

Original Article

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

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Abstract: Agomelatine is a melatonin (MT1/MT2) receptor agonist and serotonin (5-HT_{2C}) receptor antagonist. To study the effects of agomelatine on myocardial ischemia reperfusion injury (MIRI), an isolated rat heart model was utilized. To induce MIRI, rat hearts were isolated and subjected to 30 min of ischemia followed by 120 min of reperfusion. Rats were intraperitoneally injected with agomelatine (10, 20 or 40 mg/kg) 1 h before heart isolation. Agomelatine (20 mg/kg and 40 mg/kg) significantly improved cardiac function, alleviated pathological changes in the ischemic myocardium, reduced myocardial infarct size and decreased release of creatine kinase-MB and lactate dehydrogenase. Heart tissue from agomelatine-treated rats retained higher NAD⁺ content and was more resistant to Ca²⁺, indicating inhibition of mitochondrial permeability transition pore (MPTP) opening. Notably, agomelatine's protective effects were abrogated by atractyloside, a MPTP opener. We also found that agomelatine significantly enhanced GSK-3 β phosphorylation and decreased expression of cytochrome C, cleaved caspase 9 and cleaved caspase 3, resulting in a decreased apoptosis rate. These findings demonstrate that agomelatine protects against MIRI by inhibiting MPTP opening.

Keywords: Myocardial ischemia reperfusion injury, agomelatine, melatonin, mitochondrial permeability transition pore, apoptosis

Introduction

Ischemic heart disease (IHD) is the leading cause of death worldwide [1]. Restoring blood flow, or reperfusion, is considered the optimal method to treat ischemic heart disease, especially acute myocardial infarction. However, reperfusion can also aggravate the degree of myocardial injury due to so called myocardial ischemia reperfusion injury (MIRI) [2]. MIRI is a major focus of cardiovascular research, and several therapeutic targets have already been demonstrated in recent studies [3].

Mitochondrial permeability transition pore (MPTP), a non-specific pore located in the mitochondrial inner membrane, is a key effector under the condition of MIRI [4]. Research has found that MPTP remains closed during ischemia, but rapidly opens once the reperfusion starts [5]. MPTP opening leads to mitochondrial depolarization, swelling, cell apoptosis and necrosis [6]. Moreover, blocking MPTP opening

with a specific inhibitor, such as cyclosporin A, can attenuate MIRI [7, 8]. Thus, MPTP is considered an important therapeutic target for preventing MIRI.

Melatonin was originally identified as a hormone produced by the pineal gland to regulate sleep and wakefulness [9]. Melatonin has many biological effects, including antioxidant [10], anti-inflammatory [11], and anti-tumor effects [12, 13]. Recent studies provide direct evidence that melatonin protected against MIRI by inhibiting MPTP opening [14, 15], which implies that melatonin or melatonin receptor agonists might possess protective effects on MIRI.

Agomelatine (AGO) is a small molecule that is structurally similar to melatonin, and acts as both a melatonin (MT1/MT2) receptor agonist and serotonin 5-HT_{2C} receptor antagonist [16]. AGO has been shown to protect brain and ovaries from ischemia reperfusion injury via enhancing antioxidant properties and inhibiting

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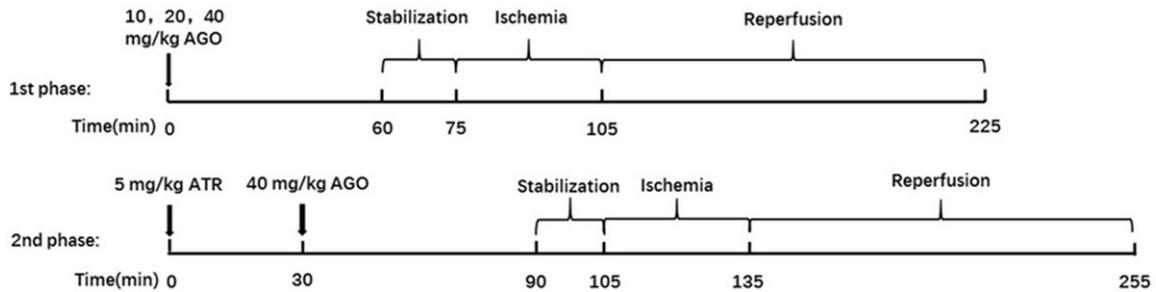


Figure 1. Experimental protocol. AGO: agomelatine, ATR: atractyloside.

apoptosis [17, 18]. Furthermore, another type of melatonin receptor agonist, Neu-p11, has been demonstrated to possess protective effects on MIRI *in vitro* [19]. Therefore, we hypothesized that AGO could protect the heart against ischemia reperfusion injury (IRI). The aim of our study was to test the effects of AGO on MIRI in an isolated rat heart model and to further explore the role of MPTP in this context.

Materials and methods

Animals and care

130 healthy male Wistar rats, weighing 250 ± 10 g were purchased from Changsheng Biotechnology Co., Ltd. (Benxi, Liaoning). All rats were treated and used abiding by the Guide for the Care and Use of Laboratory Animals (NIH, USA). The experimental protocol was approved by the institutional ethics committee of China Medical University.

Drug

Agomelatine, hydroxyethylcellulose, atractyloside (ATR) and 2, 3, 5-triphenyltetrazolium chloride (TTC) were purchased from Sigma-Aldrich (St. Louis, MO, USA). Agomelatine powder was dissolved in 1% hydroxyethylcellulose in preparation for injection.

Heart preparation

Heart preparation was performed as described previously [20]. In brief, pentobarbital sodium at the dose of 100 mg/kg was administered intravenously to anesthetize rats. Meanwhile, heparin (1,500 IU/kg) was injected intravenously to block intracoronary clot formation. After opening the thoracic cavity, the heart was swiftly removed and immediately immersed in

ice cooling heparinized Krebs-Henseleit (K-H) solution [21]. Then, the isolated heart was hung on a Langendorff apparatus from the root of the aorta followed by perfusion with K-H solution saturated with 95% O₂+5% CO₂ at a constant temperature of 37°C. The 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 15 min of stabilization before starting ischemia.

Experimental protocol

The experimental protocol consisted of two phases (**Figure 1**). All isolated rat hearts were subjected to 30 min of global ischemia followed by 120 min of reperfusion to induce MIRI model.

At the first phase, 50 rats were divided into 5 groups with 10 rats per group as follows: (1) Ischemia reperfusion group (IR): described above; (2) Vehicle group (Vehicle): 1% hydroxyethylcellulose at the dose of 1 ml/kg was injected intraperitoneally to the rats 1 h before heart isolation; (3) 10 mg/kg AGO treatment group (AGO10): AGO at the dose of 10 mg/kg was injected intraperitoneally to the rats 1 h before heart isolation; (4) 20 mg/kg AGO treatment group (AGO20): AGO at the dose of 20 mg/kg was injected to the rats as described as AGO10; (5) 40 mg/kg AGO treatment group (AGO40): AGO at the dose of 40 mg/kg was injected to the rats as described as the AGO10.

According to the results of first phase, 40 mg/kg AGO was determined to be used in the second phase. Specifically, 80 rats were further divided into 4 groups with 20 rats per group at the second phase as follow: (I) IR group; (II) Vehicle group; (III) AGO40 group. These three

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groups were as described earlier in the first phase. (IV) 40 mg/kg AGO in combination with ATR treatment group (AGO40+ATR): ATR, a MPTP opener [22], at the dose of 5 mg/kg was injected intraperitoneally to the rats 30 min ahead of AGO injection and the rest of the procedure was the same as the AGO40 group.

Cardiac function monitoring

To evaluate the change of cardiac function, heart rate (HR), left ventricular developed pressure (LVDP), positive first order derivative of ventricular pressure (+dp/dt) and negative first order derivative of ventricular pressure (-dp/dt) were continuously recorded by a homodynamic system (BIOPAC MP150, USA) throughout the experimental protocol.

HE staining

At the end of reperfusion, the heart was harvested. The heart tissues were undertaken by routine fixation, dehydration and paraffin embedding and were subsequently made into 4 μ m paraffin sections. For HE staining, the paraffin sections were successively de-waxed by xylene, hydrated by a descending series of ethanol, subsequently stained by hematoxylin-eosin, then dehydrated by ascending series of ethanol, cleared by xylene and finally mounted by neutral balsam. The dyed sections were observed by light microscope (Olympus BX51, Japan) to assess the pathological changes of myocardium.

Measurement of infarct size

Myocardial infarct size was determined by TTC stain as described previously [20]. Briefly, at the end of reperfusion, the hearts were removed and frozen at -20°C for 1 h. The freezing hearts were rapidly cut into 1-2 mm sections and incubated in 1% TTC at 37°C for 20 min, followed by methanol fixation overnight. Viable myocardium was stained red and infarct myocardium was unstained. The myocardium sections were finally photographed using a digital camera. Myocardial infarct size was expressed as the ratio of infarct areas to the whole areas.

Release of myocardial enzymes

At 120 min of reperfusion, 1 ml coronary effluent samples per heart were collected for mea-

suring the release of CK-MB and LDH using Creatine Kinase Isoenzyme Test Kit (Jiancheng Bioengineering Institute, Nanjing, China) and Lactate Dehydrogenase Test Kit (Jiancheng Bioengineering Institute, Nanjing, China) according to the manufacturer's instructions.

Myocardial mitochondria observation by electron microscopy

At 120 min of reperfusion, the $1\text{ mm} \times 1\text{ mm} \times 1\text{ mm}$ size of cardiac tissue was isolated from the left ventricle and further made into electron microscopic sections as previous described [21]. The morphologic changes on mitochondria were observed by transmission electron microscopy (JEM-1200EX, JEOL, Japan).

Measurement of apoptosis

Apoptosis was detected by Terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) assay using In Situ Cell Death Detection Kit (Roche, USA) according to the manufacturer's instructions. Each sample was prepared for 6 sections at least, and 5 fields in each section under microscope (Olympus BX51 microscope, Japan) were randomly photographed. The number of apoptotic cell nuclei was evaluated by two independent observers. The percentage of apoptotic cell was calculated as the number of TUNEL-positive apoptotic nuclei divided by the total number of nuclei.

Measurement of NAD⁺

In this study, NAD⁺ was extracted from left ventricle tissues harvested at 15 min of reperfusion using the Klingenberg method. Concentrations of NAD⁺ was measured by a spectrophotometer (DU 640, Beckman Coulter, Fullerton, CA, USA) as described in previous article [23].

Sensitivity of MPTP to calcium

The mitochondria were isolated from heart tissues using Tissue Mitochondria Isolation Kit (Beyotime, Shanghai, China) according to the manufacturer's instructions. 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 instructions.

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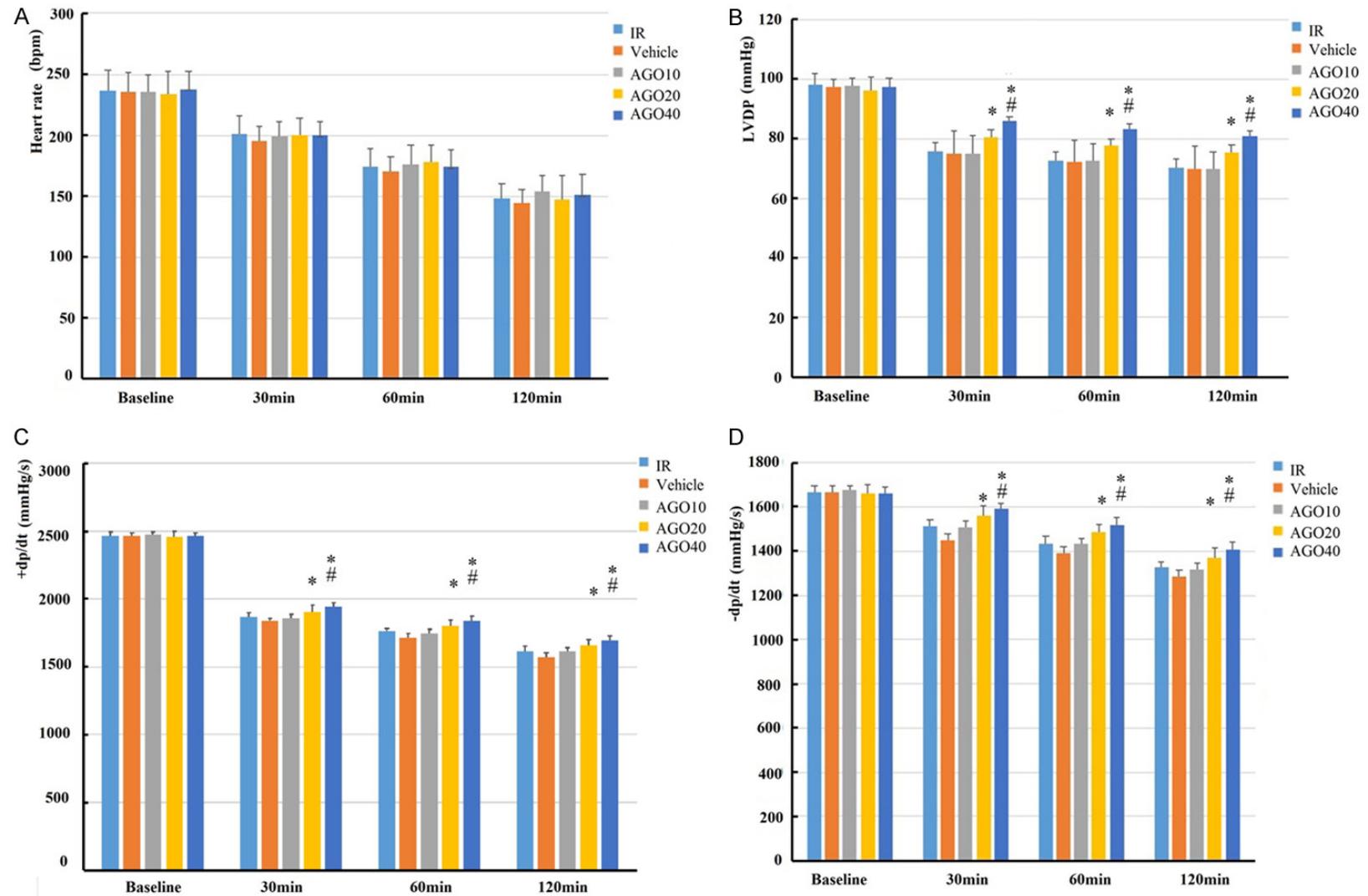


Figure 2. The effect of agomelatine on cardiac function. A. The changes in heart rate. B. The changes in left ventricular developed pressure (LVDP). C. The changes in positive first order derivative of ventricular pressure (+dp/dt). D. The changes in negative first order derivative of ventricular pressure (-dp/dt). IR: ischemia reperfusion group, Vehicle: Vehicle group, AGO10: 10 mg/kg agomelatine treatment group, AGO20: 20 mg/kg agomelatine treatment group, AGO40: 40 mg/kg agomelatine treatment group. *represents $P < 0.05$ vs. IR; #represents $P < 0.05$ vs. AGO20, $n = 8-10$ per group.

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Western blotting

Cytosolic and mitochondrial proteins were separately extracted from left ventricle tissues using Mitochondrial/Cytoplasmic Protein Extraction Kit (Bioco Laibo Technology Co. Ltd, Beijing, China) according to the manufacturer's instructions. The concentration of protein was measured using Enhanced BCA Protein Assay Kit (Beyotime, Shanghai, China). For Western blotting analysis, 50 µg protein denatured by heating was subjected to 10% SDS polyacrylamide gels for separation and then transferred to PVDF membranes. The membrane was blocked using 5% skim milk for 1 h and then incubated overnight at 4°C with the specific primary antibodies (Abcam, Hongkong, China) including monoclonal anti-GSK-3β (1:1,000), anti-p-GSK-3β (1:1,000), anti-Cytochrome C (1:1,000), anti-cleaved caspase 9 (1:1,000), anti-cleaved caspase-3 (1:1,000), anti-GAPDH (1:2,000) and anti-COXIV (1:2,000). After this, the membranes were incubated with horseradish peroxidase-conjugated goat anti-rabbit or rabbit anti-mouse immunoglobulin G (1:2000; Santa Cruz, California, USA) at 37°C for 2 h. Detection of protein band was performed using an enhanced chemi-luminescence (ECL) for Western blotting kit (Santa Cruz, California, USA) according to the manufacturer's instructions. The levels of phosphorylated proteins were normalized to their corresponding total protein levels. Relative densitometry was calculated using Image J2x analysis software (NIH, USA).

Statistical analysis

All data were expressed as mean ± SD and statistically analyzed using software SPSS 17.0 version (SPSS, Inc., Chicago, USA). Differences between groups were first evaluated using one-way analysis of variance (ANOVA), and if the differences were significant, multiple comparison analysis was further performed using Fisher's Least Significant Difference (LSD) test. All *P* values less than 0.05 were considered statistically significant.

Results

AGO improved the recovery of cardiac function after ischemia reperfusion

AGO (20 mg/kg and 40 mg/kg) treatment significantly increased the values of LVDP and ±

dp/dt as compared with the IR group at 30, 60 and 120 min of reperfusion. Furthermore, the AGO40 group (40 mg/kg) retained better cardiac function after ischemia reperfusion than the AGO20 group (20 mg/kg). However, no significant changes in the values of LVDP and ± dp/dt were observed in the AGO10 (10 mg/kg) group. There was no significant change in the value of HR before or after ischemia reperfusion among the different groups (**Figure 2**).

AGO attenuated MIRI in a dose-dependent manner

AGO treatment at 20 mg/kg and 40 mg/kg significantly alleviated pathological changes in ischemic myocardium, but AGO treatment at 10 mg/kg did not show any changes (**Figure 3A**). To further quantify the cardioprotective effect of AGO, we measured the changes of infarct size and the release of myocardial enzymes. AGO treatment at 20 mg/kg and 40 mg/kg significantly reduced myocardial infarct size (AGO20 vs. IR: 39.1% ± 2.7% vs. 46.1% ± 6.9%, *P* = 0.01; AGO40 vs. IR: 33.7% ± 4.0% vs. 46.1% ± 6.9%, *P* < 0.001) (**Figure 3B** and **3C**) and decreased the release of creatine kinase-MB (CK-MB) (AGO20 vs. IR: 171.5 ± 14.9 IU/L vs. 208.8 ± 25.7 IU/L, *P* < 0.001; AGO40 vs. IR: 150.6 ± 16.3 IU/L vs. 208.8 ± 25.7 IU/L, *P* < 0.001) (**Figure 3D**) and lactate dehydrogenase (LDH) (AGO20 vs. IR: 274.6 ± 11.7 IU/L vs. 304.5 ± 30.2 IU/L, *P* = 0.006; AGO40 vs. IR: 252.5 ± 19.3 IU/L vs. 304.5 ± 30.2 IU/L, *P* < 0.001) (**Figure 3E**). AGO treatment at 10 mg/kg did not reduce myocardial infarct size and did not decrease the release of myocardial enzymes (*P* > 0.05). Because the AGO40 group demonstrated the greatest cardioprotective effects, we used the 40 mg/kg AGO dose for the second stage of our study.

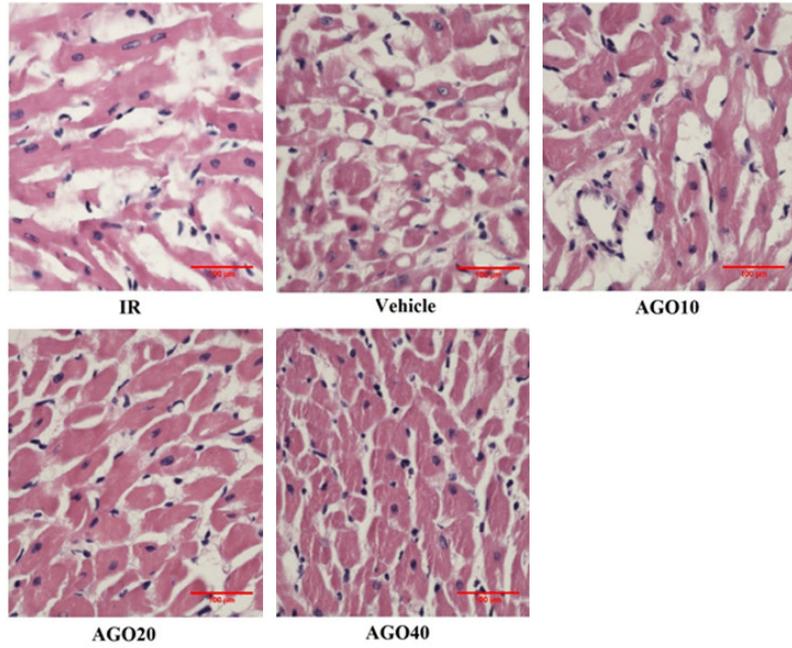
AGO inhibited the opening of MPTP

In IR and vehicle groups, the majority of mitochondria were subjected to swelling, vacuolating and mitochondrial cristae were broken. AGO treatment alleviated this mitochondrial damage. However, no alleviation of mitochondrial damage was observed when treated with ATR (**Figure 4A**).

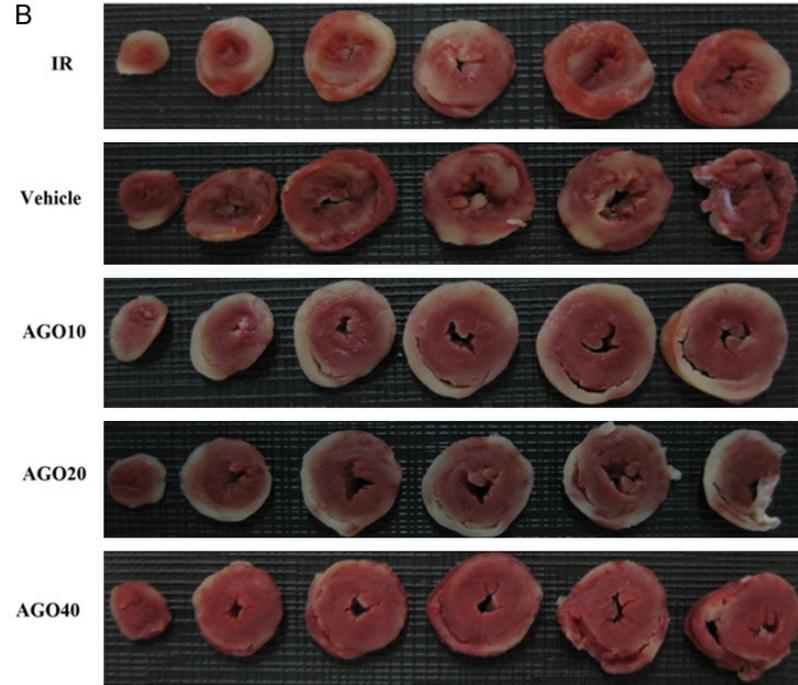
Low nicotinamide adenine dinucleotide (NAD⁺) content in the myocardium is considered a marker of MPTP opening. NAD⁺ is released from inactive and dysfunctional mitochondria upon

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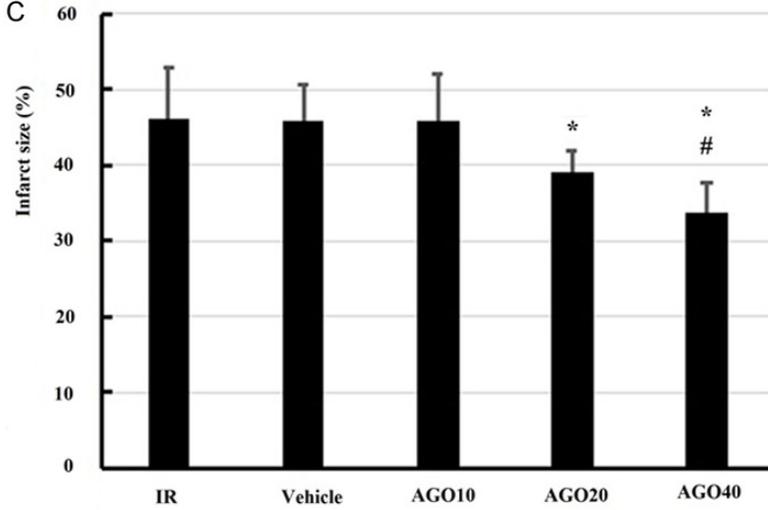
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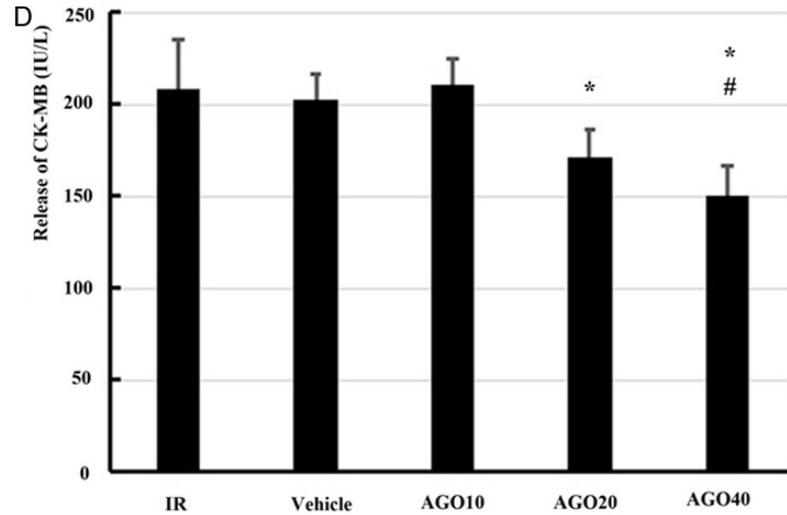
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Agomelatine protects against MIRI

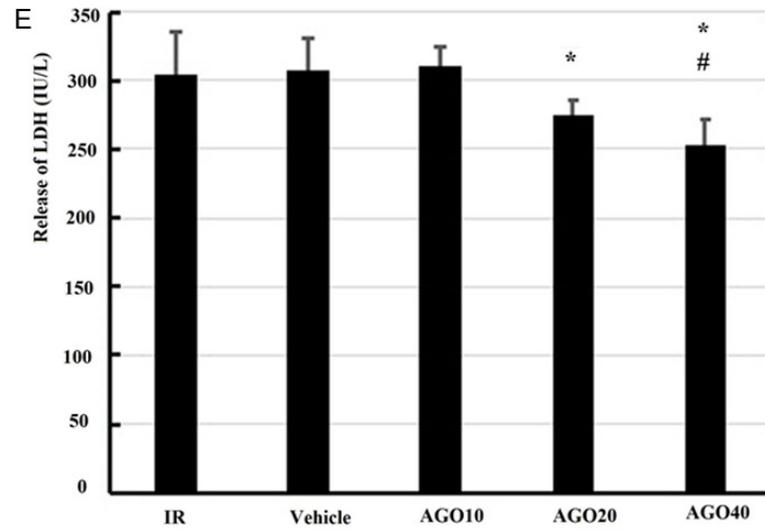


Figure 3. The effect of agomelatine on myocardial ischemia reperfusion injury. A. The pathological changes of ischemic myocardium stained by hematoxylin-eosin ($\times 400$). B. Image of myocardial infarct size stained by TTC. C. Statistic for myocardial infarct size. D. Statistic for the release of CK-MB in coronary effluent. E. Statistic for the release of LDH in coronary effluent. IR: ischemia reperfusion group, Vehicle: Vehicle group, AGO10: 10 mg/kg agomelatine treatment group, AGO20: 20 mg/kg agomelatine treatment group, AGO40: 40 mg/kg agomelatine treatment group. *represents $P < 0.05$ vs. IR; #represents $P < 0.05$ vs. AGO20, $n = 6$ per group.

Agomelatine protects against MIRI

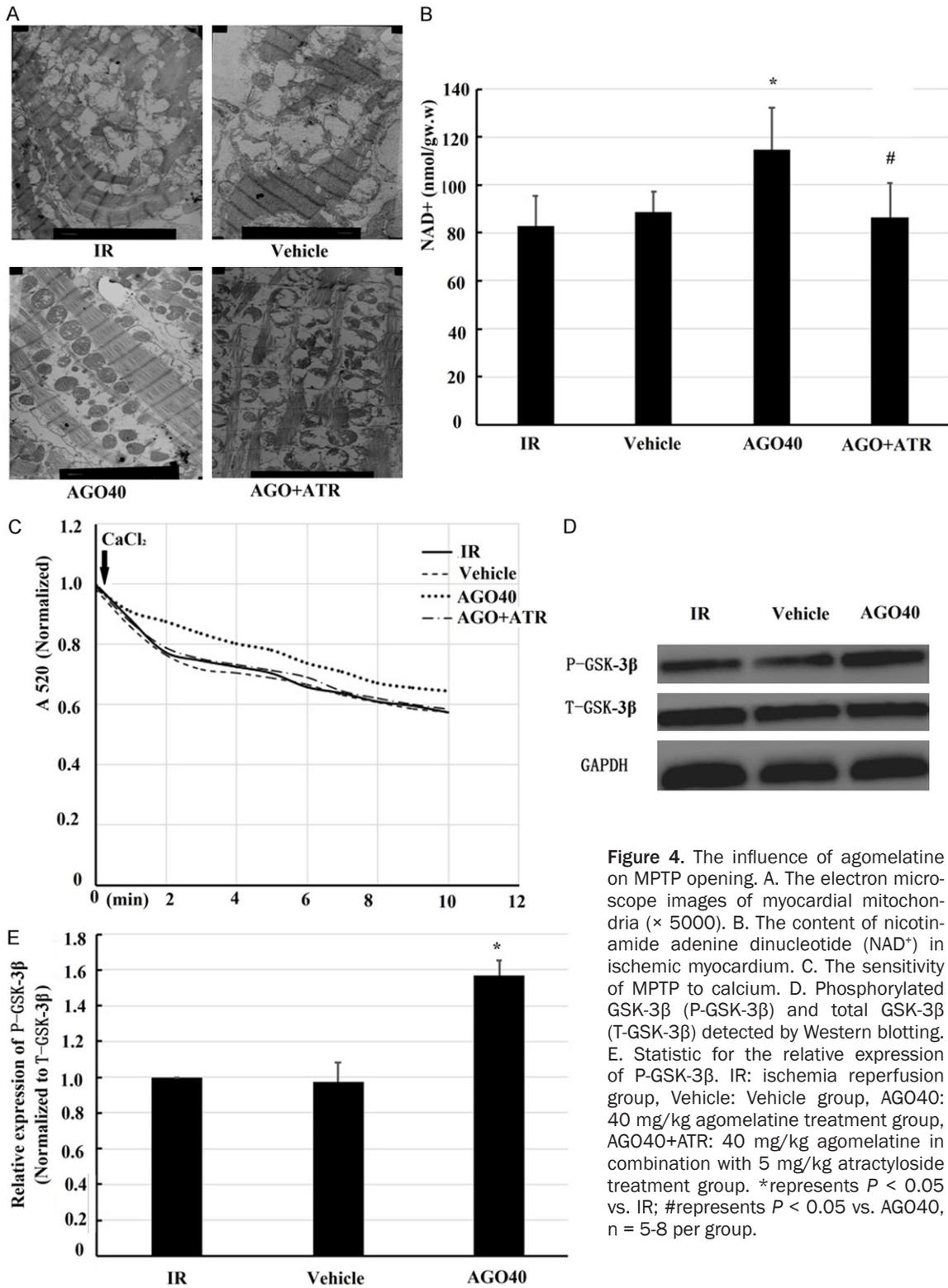


Figure 4. The influence of agomelatine on MPTP opening. A. The electron microscope images of myocardial mitochondria ($\times 5000$). B. The content of nicotinamide adenine dinucleotide (NAD^+) in ischemic myocardium. C. The sensitivity of MPTP to calcium. D. Phosphorylated GSK-3 β (P-GSK-3 β) and total GSK-3 β (T-GSK-3 β) detected by Western blotting. E. Statistic for the relative expression of P-GSK-3 β . IR: ischemia reperfusion group, Vehicle: Vehicle group, AGO40: 40 mg/kg agomelatine treatment group, AGO40+ATR: 40 mg/kg agomelatine in combination with 5 mg/kg atractyloside treatment group. *represents $P < 0.05$ vs. IR; #represents $P < 0.05$ vs. AGO40, $n = 5-8$ per group.

MPTP opening during reperfusion [24]. The ischemic myocardium retained a higher NAD^+ in the AGO40 group as compared to the IR group (AGO40 vs. IR: 114.5 ± 17.6 nmol/gw.w vs.

82.8 ± 12.4 nmol/gw.w, $P = 0.001$). NAD^+ decreased when treated with ATR (AGO40+ATR vs. AGO40: 86.5 ± 14.3 nmol/gw.w vs. 114.5 ± 17.6 nmol/gw.w, $P = 0.002$) (Figure 4B).

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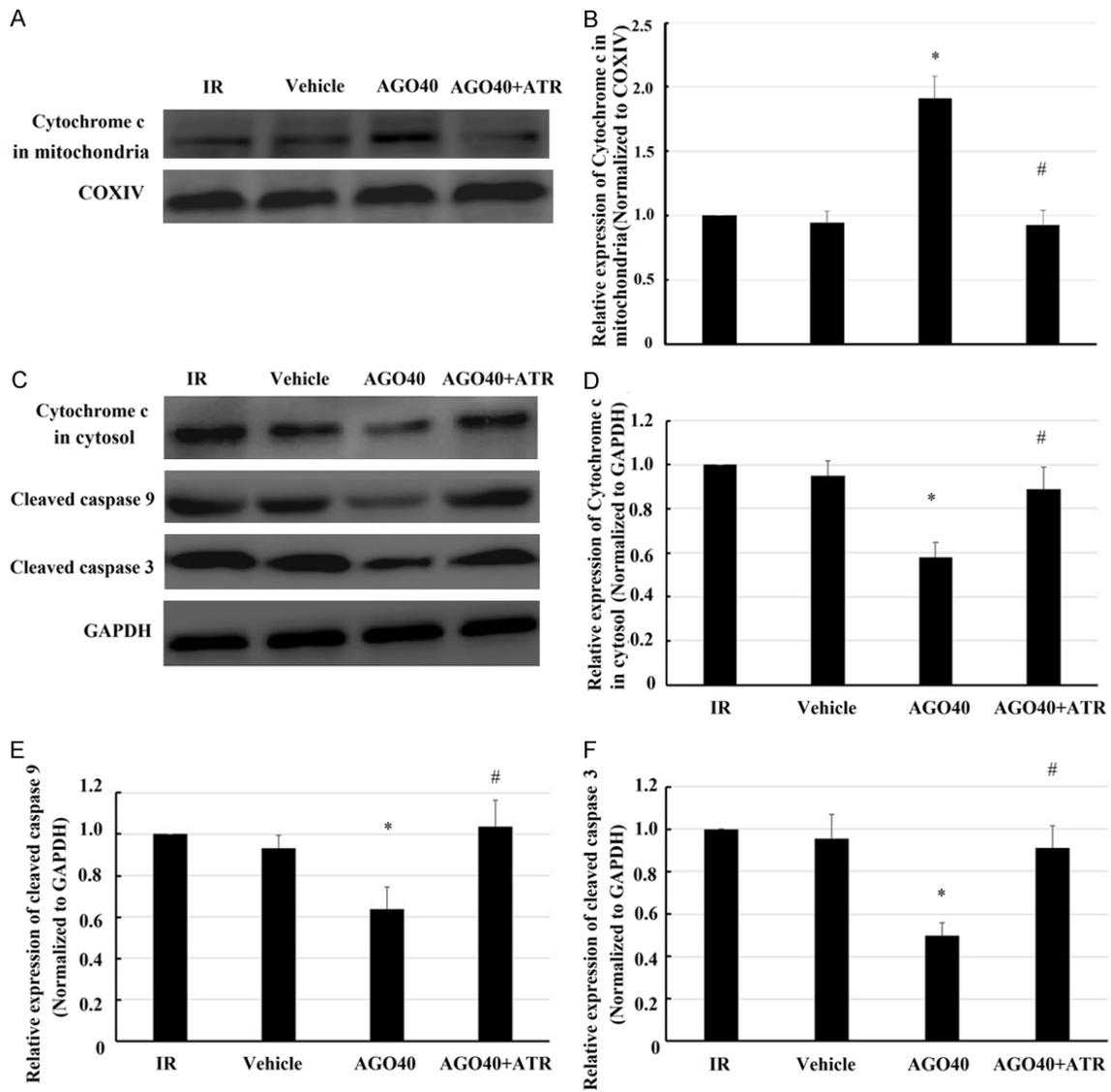


Figure 5. The effect of agomelatine on the mitochondrial apoptotic pathway. A. Mitochondrial Cytochrome C detected by Western blotting. B. Statistic for the relative expression of Cytochrome C in mitochondria. C. Cytosolic Cytochrome C, cleaved-caspase 9 and cleaved-caspase 3 detected by Western blotting. D. Statistic for the relative expression of Cytochrome C in cytosol. E. Statistic for the relative expression of cleaved-caspase 9. F. Statistic for the relative expression of cleaved-caspase 3. IR: ischemia reperfusion group, Vehicle: Vehicle group, AGO40: 40 mg/kg agomelatine treatment group, AGO40+ATR: 40 mg/kg agomelatine in combination with 5 mg/kg atractyloside treatment group. *represents $P < 0.05$ vs. IR; #represents $P < 0.05$ vs. AGO40, $n = 5$ per group.

MPTP opening is usually induced by calcium, and the sensitivity of MPTP to calcium is an indicator of MPTP opening [25]. In our study, isolated mitochondria in the AGO40 group were more resistant to Ca^{2+} stimulation, but the resistance to Ca^{2+} was decreased when treated with ATR (Figure 4C). These two results suggest that AGO inhibited the opening of MPTP.

AGO enhanced the phosphorylation of GSK-3 β

GSK-3 β is a key upstream regulator of MPTP opening, and GSK-3 β phosphorylation is nega-

tively correlated with the opening of MPTP [26]. In this study, we found that 40 mg/kg AGO treatment could significantly enhance the GSK-3 β phosphorylation in ischemic myocardium (Figure 4D and 4E).

AGO inhibited the mitochondrial apoptotic pathway

Western blot analysis showed that AGO at the dose of 40 mg/kg treatment significantly increased the expression of cytochrome C in

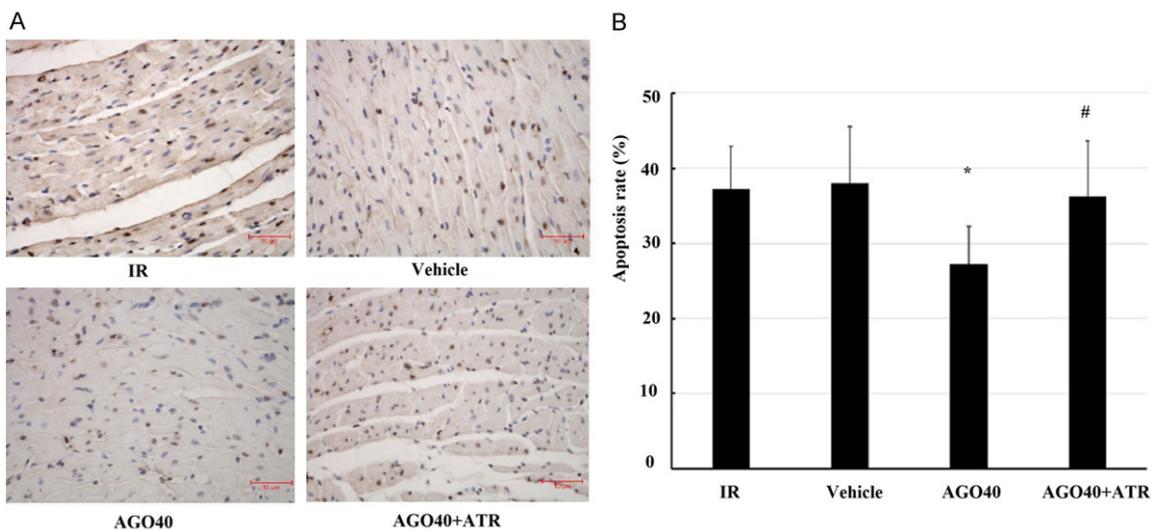


Figure 6. The effect of agomelatine on cell apoptosis. A. Apoptosis detected by TUNEL assay ($\times 400$), the apoptotic cell nuclei were stained brown and the living cell nuclei were stained blue. B. Statistic for the apoptosis rate. IR: ischemia reperfusion group, Vehicle: Vehicle group, AGO40: 40 mg/kg agomelatine treatment group, AGO40+ATR: 40 mg/kg agomelatine in combination with 5 mg/kg atractyloside treatment group. *represents $P < 0.05$ vs. IR; #represents $P < 0.05$ vs. AGO40, $n = 6$ per group.

the mitochondria (Figure 5A and 5B), decreased the expression of cytochrome C in the cytosol (Figure 5D), cleaved-caspase 9 (Figure 5E) and cleaved-caspase 3 (Figure 5F) and decreased the apoptosis rate (Figure 6) as compared to the IR group. However, when treated with a combination of AGO and ATR, the expression of cytochrome C in mitochondria was decreased and the expression of cytochrome C in cytosol, cleaved-caspase 9, cleaved-caspase 3 and apoptosis rate were significantly increased.

The protective effects of AGO were abrogated by ATR

To reconfirm the role of MPTP on the protective effect of AGO, we tested the influence of ATR, a MPTP opener, on the cardioprotection of AGO. The results showed that myocardial infarct size and the release of myocardial enzymes were significantly increased in the AGO40+ATR group as compared with the AGO40 group (Figure 7), suggesting that ATR completely abrogated the cardioprotection of AGO.

Discussion

Ischemic preconditioning (IPC), repetitive short periods of ischemia and reperfusion performed ahead of sustained ischemia, has been found to enhance the tolerance to ischemia insult and

protect against MIRI in several animal models [27-29] and in humans [30]. Similarly, some pharmacological agents, such as adenosine, nicorandil and other agents, have been found to mimic the protective effect of IPC and be helpful to alleviate MIRI, which was termed as pharmacological preconditioning [31, 32]. Melatonin has also been found to be a pharmacological agent to induce pharmacological preconditioning against ischemia reperfusion injury. Melatonin is involved with the activation of Akt and ERK1/2 [33], inhibition of MPTP opening [14] and reinforcement of anti-oxidative properties [34]. AGO is the first melatonergic antidepressant that activates melatonin MT1 and MT2 receptors and simultaneously blocks serotonin 5-HT_{2C} receptor. It is also believed that the protective cardiovascular effects of melatonin are partly mediated via MT1/MT2 receptors [35, 36]. However, until now, there is no data concerning the effect of AGO on MIRI.

Our study is the first to find that AGO could alleviate myocardial ischemia injury and improve recovery of cardiac function after reperfusion. Moreover, AGO's cardioprotective effect appears to be dose-dependent, which coincides with AGO's neuroprotective effects [17]. Taken together, we considered that AGO could induce preconditioning effects to protect heart against ischemia reperfusion injury.

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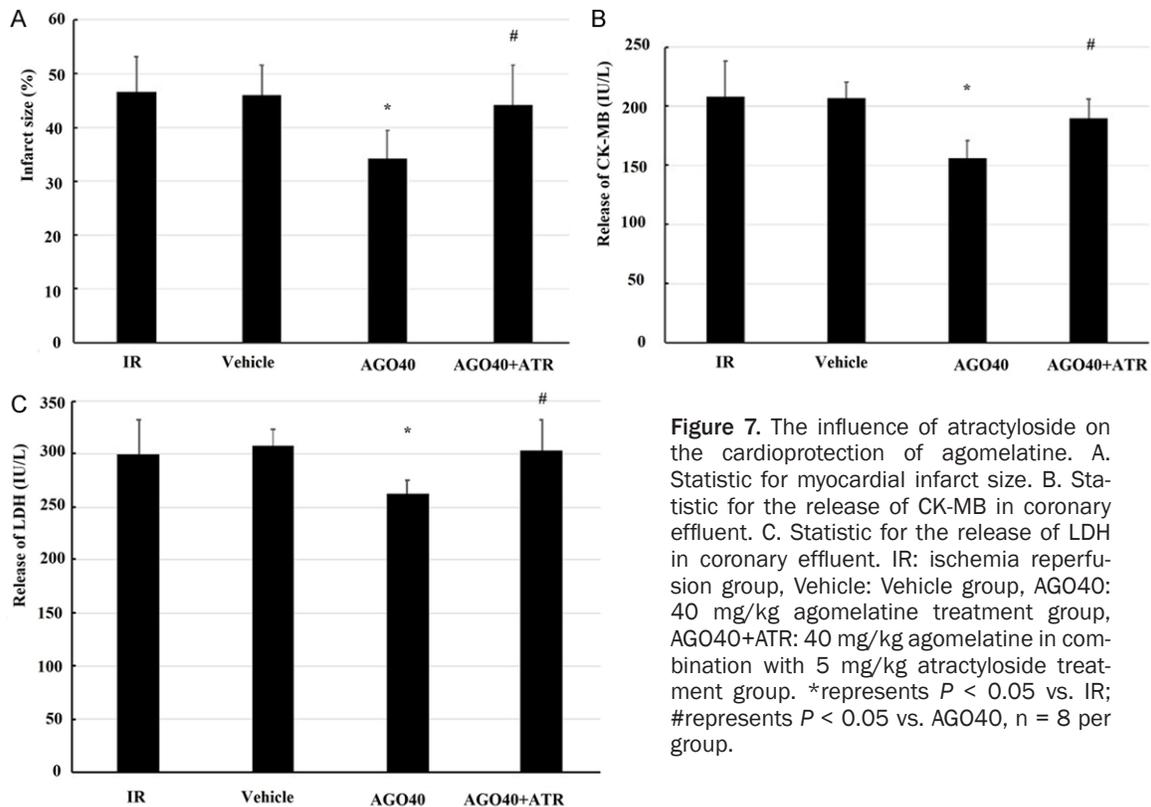


Figure 7. The influence of atractyloside on the cardioprotection of agomelatine. A. Statistic for myocardial infarct size. B. Statistic for the release of CK-MB in coronary effluent. C. Statistic for the release of LDH in coronary effluent. IR: ischemia reperfusion group, Vehicle: Vehicle group, AGO40: 40 mg/kg agomelatine treatment group, AGO40+ATR: 40 mg/kg agomelatine in combination with 5 mg/kg atractyloside treatment group. *represents $P < 0.05$ vs. IR; #represents $P < 0.05$ vs. AGO40, $n = 8$ per group.

To our knowledge, MPTP includes at least Voltage Dependent Anion Channel (VDAC), Adenine Nucleotide Translocase (ANT) and cyclophilin-D [37]. MPTP remains closed or seldom opens under physiological conditions, but its excessive opening can be induced by Ca^{2+} , free radicals and inorganic phosphate [38]. Several studies have already demonstrated that MIRI is closely associated with the opening of MPTP [4-6]. Moreover, MPTP has been identified as the end effector of IPC, including pharmacological preconditioning [39], while little has been known about the association between the protective effect of AGO and MPTP.

In this study, we provided evidence for the first time that AGO could inhibit MPTP opening during the process of MIRI. First, the content of NAD^+ in heart tissues and the sensitivity of MPTP to Ca^{2+} are negatively correlated with the degree of MPTP opening [24, 25]. We found that rat hearts treated with AGO retained a high content of NAD^+ and was more resistant to stimulation with Ca^{2+} , which indicates that AGO inhibited the degree of MPTP opening. Second, GSK-3 β is a key upstream regulator of MPTP opening. The phosphorylation of GSK-3 β at

Ser9 will decrease the activation of GSK-3 β , subsequently increase the threshold of MPTP opening and then lead to a decrease in MPTP opening [26]. We found that AGO treatment enhanced the phosphorylation of GSK-3 β , which agrees with the findings of Musazzi *et al.* in the hippocampus and prefrontal/frontal cortex of male rats [40]. This result implies that AGO inhibited MPTP opening likely through GSK-3 β . Moreover, MPTP is identified as a regulator of cell apoptosis. MPTP opening usually promotes cytochrome C to release into the cytoplasm, which then activates caspases and ultimately leads to cell apoptosis [41]. In this study, we showed that AGO treatment reduced the expression of cytochrome C, cleaved-caspase9, cleaved-caspase3 and decreased the rate of cell apoptosis. This is consistent with the findings of Akpinar *et al.* in PC-12 neuronal cells [42]. This result also provided indirect evidence for the inhibition of MPTP opening by AGO treatment. Most importantly, we discovered that all the protective effects of AGO were abolished by treatment with a MPTP opener. Taken together, we suggested that AGO protected against MIRI by inhibiting MPTP opening.

There still exists two limitations in our study. First, the isolated rat heart model used in this study was deprived of neural and humoral regulation and could not completely mimic the pathophysiological changes under the condition of MIRI. Future studies of the cardioprotection of AGO should be performed *in vivo*. Second, we only demonstrated that AGO inhibited MPTP opening via GSK-3 β , but more upstream pathways of MPTP, such as PI3K-Akt and MAPK/ERK1/2 pathways, were not confirmed in this study. Although Musazzi *et al.* have demonstrated that AGO could activate PI3K-Akt and MAPK/ERK1/2 in a time-dependent manner [40], we did not provide direct evidence for the activation of PI3K-Akt and MAPK/ERK1/2 in the cardioprotection of AGO.

In conclusion, our study demonstrated, for the first time, that AGO protected against MIRI in a dose-dependent manner and that its protective effect involved inhibiting MPTP opening.

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Disclosure of conflict of interest

None.

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References

- [1] Moran AE, Forouzanfar MH, Roth GA, Mensah GA, Ezzati M, Murray CJ and Naghavi M. Temporal trends in ischemic heart disease mortality in 21 world regions, 1980 to 2010: the Global Burden of Disease 2010 study. *Circulation* 2014; 129: 1483-1492.
- [2] Hausenloy DJ and Yellon DM. Myocardial ischemia-reperfusion injury: a neglected therapeutic target. *J Clin Invest* 2013; 123: 92-100.
- [3] Bulluck H, Yellon DM and Hausenloy DJ. Reducing myocardial infarct size: challenges and future opportunities. *Heart* 2016; 102: 341-348.
- [4] Morciano G, Bonora M, Campo G, Aquila G, Rizzo P, Giorgi C, Wieckowski MR and Pinton P. Mechanistic role of mPTP in ischemia-reperfusion injury. *Adv Exp Med Biol* 2017; 982: 169-189.
- [5] Bopassa JC, Michel P, Gateau-Roesch O, Ovize M and Ferrera R. Low-pressure reperfusion alters mitochondrial permeability transition. *Am J Physiol Heart Circ Physiol* 2005; 288: 2750-2755.
- [6] Lemasters JJ, Theruvath TP, Zhong Z and Nieminen AL. Mitochondrial calcium and the permeability transition in cell death. *Biochim Biophys Acta* 2009; 1787: 1395-1401.
- [7] Xie JR and Yu LN. Cardioprotective effects of cyclosporine A in an *in vivo* model of myocardial ischemia and reperfusion. *Acta Anaesthesiol Scand* 2007; 51: 909-913.
- [8] Duan X, Ji B, Yu K, Hei F, Liu J and Long C. Acidic buffer or plus cyclosporine A post-conditioning protects isolated rat hearts against ischemia-reperfusion injury. *Perfusion* 2011; 26: 245-252.
- [9] Hardeland R, Pandi-Perumal SR and Cardinali DP. Melatonin. *Int J Biochem Cell Biol* 2006; 38: 313-316.
- [10] Özdem M, Kırzioğlu FY, Yılmaz HR, Vural H, Fentoğlu Ö, Uz E, Koçak A and Yiğit A. Antioxidant effects of melatonin in heart tissue after induction of experimental periodontitis in rats. *J Oral Sci* 2017; 59: 23-29.
- [11] Chen Y, Zhang J, Zhao Q, Chen Q, Sun Y, Jin Y and Wu J. Melatonin induces anti-inflammatory effects to play a protective role via endoplasmic reticulum stress in acute pancreatitis. *Cell Physiol Biochem* 2016; 40: 1094-1104.
- [12] Yang CY, Lin CK, Tsao CH, Hsieh CC, Lin GJ, Ma KH, Shieh YS, Sytwu HK and Chen YW. Melatonin exerts anti-oral cancer effect via suppressing LSD1 in patient-derived tumor xenograft models. *Oncotarget* 2017; 8: 33756-33769.
- [13] Nooshinfar E, Bashash D, Safaroghli-Azar A, Bayati S, Rezaei-Tavirani M, Ghaffari SH and Akbari ME. Melatonin promotes ATO-induced apoptosis in MCF-7 cells: proposing novel therapeutic potential for breast cancer. *Biomed Pharmacother* 2016; 83: 456-465.
- [14] Petrosillo G, Colantuono G, Moro N, Ruggiero FM, Tiravanti E, Di Venosa N, Fiore T and Paradies G. Melatonin protects against heart ischemia-reperfusion injury by inhibiting mitochondrial permeability transition pore opening. *Am J Physiol Heart Circ Physiol* 2009; 297: 1487-1493.
- [15] Liu LF, Qian ZH, Qin Q, Shi M, Zhang H, Tao XM and Zhu WP. Effect of melatonin on oncosis of myocardial cells in the myocardial ischemia/reperfusion injury rat and the role of the mito-

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- chondrial permeability transition pore. *Genet Mol Res* 2015; 14: 7481-7489.
- [16] Fuchs E, Simon M and Schmelting B. Pharmacology of a new antidepressant: benefit of the implication of the melatonergic system. *Int Clin Psychopharmacol* 2006; 21 Suppl 1: 17-20.
- [17] Chumboatong W, Thummayot S, Govitrapong P, Tocharus C, Jittiwat J and Tocharus J. Neuroprotection of agomelatine against cerebral ischemia/reperfusion injury through an antiapoptotic pathway in rat. *Neurochem Int* 2017; 102: 114-122.
- [18] Yapca OE, Borekci B, Turan MI and Gulapoglu M. The effect of agomelatine on oxidative stress induced with ischemia/reperfusion in rat ovaries. *Adv Clin Exp Med* 2014; 23: 715-721.
- [19] Yu J, Wei J, Ji L and Hong X. Exploration on mechanism of a new type of melatonin receptor agonist Neu-p11 in hypoxia-reoxygenation injury of myocardial cells. *Cell Biochem Biophys* 2014; 70: 999-1003.
- [20] Wu N, Li W, Shu W and Jia D. Protective effect of picoside II on myocardial ischemia reperfusion injury in rats. *Drug Des Devel Ther* 2014; 8: 545-854.
- [21] Jia D. The protective effect of mitochondrial ATP-sensitive K⁺ channel opener, nicorandil, combined with Na⁺/Ca²⁺ exchange blocker KB-R7943 on myocardial ischemia-reperfusion injury in rat. *Cell Biochem Biophys* 2011; 60: 219-224.
- [22] Feng J, Lucchinetti E, Ahuja P, Pasch T, Perriard JC and Zaugg M. Isoflurane postconditioning prevents opening of the mitochondrial permeability transition pore through inhibition of glycogen synthase kinase 3beta. *Anesthesiology* 2005; 103: 987-995.
- [23] Wu N, Zhang X, Guan Y, Shu W, Jia P and Jia D. Hypercholesterolemia abrogates the cardioprotection of ischemic postconditioning in isolated rat heart: roles of glycogen synthase kinase-3 β and the mitochondrial permeability transition pore. *Cell Biochem Biophys* 2014; 69: 123-130.
- [24] Di Lisa F and Ziegler M. Pathophysiological relevance of mitochondria in NAD (+) metabolism. *FEBS Lett* 2001; 492: 4-8.
- [25] Endlicher R, Křiváková P, Lotková H, Milerová M, Drahotka Z and Červinková Z. Tissue specific sensitivity of mitochondrial permeability transition pore to Ca²⁺ ions. *Acta Medica* 2009; 52: 69-72.
- [26] Juhaszova M, Zorov DB, Kim SH, Pepe S, Fu Q, Fishbein KW, Ziman BD, Wang S, Ytrehus K, Antos CL, Olson EN and Sollott SJ. Glycogen synthase kinase-3beta mediates convergence of protection signaling to inhibit the mitochondrial permeability transition pore. *J Clin Invest* 2004; 113: 1535-1549.
- [27] Iwamoto T, Miura T, Adachi T, Noto T, Ogawa T, Tsuchida A and Imura O. Myocardial infarct size-limiting effect of ischemic preconditioning was not attenuated by oxygen free-radical scavengers in the rabbit. *Circulation* 1991; 83: 1015-1022.
- [28] Hagar JM, Hale SL and Kloner RA. Effect of preconditioning ischemia on reperfusion arrhythmias after coronary artery occlusion and reperfusion in the rat. *Circ Res* 1991; 68: 61-68.
- [29] Murry CE, Jennings RB and Reimer KA. Preconditioning with ischemia: a delay of lethal cell injury in ischemic myocardium. *Circulation* 1986; 74: 1124-1136.
- [30] Cribier A, Korsatz L, Koning R, Rath P, Gamra H, Stix G, Merchant S, Chan C and Letac B. Improved myocardial ischemic response and enhanced collateral circulation with long repetitive coronary occlusion during angioplasty: a prospective study. *J Am Coll Cardiol* 1992; 20: 578-586.
- [31] Andreadou I, Iliodromitis EK, Koufaki M and Kremastinos DT. Pharmacological pre- and post-conditioning agents: reperfusion-injury of the heart revisited. *Mini Rev Med Chem* 2008; 8: 952-959.
- [32] Sivaraman V and Yellon DM. Pharmacologic therapy that simulates conditioning for cardiac ischemic/reperfusion injury. *J Cardiovasc Pharmacol Ther* 2014; 19: 83-96.
- [33] Hadj Ayed Tka K, Mahfoudh Boussaid A, Zaouali MA, Kammoun R, Bejaoui M, Ghoul Mazgar S, Rosello Catafau J, Ben Abdennebi H. Melatonin modulates endoplasmic reticulum stress and Akt/GSK3-beta signaling pathway in a rat model of renal warm ischemia reperfusion. *Anal Cell Pathol (Amst)* 2015; 2015: 635172.
- [34] Liu LF, Qin Q, Qian ZH, Shi M, Deng QC, Zhu WP, Zhang H, Tao XM and Liu Y. Protective effects of melatonin on ischemia-reperfusion induced myocardial damage and hemodynamic recovery in rats. *Eur Rev Med Pharmacol Sci* 2014; 18: 3681-3686.
- [35] Paulis L, Simko F and Laudon M. Cardiovascular effects of melatonin receptor agonists. *Expert Opin Investig Drugs* 2012; 21: 1661-1678.
- [36] Genade S, Genis A, Ytrehus K, Huisamen B and Lochner A. Melatonin receptor-mediated protection against myocardial ischaemia/reperfusion injury: role of its anti-adrenergic actions. *J Pineal Res* 2008; 45: 449-458.
- [37] Halestrap AP, McStay GP and Clarke SJ. The permeability transition pore complex: another view. *Biochimie* 2002; 84: 153-166.
- [38] Halestrap AP, Clarke SJ and Javadov SA. Mitochondrial permeability transition pore opening during myocardial reperfusion—a target for cardioprotection. *Cardiovasc Res* 2004; 61: 372-385.

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- [39] Ferdinandy P, Hausenloy DJ, Heusch G, Baxter GF and Schulz R. Interaction of risk factors, comorbidities, and comedications with ischemia/reperfusion injury and cardioprotection by preconditioning, postconditioning, and remote conditioning. *Pharmacol Rev* 2014; 66: 1142-1174.
- [40] Musazzi L, Seguini M, Mallei A, Treccani G, Pelizzari M, Tornese P, Racagni G and Tardito D. Time-dependent activation of MAPK/Erk1/2 and Akt/GSK3 cascades: modulation by agomelatine. *BMC Neurosci* 2014; 15: 119.
- [41] Petit PX, Susin SA, Zamzami N, Mignotte B and Kroemer G. Mitochondria and programmed cell death: back to the future. *FEBS Lett* 1996; 396: 7-13.
- [42] Akpınar A, Uğuz AC and Nazıroğlu M. Agomelatine and duloxetine synergistically modulates apoptotic pathway by inhibiting oxidative stress triggered intracellular calcium entry in neuronal PC12 cells: role of TRPM2 and voltage-gated calcium channels. *J Membr Biol* 2014; 247: 451-459.