the HT 100 μM group (52.8 ± 7.8 vs. $30.9 \pm 2.9\%$; $P < 0.05$; Fig. 3B and C).

Effects of HT on MPTP opening. MPTP opening is generally induced by Ca^{2+} and the sensitivity of MPTP to Ca^{2+} is an indicator for MPTP opening (24-26). The results indicated that changes in Ca^{2+} induced MPTP opening. Isolated mitochondria from the HT 100 μM group were significantly more resistant to

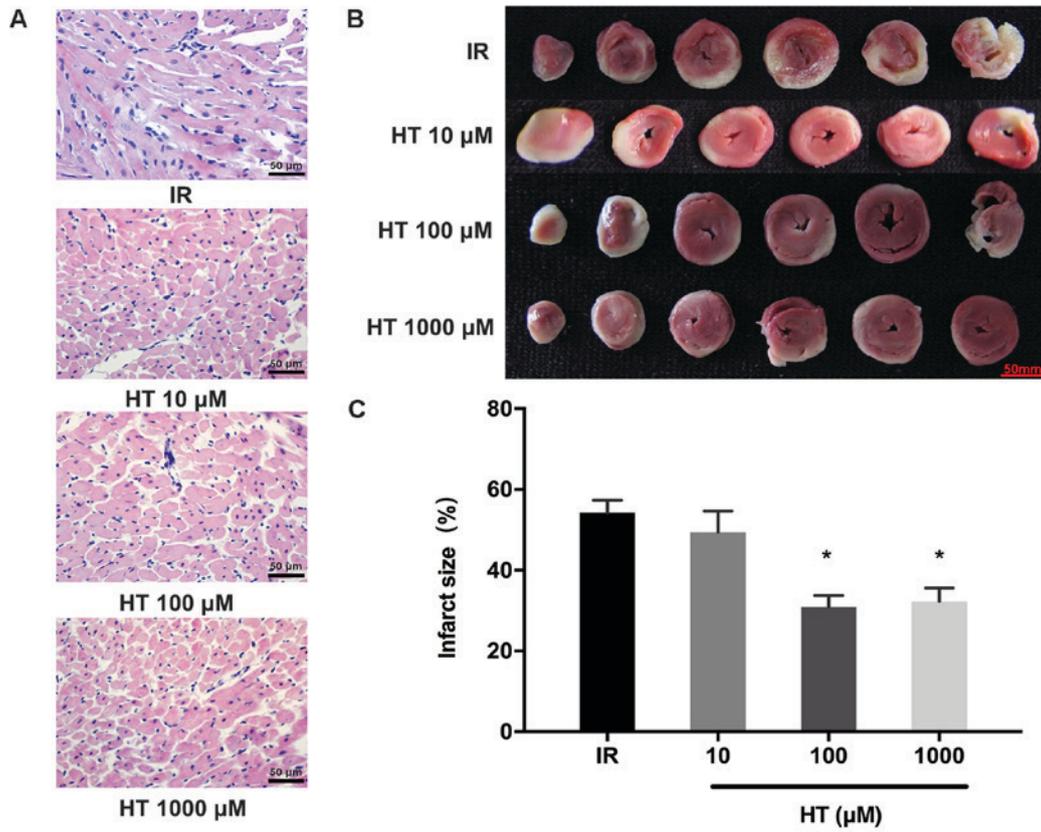


Figure 2. Effects of HT on myocardial ischemia reperfusion injury. (A) Pathological changes of the ischemic myocardium stained by hematoxylin-eosin (magnification, x400; scale bar, 50 μm). (B) Heart tissue slices stained with 2,3,5-triphenyltetrazolium chloride visualizing the size of the myocardial infarct (scale bar, 50 mm). (C) Statistical analysis of the myocardial infarct size (n=6). *P<0.05 vs. IR. IR, ischemia reperfusion; HT, hydroxytyrosol.

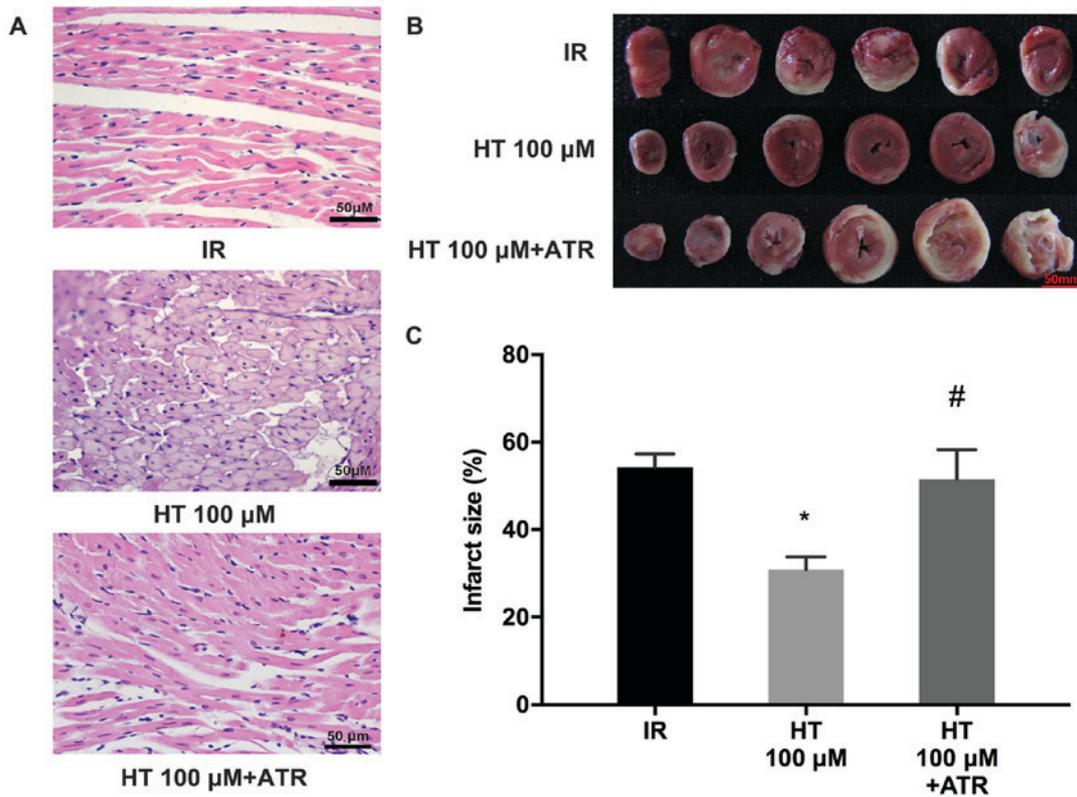


Figure 3. Effects of ATR on HT cardioprotection in myocardial ischemia reperfusion injury. (A) Pathological changes of the ischemic myocardium stained with hematoxylin-eosin (magnification, x400; scale bar, 50 μm). (B) Heart tissue slices stained with 2,3,5-triphenyltetrazolium chloride visualizing the size of the myocardial infarct (scale bar, 50 mm). (C) Statistical analysis of the myocardial infarct sizes (n=6). *P<0.05 vs. IR and #P<0.05 vs. 100 μM HT. IR, ischemia reperfusion; HT, hydroxytyrosol; ATR, atractyloside.

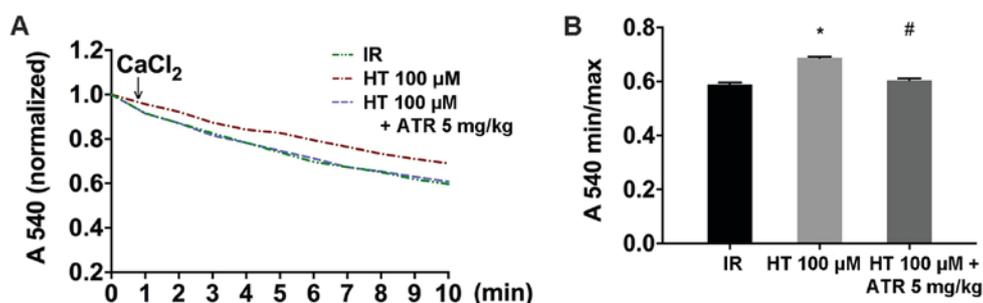


Figure 4. Effects of HT on MPTP opening. Ca^{2+} sensitivity of MPTP was determined using purified mitochondrial membrane pore channel colorimetric assay. (A) Sensitivity of MPTP opening to Ca^{2+} . (B) Alterations in MPTP opening in response to Ca^{2+} (n=6). * $P < 0.05$ vs. IR and # $P < 0.05$ vs. 100 μM HT. IR, ischemia reperfusion; HT, hydroxytyrosol; ATR, atractyloside.

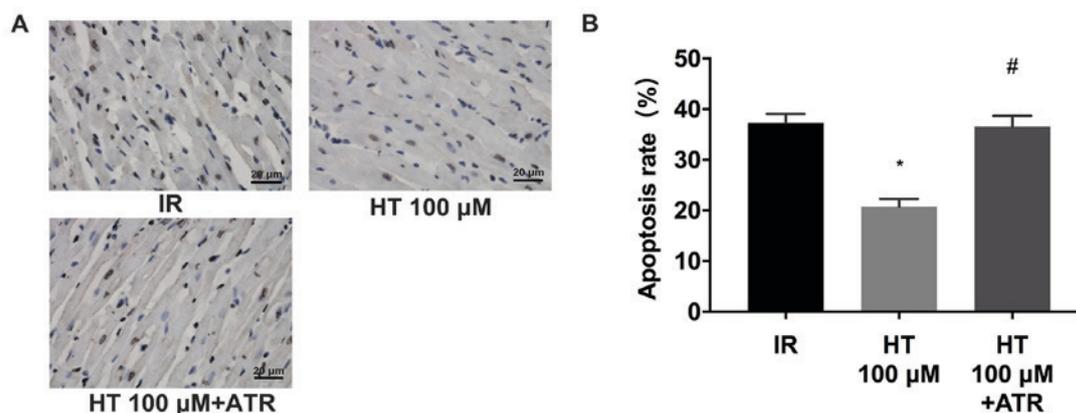


Figure 5. Effects of HT on apoptosis. (A) Apoptosis was detected by terminal deoxynucleotidyl-transferase-mediated dUTP nick end labeling assay (magnification, $\times 400$; scale bar, 20 μm). (B) Statistical analysis of apoptosis rates (n=6). * $P < 0.05$ vs. IR and # $P < 0.05$ vs. 100 μM HT. IR, ischemia reperfusion; HT, hydroxytyrosol; ATR, atractyloside.

stimulation by Ca^{2+} compared with the IR group, suggesting that HT inhibited MPTP opening (Fig. 4A and B). Additionally, the resistance of the isolated mitochondria to Ca^{2+} was decreased in the HT 100 μM + ATR group compared with the HT 100 μM group (Fig. 4B).

Effects of HT on the mitochondrial apoptotic pathway. MPTP opening leads to the release of cytochrome *c* and activation of caspase-9 and -3, which results in apoptosis (27). Results from the TUNEL assay revealed that 100 μM HT significantly decreased the rate of apoptosis ($P < 0.05$; Fig. 5A and B). In addition, compared with HT 100 μM group, the apoptosis rate in the HT 100 μM + ATR group increased by 15.8% (Fig. 5). Similarly, western blot analysis revealed that 100 μM HT significantly decreased cytochrome *c*, cleaved caspase-9 and -3 protein levels compared with the IR group ($P < 0.05$; Fig. 6). In addition, the cytochrome *c*, cleaved caspase-9 and -3 levels in the HT 100 μM + ATR group were significantly increased compared with the 100 μM HT group (Fig. 6). The rate of apoptosis and expression level of apoptosis-associated proteins was not significantly different in the ATR-treated group compared with the IR group, suggesting that ATR reversed cardioprotective effects exerted by HT.

Effects of HT on Bax and Bcl-2 protein expression. The Bcl-2 protein family is a key regulator in MPTP opening (28).

Western blot analysis demonstrated that treatment with 100 μM HT significantly decreased Bax expression and Bax/Bcl-2 compared with the IR group ($P < 0.05$; Fig. 7). Additionally, Bcl-2 expression was significantly increased compared with the IR group ($P < 0.05$; Fig. 7).

Discussion

In the present study, treatment with the pharmacological agent HT at 100 or 1,000 μM was revealed to reduce the myocardial infarction area and damage to the myocardium in rats compared with the untreated animals, which suggested that HT may protect against MIRI. However, there was no effect for 10 μM HT. Additionally, there was no significant difference in the cardioprotection exerted by 100 and 1,000 μM HT. Therefore, it was suggested that a dose-associated effect of HT occurred at lower doses (10–100 μM), which is consistent with a previous study by Pan *et al* (18). Pei *et al* (20) used SD rats to perform *in vivo* cardiac ischemia for 30 min followed by reperfusion for 3 h. In their study, rats were intraperitoneally injected with HT at a concentration of 20 mg/kg during ischemia. The results revealed that HT attenuated MIRI via the PI3K/Akt signaling pathway, and protected functional parameters of the heart. However, in the present study, no difference in HR or CF of rats from various groups was observed at various times. Reasons for this disparity may include differences between *in vivo* and

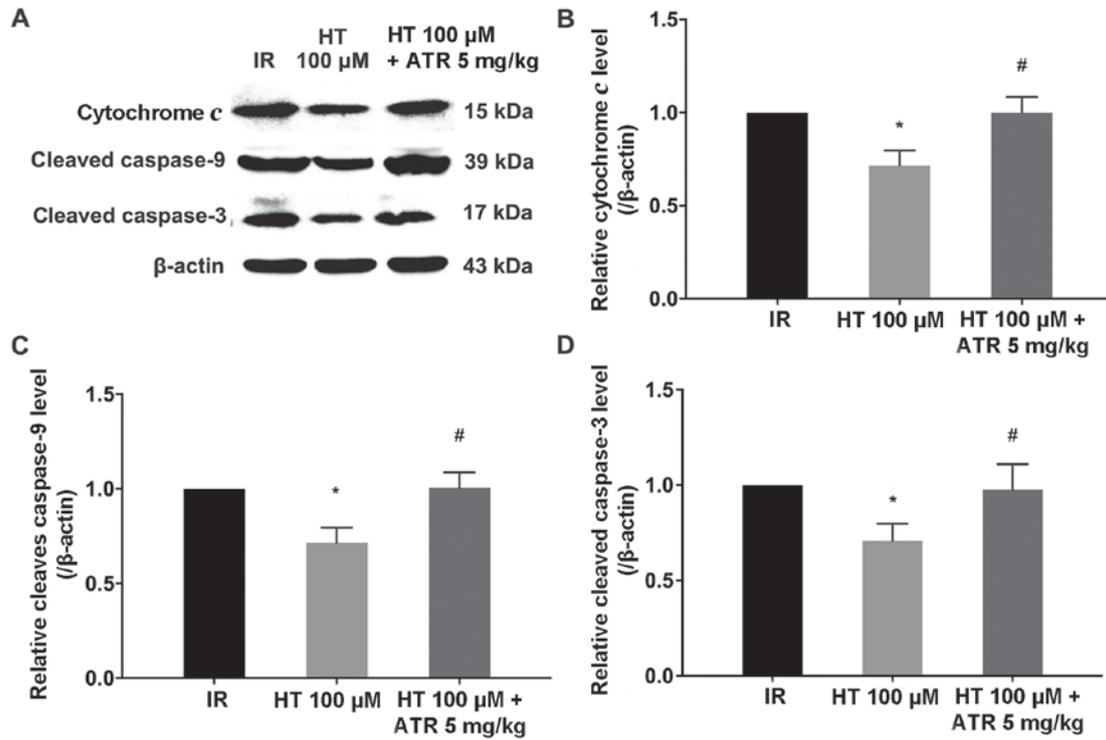


Figure 6. Effects of HT on the mitochondrial apoptotic pathway. (A) Cytochrome *c*, cleaved caspase-9 and -3 levels detected by western blotting. Statistical analysis of the relative expression of (B) cytochrome *c*, (C) cleaved caspase-9 and (D) cleaved caspase-3 (n=6). *P<0.05 vs. IR and #P<0.05 vs. 100 μM HT. IR, ischemia reperfusion; HT, hydroxytyrosol; ATR, atractyloside.

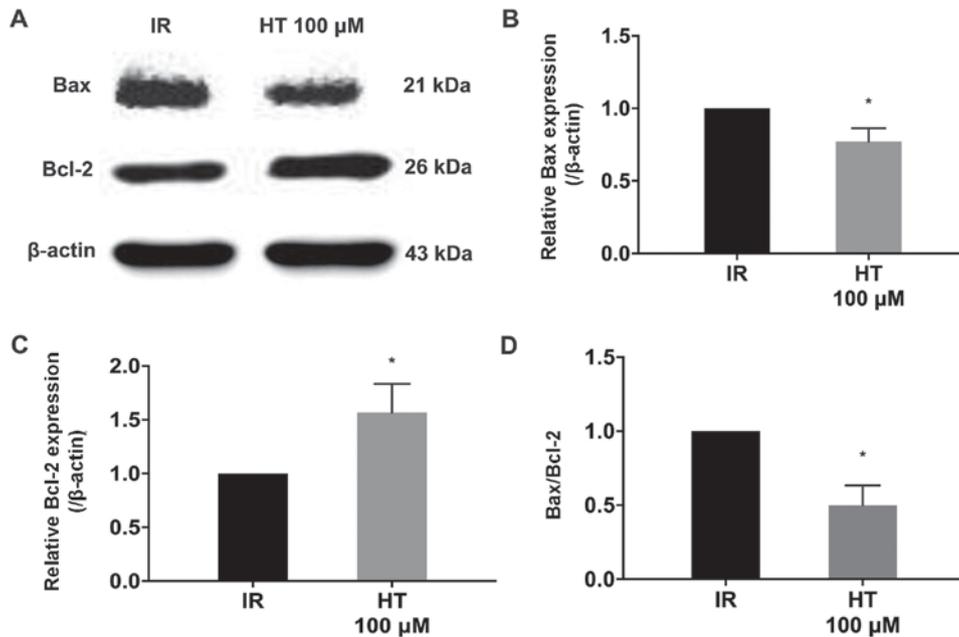


Figure 7. Effects of HT on the Bcl-2 protein family. (A) Bax and Bcl-2 levels detected by western blotting. Statistical analysis of the relative expression of (B) Bax, (C) Bcl-2 and (D) Bax/Bcl-2 (n=6). *P<0.05 vs. IR. IR, ischemia reperfusion; HT, hydroxytyrosol; Bcl-2, B cell lymphoma-2; Bax, Bcl-2-like protein 4.

in vitro models, differences in rat species, duration of procedures and dosing methods.

To the best of our knowledge, this is the first study to demonstrate the effect of HT on the inhibition of MPTP in MIRI. The opening of MPTP causes irreversible damage to the heart (29). According to previous studies, core components of MPTP are voltage-dependent anion channels (VDAC), adenine

nucleotide transporter and cyclophilin-D (30,31). The opening of MPTP is induced by insufficient intracellular adenosine triphosphate synthesis, reactive oxygen species-induced oxidative stress, and Ca²⁺ and phosphate accumulation (32,33). Several studies have revealed that MIRI is closely associated to MPTP opening (34-36). Pretreatment with several pharmacological agents, including irisin, melatonin and carnosic

acid, have been demonstrated to alleviate MIRI via inhibition of MPTP opening (37-39). In the present study, it was demonstrated that HT inhibited MPTP opening during MIRI. Ca^{2+} treatment induces MPTP opening (40); compared with the IR group, it was revealed that isolated mitochondria from rat hearts pretreated with HT had a higher resistance to Ca^{2+} stimulation, which indicated that HT inhibited MPTP opening. Additionally, it was revealed that HT pretreatment reduced cytochrome *c*, cleaved caspase-9 and -3 levels and decreased the rate of apoptosis. These observations were similar to results reported by Soni *et al* (41) studying rat brains. All protective effects of HT were abolished with ATR treatment, which strongly suggested that HT protected against MIRI by inhibiting MPTP opening.

The Bcl-2 protein family is an important constituent of the apoptotic pathway (42,43) and serves an important regulatory role in MPTP opening (44). Members of the Bcl-2 family include the anti-apoptotic protein Bcl-2 and the pro-apoptotic protein Bax (45,46). Interactions of Bcl-2 and Bax with VDAC regulate MPTP opening; Bax facilitates MPTP opening by binding to VDAC, while Bcl-2 inhibits binding of Bax to VDAC (28). Bcl-2 and Bax co-express in tissue cells and MPTP opening is closely associated with the ratio of Bax to Bcl-2 (47). In the present study, it was revealed that HT pretreatment enhanced Bcl-2 expression in MIRI and decreased Bax expression and Bax/Bcl-2 levels. This demonstrated that HT inhibited MPTP opening by regulating Bcl-2 and Bax expression, which is consistent with a previous study by Liu and Dong (39). Notably, a recent study suggested that phosphorylated-Akt inhibits MPTP opening by regulating the Bcl-2 protein family (48). Furthermore, Pei *et al* (20) demonstrated that HT protects the rat myocardium from MIRI via direct activation of the PI3K/Akt signaling pathway. It was therefore further suggested that the PI3K/Akt/Bcl-2 signaling pathway may serve an important role in the inhibition of MPTP opening by HT. This hypothesis requires to be investigated in further studies for confirmation.

There are limitations to the present study. The isolated rat heart model used was deprived of neural and humoral regulation and may not completely mimic pathophysiological changes that occur during MIRI and *in vivo* cardioprotective effects of HT require further validation. The present study solely demonstrated that HT inhibited MPTP opening via Bcl-2; upstream targets of the MPTP pathway, including PI3K/Akt, glycogen synthase kinase β and Janus kinase/signal transducer and activator of transcription pathways require further investigation.

In conclusion, the present study demonstrated for the first time that HT protected against MIRI by inhibiting MPTP opening and thereby providing a pharmacological basis for future research and treatment of MIRI.

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Availability of data and materials

All datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Authors' contributions

DJ and NW designed the experiments. JM, SL, ZH and XL performed the experiments. JM, PJ and YG analyzed the data. JM prepared the manuscript. NW revised the manuscript. All authors read and approved the manuscript and agree to be accountable for all aspects of the research in ensuring that the accuracy or integrity of any part of the work are appropriately investigated and resolved.

Ethics approval and consent to participate

All rats were treated in accordance with the Guide for the Care and Use of Laboratory Animals. The experimental protocol was approved by the Institutional Ethics Committee of China Medical University (Shenyang, China).

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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