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Melatonin protects circulatory death heart from ischemia/reperfusion injury *via* the JAK2/STAT3 signalling pathway

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ABSTRACT

Aims: The shortage of donor hearts could be alleviated with the use of the allografts from donation after circulatory death (DCD). Here, we evaluated the protective effect of melatonin on myocardial ischemia/reperfusion (MI/R) injury in a DCD heart model after *ex vivo* perfusion.

Main methods: Donor hearts were harvested from DCD model rats pre-treated with or without melatonin and subjected to 30 min of *ex vivo* perfusion, followed by transplantation. Tissue samples were obtained at 3, 12, and 24 h after heart transplantation. Myocardial oedema was evaluated based on the water content and wet/dry ratio, while inflammation was examined with hematoxylin & eosin staining. The expression levels of matrix metalloproteinase-9, interleukin-6, and tumour necrosis factor-α were evaluated. Oxidative stress level was determined from the content of malondialdehyde, activities of superoxide dismutase and glutathione peroxidase, and expression of Nrf2, NQO1 and cytochrome-C. Myocardial apoptosis was detected with TUNEL assay and measurement of the expression levels of Bax, Bcl-2, caspase-3, and cleaved caspase-3. The activation of the JAK2/STAT3 signalling pathway was evaluated by determining the levels of p-JAK2 and p-STAT3.

Key findings: Melatonin pre-treatment protected the heart from MI/R by reducing myocardial oedema and inflammation, attenuating oxidative stress, and decreasing myocardial apoptosis. Furthermore, the JAK2/STAT3 signalling pathway was activated after melatonin treatment during MI/R. The protective effects of melatonin were abolished by AG490.

Significance: Melatonin pre-treatment protected the heart from MI/R in a DCD heart model after *ex vivo* perfusion. Melatonin exerted cardioprotective effects through the activation of the JAK2/STAT3 signalling pathway.

1. Introduction

Heart transplantation is the most effective therapy for patients with end-stage heart disease [1]. Heart transplantation has been performed using brain-dead donors after the establishment of the brain-death criteria. However, the mortality rate of patients awaiting heart

Abbreviations: DCD, donation after circulatory death; MI/R, myocardial ischemia/reperfusion; HE, hematoxylin & eosin; TUNEL, terminal deoxynucleotidyl transferase dUTP nick end labelling; MMP-9, matrix metalloproteinase-9; IL-6, interleukin-6; TNF-α, tumour necrosis factor-α; MDA, malondialdehyde; SOD, superoxide dismutase; GPX, glutathione peroxidise; BCA, bicinchoninic acid; RIPA, radioimmunoprecipitation assay; Nrf2, nuclear factor (erythroid-derived 2)-like 2; NQO1, NAD(P)H: quinone oxidoreductase 1; Cyto-C, cytochrome-C; Bax, Bcl-2 associated X protein; Bcl-2, B cell lymphoma/lewkmia-2; C-caspase-3, cleaved caspase-3; JAK2/STAT3, Janus kinase 2/signal transducer and activator of transcription 3; p-JAK2, phospho-JAK2; p-STAT3, phospho-STAT3; t-JAK2, total JAK2; t-STAT3, total STAT3; TTC, triphenyl tetrazolium chloride; OCS, organ care system; EVHP, *ex vivo* heart perfusion; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; SEM, standard errors of the means; MV, mechanical ventilation; DMV, discontinue mechanical ventilation; NF-κB, nuclear factor-κB; DNA, deoxyribonucleic acid; Bim, Bcl-2 interacting mediator of cell death; AMPK/SERCA2a, 5′-AMP-activated protein kinase/sarcoplasmic/endoplasmic reticulum calcium ATPase 2a; siRNA, short interfering RNA; MPTP, mitochondrial permeability transition pore; TOM20, Translocase of the outer membrane 20; PKB/Akt, protein kinase B; ERK, extracellular signal-regulated kinase; GSK, glycogen synthase kinase; SIRT1, sirtuin 1; SIRT3, sirtuin 3; PGC1α, peroxisome proliferator-activated receptor gamma coactivator 1-alpha

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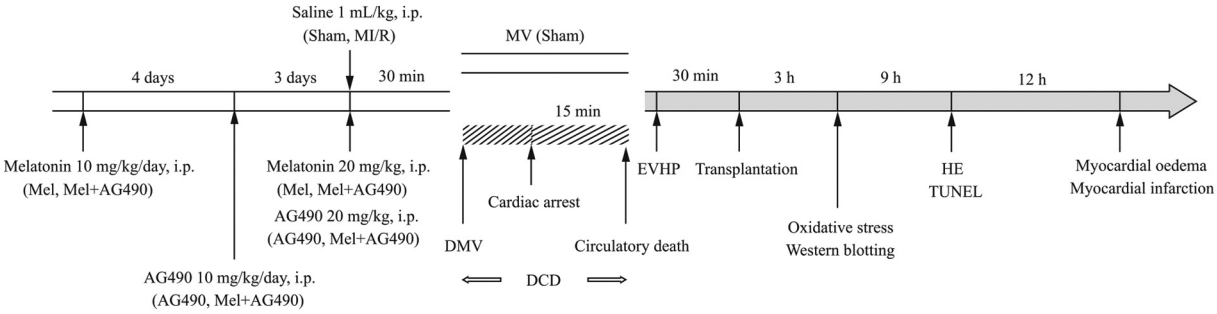

Fig. 1. Experimental design.
Experimental protocol for treatment, DCD, EVHP, and transplantation in rat hearts. DCD, donation after circulatory death; EVHP, *ex vivo* heart perfusion; AG490, a specific inhibitor of JAK2; MV, mechanical ventilation; DMV, discontinue mechanical ventilation; HE, hematoxylin & eosin; TUNEL, terminal deoxynucleotidyl transferase dUTP nick end labelling.

transplantation is as high as 15% every year, owing to the lack of suitable donor hearts [2]. This critical shortage of suitable organs from donors has prompted interest in heart donation after circulatory death (DCD) to expand the donor pool. However, the redundant warm ischemia time causes severe inflammation, oxidative stress, and apoptosis during myocardial ischemia/reperfusion (MI/R). MI/R is a characteristic and crucial prognostic factor for DCD hearts. Therefore, strategies that alleviate MI/R in DCD heart transplantation have gained popularity in cardioprotective research.

Melatonin (*N*-acetyl-5-methoxytryptamine), is well-known for its free radical-scavenging [3], anti-inflammatory, and antioxidative activities. This hormone is produced mainly by the pineal gland in mammals and humans. Melatonin plays a role in a wide range of tissues and cells and could cross the cell membrane and nuclear membrane owing to its hydrophilic and lipophilic properties. In addition, melatonin is known to affect the organs under various pathological conditions such as infection [4], neurodegeneration [5], and diabetes [6]. The cardioprotective effects of melatonin have been previously evaluated, and MI/R injury was shown to be alleviated upon melatonin treatment. However, the mechanisms underlying the protective effect of melatonin during MI/R injury in DCD hearts remain unclear.

The Janus kinase/signal transducer and activator of transcription (JAK/STAT) signalling pathway is involved in a variety of biological processes, including inflammation, differentiation, proliferation, and tumour metastasis. The activation of the JAK2/STAT3 signalling pathway could reduce oxidative stress and apoptosis during ischemia/reperfusion. In addition, the JAK2/STAT3 pathway is known to be involved in imparting cardioprotection against many drugs in ischemia/reperfusion injury [7,8]. Whether this signalling pathway plays a crucial role in the melatonin-mediated cardioprotection of DCD heart is, however, unknown.

In the present study, we established a DCD heart model to investigate the effects of melatonin pre-treatment on MI/R and explored the mechanism underlying the role of JAK2/STAT3 signalling in mediating cardioprotective effects of melatonin.

2. Materials and methods

2.1. Animals

All animal experimental procedures were approved by the Tongji Hospital, Tongji Medical College, Huazhong University of Science and Technology Review Board in Wuhan, China. A total of 120 male Sprague-Dawley rats weighing 300–350 g were selected for the study. All animals were housed under pathogen-free conditions on a 12:12-h light/dark cycle at 22–24 °C and fed a regular pellet diet *ad libitum*.

2.2. Reagents

Melatonin and triphenyl tetrazolium chloride (TTC) were purchased from Sigma-Aldrich (St. Louis, MO, USA) and AG490, from APExBIO Technology (Houston, TX, USA). Commercially available kits to evaluate malondialdehyde (MDA) content, superoxide dismutase (SOD) activity, and glutathione peroxidase (GPX) activity, and bicinchoninic acid (BCA) protein assay kit were supplied by Beyotime Biotechnology (Shanghai, China). Radioimmunoprecipitation assay (RIPA) lysis buffer (50 mM Tris-HCl, 150 mM sodium chloride, 1% Triton X-100, 1% sodium deoxycholate, and 0.1% sodium dodecyl sulphate) and phenylmethanesulfonyl fluoride were procured from Servicebio (Wuhan, China). Antibodies against tumour necrosis factor-α (TNF-α; 17590-1-ap), nuclear factor-like 2 (Nrf2; 16396-1-AP), and caspase-3 (66470-2-lg) were provided by Proteintech Group (Wuhan, China). Antibodies against Bax (GB11007), interleukin-6 (IL-6; GB11117), matrix metalloproteinase-9 (MMP-9; GB12132-1), NAD(P)H quinone dehydrogenase 1 (NQO1; GB11282), Cytochrome-C (Cyto-C; GB11080), JAK2 (GB11325), STAT3 (GB11176), and glyceraldehyde 3-phosphate dehydrogenase (GAPDH; GB12002) were obtained from Servicebio (Wuhan, China). Antibodies against p-STAT3 (RLP0250) and Bcl-2 (ab59348) were purchased from Ruiying Biotechnology (Suzhou, China) and Abcam (Cambridge, UK), respectively, while those against cleaved caspase-3 (C-caspase-3; 9661) and p-JAK2 (3771S) were obtained from Cell Signaling Technology (Danvers, MA, USA).

2.3. Experimental groups

The rats (n = 120) were randomly and equally divided into five groups; each group comprised rats for blood donation, heart donation, and transplantation. The procedure of blood donation and heart transplantation was uniform in all groups. Heart donation was performed as follows (Fig. 1): (1) The rats from MI/R group received sterile saline solution (1 mL/kg, intraperitoneal [i.p.]) 30 min before surgery. The DCD model was induced through the cessation of the mechanical ventilation, and the donor hearts were harvested after the establishment of DCD. Thirty minutes of *ex vivo* perfusion was performed followed by heart transplantation; (2) In the Sham group, the rats received sterile saline solution (1 mL/kg, i.p.) 30 min prior to surgery, and the surgical procedure was similar to that adopted for MI/R group except that the mechanical ventilation was not ceased; (3) The rats from the melatonin (Mel) group were treated with melatonin ($10\ mg{\cdot}kg^{-1}\ day^{-1}$, i.p.) for 7 consecutive days prior to surgery. Melatonin (20 mg/kg, i.p.) was administered once again 30 min before DCD, and the surgical procedure was identical to that adopted for the rats from the MI/R group; (4) In melatonin + AG490 (Mel + AG490) group, the rats were treated with AG490 ($10\ mg{\cdot}kg^{-1}\ day^{-1}$, i.p.) for 3 consecutive days prior to surgery. AG490 (20 mg/kg, i.p.) was administered once again 30 min before DCD, and the remaining steps

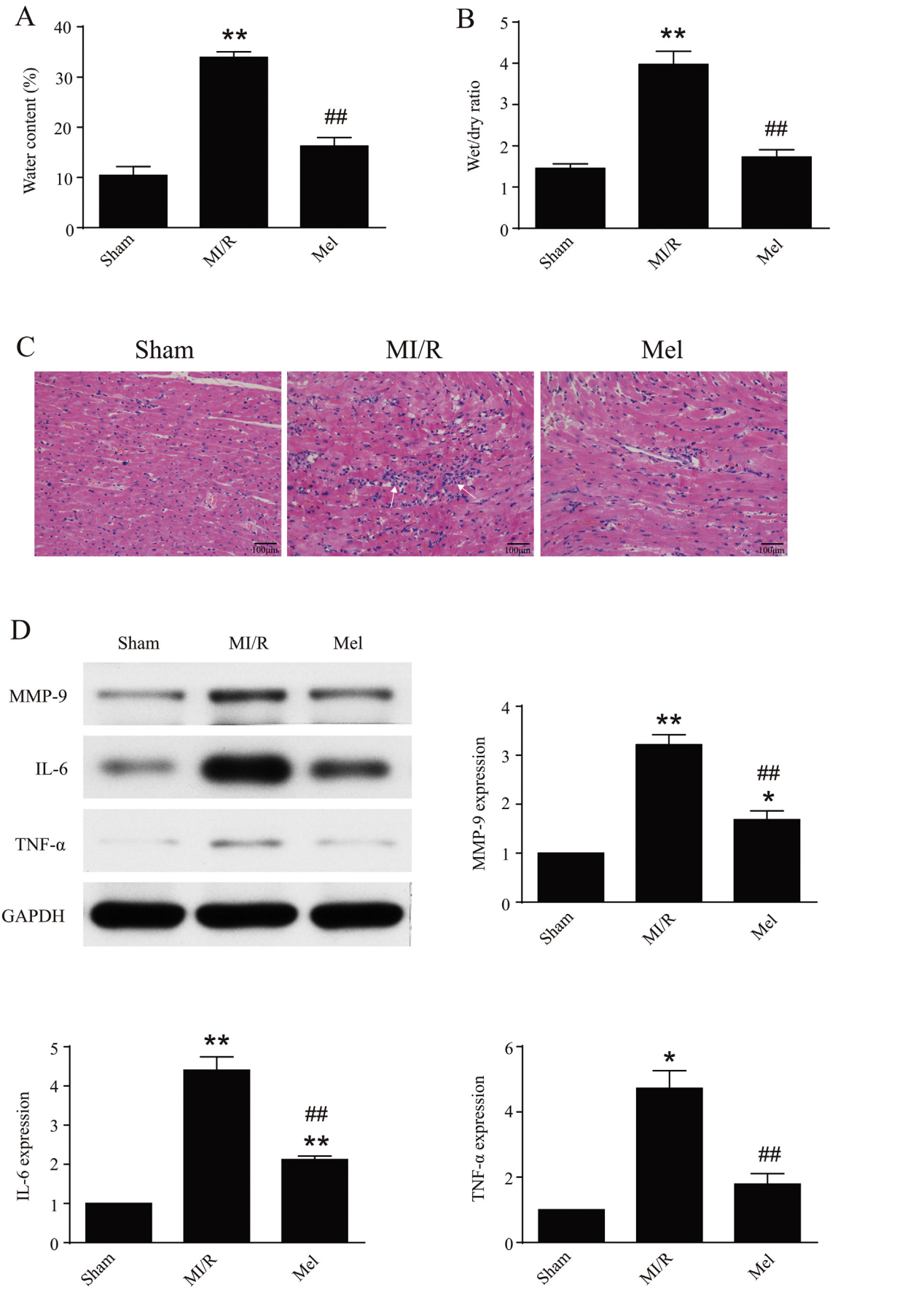

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Fig. 2. Myocardial oedema and inflammation by melatonin pre-treatment in ischemia/reperfusion injured hearts.
(A) Water content was detected by weighing the heart before EVHP and at 24 h after transplantation. (B) Wet/dry ratio was evaluated in the left ventricular myocardial sample. (C) Inflammatory changes in the heart were assessed by HE staining. Inflammatory cell infiltration is indicated by a white arrow. (D) The expression levels of MMP9, IL-6, and TNF-α were detected by western blotting at 3 h after heart transplantation. Data are shown as the mean ± SEM. *, ** indicate $P < 0.05$, $P < 0.01$ compared to the Sham group, #, ## indicate $P < 0.05$, $P < 0.01$ compared to the MI/R group.

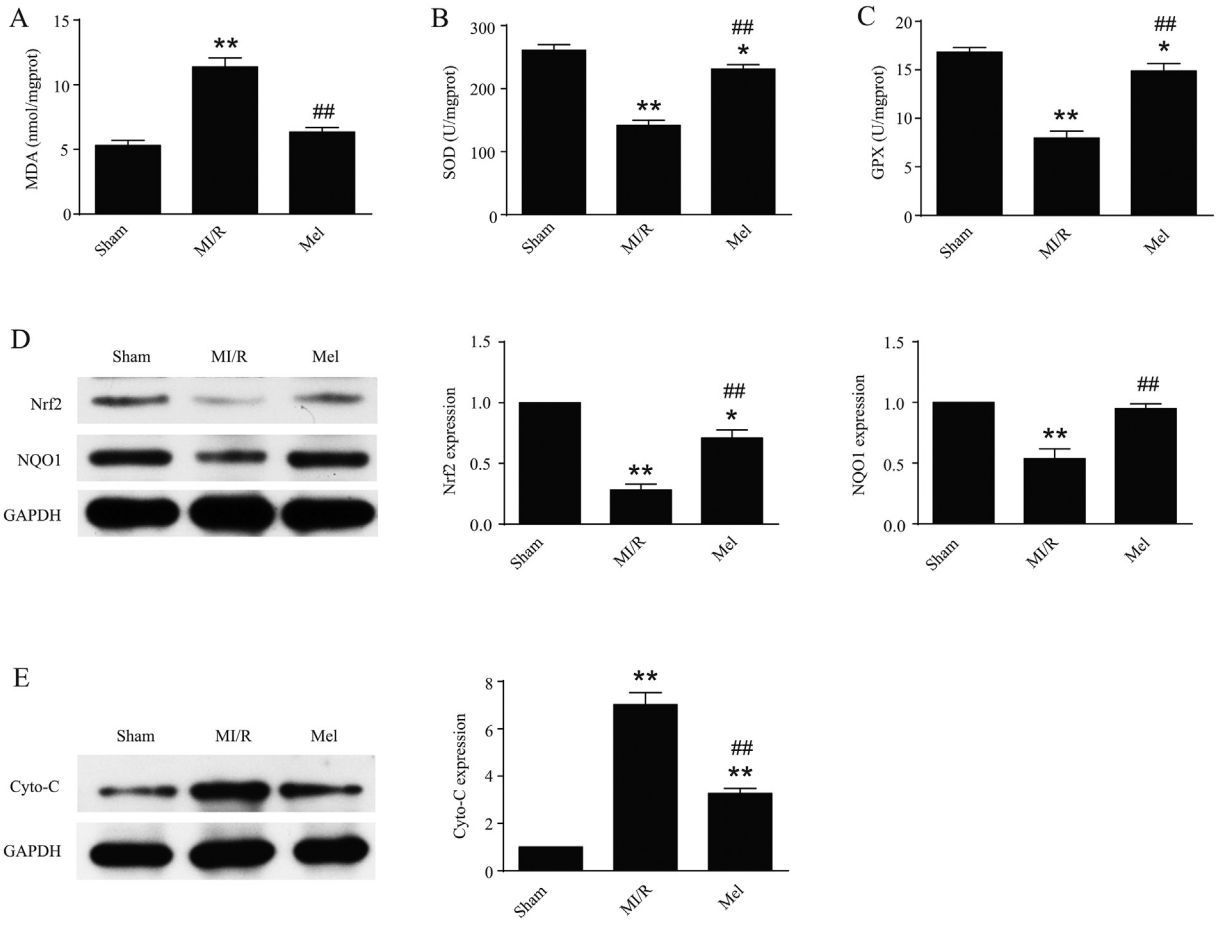

Fig. 3. Effect of melatonin pre-treatment on oxidative stress in ischemia/reperfusion injured hearts.
(A–C) MDA content, SOD activity, and GPX activity were detected at 3 h after heart transplantation with commercially available kits. (D, E) The expression levels of Nrf2, NQO1, and Cyto-C were assessed by western blotting at 3 h after heart transplantation. Data are shown as the mean ± SEM. *, ** indicate $P < 0.05$, $P < 0.01$ compared to the Sham group, #, ## indicate $P < 0.05$, $P < 0.01$ compared to the MI/R group.

were the same as those performed in the Mel group; and (5) In the rats of AG490 group, AG490 administration was similar to that adopted for Mel + AG490 group except melatonin treatment.

2.4. Blood collection

Blood donors were anesthetised with an intraperitoneal administration of 3% pentobarbital (60 mg/kg). Next, 24-G catheters were inserted into the right common carotid artery and connected to the blood reservoir. Blood collection was completed after the volume reached 12 mL. The perfusate contained 12 mL donor animal blood, 4 mL organ care system (OCS) priming solution (in mg/mL: 25 mannitol, 9.6 sodium chloride, 1.8 sodium glycerophosphate, 0.37 potassium chloride, and 0.37 magnesium sulphate heptahydrate) [9], dexamethasone sodium phosphate (25 mg/L), sodium bicarbonate (0.8 mg/L), and insulin (40 IU/L). The perfusate was oxygenated for approximately 15 min before reperfusion through a membrane oxygenator (95% O_2/5% CO_2). The temperature was maintained using a water-jacketed glassware, and the perfusate was recirculated *via* a blood reservoir.

2.5. Establishment of a DCD model

The DCD rat model was established as follows. Briefly, after the preparation of the perfusate, the rats were anesthetised with an intraperitoneal injection of 3% pentobarbital (60 mg/kg), endotracheally intubated, and ventilated with 3 cm H_2O positive-end expiratory pressure. The core temperature of the animal was maintained at 37 °C with heating pads. The adequacy of anaesthesia was monitored by detecting the corneal and withdrawal reflexes. The ventilation frequency was maintained at 60–70/min, and the tidal volume was set to 8–10 mL/kg. Subcutaneous needle electrodes were used to record the electrocardiogram. Arterial pressure was recorded through a 24-G catheter inserted into the right common carotid artery. Electrocardiogram and arterial pressure were continuously monitored. Heparin was administered (400 IU/kg) *via* the inferior vena cava.

DCD was initiated through the discontinuation of mechanical ventilation. Cardiac arrest was defined as the non-pulsatile and/or mean arterial pressure ≤ 25 mmHg. Circulatory death was declared at 15 min after the circulatory standoff period following cardiac arrest, indicative of the establishment of the DCD model. The heart was perfused with 40 mL of cold (4 °C) Custodiol solution (in mM: 15 sodium chloride, 9

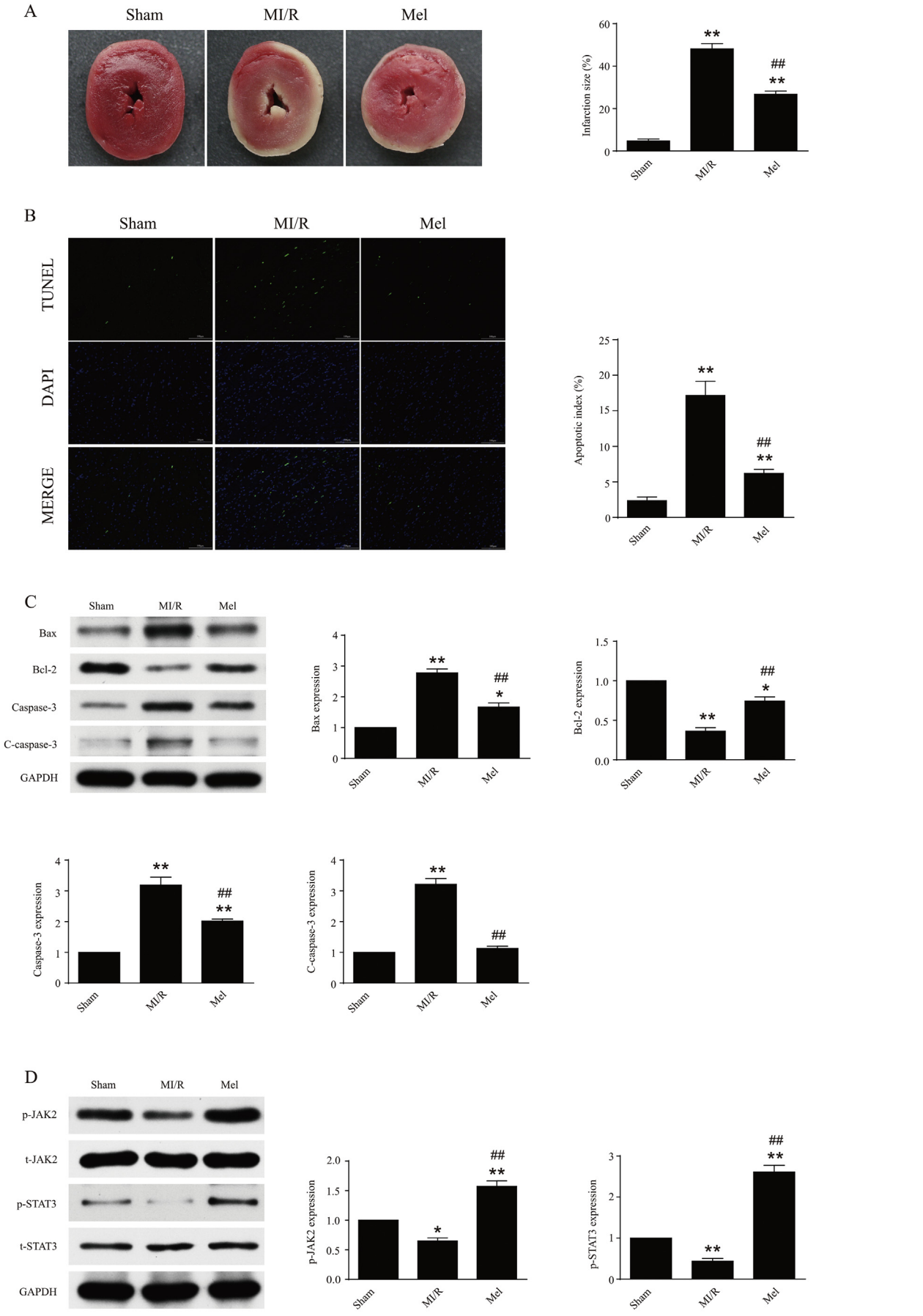

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Fig. 4. Myocardial apoptosis and activity of the JAK2/STAT3 signalling pathway by melatonin pre-treatment in ischemia/reperfusion injured hearts.
(A) Myocardial infarct size was measured by TTC staining. The myocardial infarct size was expressed as the percentage of infarct relative to the total area. (B) TUNEL assay was performed with a commercially available kit. Apoptotic cardiomyocytes were subjected to TUNEL staining (green), and the nuclei of all cardiomyocytes were stained with DAPI (blue). The apoptotic index was expressed as the percentage of apoptotic cells relative to the total cells. (C, D) The expression levels of Bax, Bcl-2, caspase-3, C-caspase-3, p-JAK2, and p-STAT3 were detected by western blotting at 3 h after heart transplantation. Data are shown as the mean ± SEM. *, ** indicate $P < 0.05$, $P < 0.01$ compared to the Sham group, #, ## indicate $P < 0.05$, $P < 0.01$ compared to the MI/R group. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

potassium chloride, 4 magnesium chloride, 18 histidine hydrochloride, 180 histidine, 2 tryptophan, 30 mannitol, 0.015 calcium chloride, and 1 potassium hydrogen 2-ketoglutarate; osmolarity 310 mOsm/kg; pH 7.20) after 15 min [10], and harvested using standard methods. Donor hearts from the rats in the Sham group were harvested without the discontinuation of mechanical ventilation.

2.6. Ex vivo perfusion apparatus

After harvest, the hearts were immediately stored in an ice-cold Custodiol solution and transported to the *ex vivo* heart perfusion (EVHP) apparatus on ice. Reperfusion of the donor heart was initiated *via* a peristaltic pump after the cannulation of the aorta. The outflow was collected in a blood reservoir and used for circular reperfusion. The reperfusion temperature was maintained at 37 °C and reperfusion pressure during EVHP was 70–80 mmHg. After 30 min reperfusion, 40 mL of ice-cold (4 °C) Custodiol solution was administered through the cannula of the aorta.

2.7. Heterotopic heart transplantation

The heart was subjected to heterotopic abdominal transplantation after 30 min reperfusion, as previously described [11]. Briefly, rats were anesthetised with an intraperitoneal injection of 3% pentobarbital (60 mg/kg). The abdominal aorta and inferior vena cava were exposed after the abdomen was opened, and end-to-side anastomosis of the recipient's abdominal aorta with the donor's ascending aorta and end-to-side anastomosis of the recipient's inferior vena cava with the donor's pulmonary artery were made. Heart transplantation was conducted within 40 min. The recipient rat was placed in a thermotank for recover.

2.8. Myocardial oedema

Myocardial oedema was assessed based on the water content and wet/dry ratio at 24 h after transplantation. The weight of the donor heart after harvest was recorded as the original weight, while the heart at 24 h after transplantation was defined as the reperfusion weight. The ventricular specimens at 24 h after transplantation were weighed and recorded as the wet weight. The specimen was wrapped in a tin foil and placed in an 80 °C oven. After 72 h, dry weight was recorded once the weight of the specimen became stable. Water content was calculated as follows:

$$\text{Water content (\%)} = (\text{Reperfusion weight} - \text{Original weight}) / (\text{Original weight}) \times 100\%$$

$$\text{Wet/dry ratio (\%)} = (\text{Wet weight} - \text{Dry weight})/(\text{Wet weight}) \times 100\%$$

2.9. Hematoxylin & eosin staining and terminal deoxynucleotidyl transferase dUTP nick end labelling assay

At 12 h after transplantation, myocardial inflammatory injury was evaluated in the left ventricle with hematoxylin & eosin (HE) staining using a standard protocol. At the end of the 12 h of transplantation, myocardial apoptosis was assessed using the Roche terminal deoxynucleotidyl transferase dUTP nick end labelling (TUNEL) assay following the manufacturer's instructions.

2.10. Mitochondrial oxidative stress evaluation

Mitochondrial oxidative stress indicators (MDA content, SOD and GPX activities) were measured at 3 h after transplantation using commercially available kits following the manufacturer's instructions.

2.11. Infarct size assessment

At 24 h after transplantation, the heart was quickly isolated and stored at −20 °C for 20 min. The heart was cut perpendicularly to the long axis, and the slices were immersed in 1% TTC diluted in phosphate buffer at 37 °C for 30 min in the dark. The slices were subsequently fixed with 4% paraformaldehyde.

2.12. Western blot analysis

Western blotting was performed using heart tissues from the left ventricle at 3 h after transplantation. Briefly, the myocardial tissue was crushed and lysed in RIPA lysis buffer containing 1 mM phenylmethanesulfonyl fluoride on ice. After homogenisation in a tissue homogeniser under 4 °C, the samples were placed on ice for 30 min. The lysate was centrifuged at 12,000 rcf for 10 min to obtain proteins. BCA protein assay kit was used to quantify proteins using a standard protein concentration curve. Proteins were prepared and separated with sodium dodecyl sulphate-polyacrylamide gel electrophoresis. The separated bands were transferred onto polyvinylidene difluoride membranes (Millipore, Billerica, MA, USA) and incubated overnight (4 °C) with antibodies against MMP-9 (1:1000), IL-6 (1:500), TNF-α (1:1000), Bcl-2 (1:1000), Bax (1:500), caspase-3 (1:500), C-caspase-3 (1:1000), Nrf2 (1:1000), NQO1 (1:1000), Cyto-C (1:1000), p-JAK2 (1:1000), total JAK2 (1:1000), p-STAT3 (1:1000), total STAT3 (1:1000), and GAPDH (1:25,000). The membranes were washed with Tris buffered saline tween (10 mM Tris-base, 100 mM sodium chloride, and 0.1% Tween-20, pH 7.50) and probed with appropriate secondary antibodies for 30 min at room temperature (20–25 °C). The protein bands were detected with the Image Lab software system (Bio-Rad, Hercules, CA, USA).

2.13. Statistical analysis

The results were expressed as the means ± standard errors of the means (SEM). Differences in the overall means between groups were assessed with analysis of variance followed by Bonferroni correction for a *post hoc t*-test. A significant difference was defined as $P < 0.05$. Analyses were performed using SPSS version 23.0 software (SPSS, Inc., Chicago, IL, USA).

3. Results

3.1. Melatonin protects the heart from MI/R by reducing myocardial oedema and inflammation

As shown in Fig. 2A–B, MI/R caused a significant increase in myocardial oedema as compared to the sham operation ($P < 0.01$). Melatonin pre-treatment alleviated myocardial oedema in the transplanted

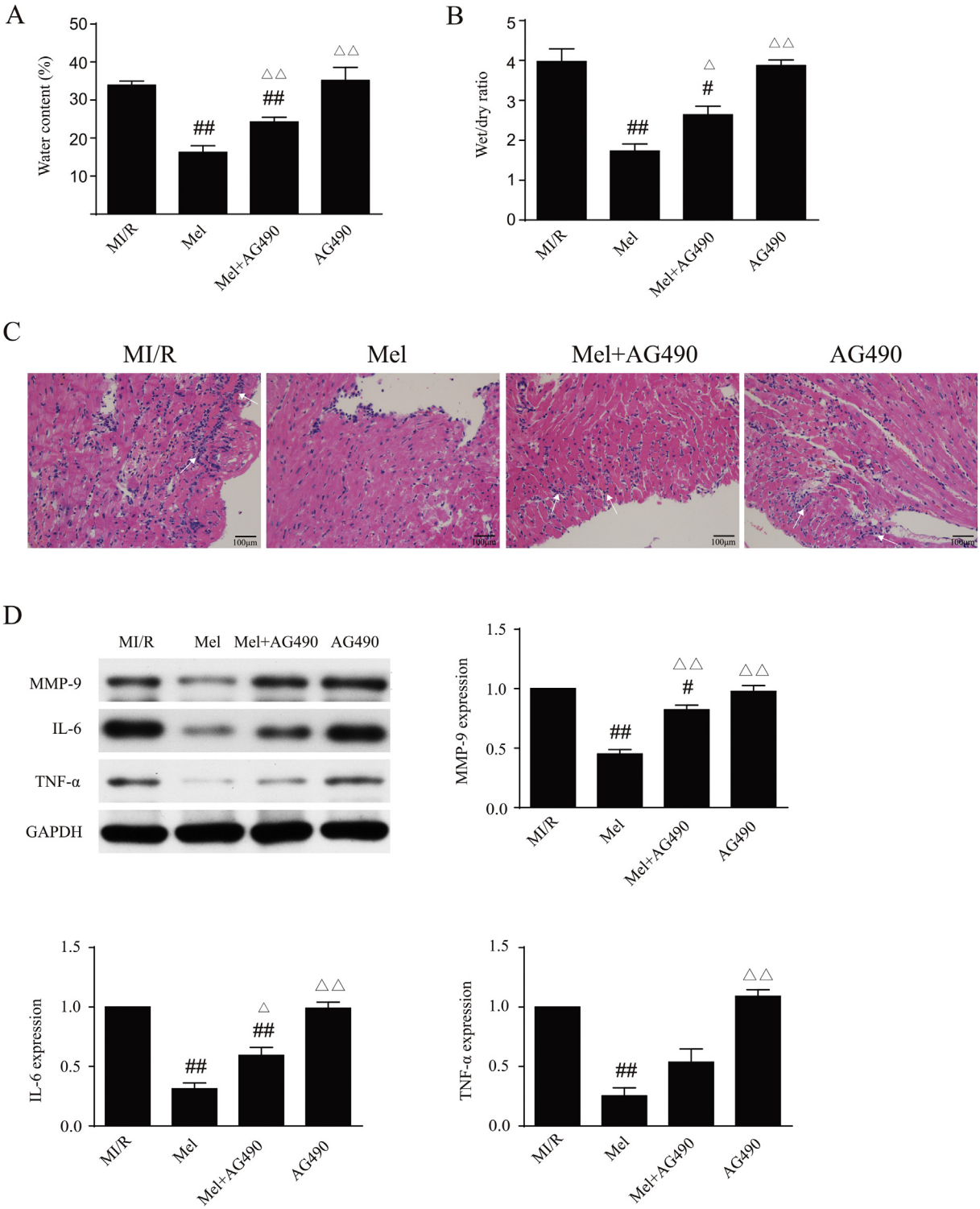

Fig. 5. Myocardial oedema and inflammation by AG490 treatment in ischemia/reperfusion injured hearts.
(A) Water content. (B) Wet/dry ratio. (C) HE staining. Inflammatory cell infiltration is indicated with a white arrow. (D) Expression levels of MMP9, IL-6, and TNF-α. Data are shown as the mean ± SEM. #, ## indicate $P < 0.05$, $P < 0.01$ compared to the MI/R group. Δ, ΔΔ indicate $P < 0.05$, $P < 0.01$ compared to the Mel group.

hearts, as confirmed with the reduced myocardial water content and wet/dry ratio in the Mel group as compared with MI/R group ($P < 0.01$).

Additionally, significant myocardial oedema and inflammation were induced by MI/R according to HE staining (Fig. 2C). In comparison with the MI/R group, the melatonin-treated group showed a significant alleviation in myocardial oedema and inflammation. As expected, the expression levels of inflammatory markers such as MMP-9, IL-6, and TNF-α increased in MI/R group ($P < 0.01$) (Fig. 2D). Treatment with melatonin protected the heart from MI/R by reducing the expression levels of MMP-9, IL-6, and TNF-α ($P < 0.01$). These results suggest that melatonin pre-treatment alleviated myocardial

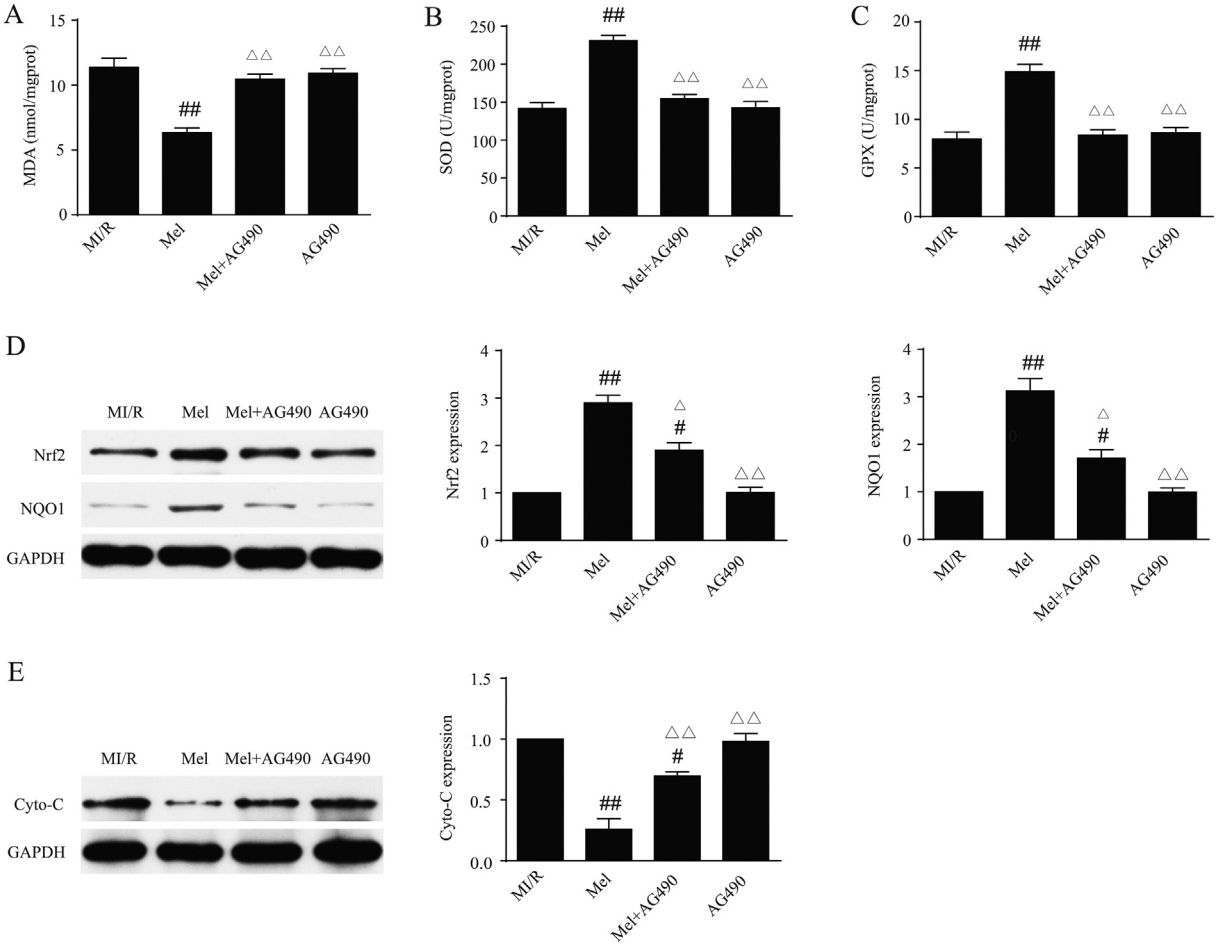

Fig. 6. Oxidative stress following AG490 treatment in ischemia/reperfusion injured hearts.
(A) MDA content. (B) SOD activity. (C) GPX activity. (D, E) Expression levels of Nrf2, NQO1, and Cyto-C. Data are shown as the mean ± SEM. #, ## indicate $P < 0.05$, $P < 0.01$ compared to the MI/R group. Δ, ΔΔ indicate $P < 0.05$, $P < 0.01$ compared to the Mel group.

oedema and inflammation following MI/R.

3.2. Melatonin alleviates the oxidative stress induced by MI/R

As shown in Fig. 3A–C, the effects of melatonin on oxidative stress in DCD heart were investigated. MI/R markedly increased the content of MDA and decreased the activities of SOD and GPX in the heart tissue ($P < 0.01$), indicating that oxidative stress occurred following MI/R injury. Melatonin pre-treatment decreased the level of MDA and increased the antioxidative capacity through the upregulation in the activities of SOD and GPX ($P < 0.01$). In comparison with the Sham group, MI/R group showed a significant decrease in the expression levels of Nrf2 and NQO1 ($P < 0.01$), the known antioxidative molecules (Fig. 3D). Nrf2 and NQO1 expression levels increased after the treatment with melatonin ($P < 0.01$). Additionally, compared to the MI/R group, melatonin pre-treatment reduced the expression level of Cyto-C ($P < 0.01$) (Fig. 3E). Cyto-C is an important component of the mitochondrion and plays a role in the electron transport of the respiratory chain. The level of cytoplasmic Cyto-C increases in response to any damage to the mitochondrial structure. Melatonin significantly decreased the cytoplasmic Cyto-C expression, indicating that melatonin preserved the integrity of the myocardial mitochondria. These results suggest that melatonin pre-treatment alleviated mitochondrial oxidative stress following MI/R.

3.3. Melatonin reduces myocardial apoptosis and activates the JAK2/STAT3 signalling pathway in MI/R

As shown in Fig. 4A–B, MI/R caused an increase in the size of the myocardial infarct consistent with an increase in the numbers of apoptotic cardiomyocytes as compared with the hearts from the Sham group. This observation was confirmed with TTC staining and TUNEL assay results ($P < 0.01$). Treatment with melatonin markedly reduced myocardial infarction and the number of apoptotic cardiomyocytes ($P < 0.01$). As shown in Fig. 4C, MI/R increased the expression levels of the pro-apoptotic factors Bax, caspase-3, and C-caspase-3 and decreased the expression of the anti-apoptotic factor Bcl-2 in the heart ($P < 0.01$). However, melatonin pre-treatment of the heart increased the level of Bcl-2 and decreased the expression levels of Bax, caspase-3, and C-caspase-3 compared with the hearts from MI/R group ($P < 0.01$).

We explored whether the cardioprotective effects of melatonin involve the JAK2/STAT3 signalling pathway. Compared to the MI/R group, melatonin pre-treatment significantly increased the expression levels of p-STAT3 and p-JAK2 ($P < 0.01$) (Fig. 4D), indicating that the JAK2/STAT3 signalling pathway was activated in the Mel group. These results suggest that melatonin pre-treatment alleviated myocardial apoptosis and activated the JAK2/STAT3 signalling pathway following MI/R.

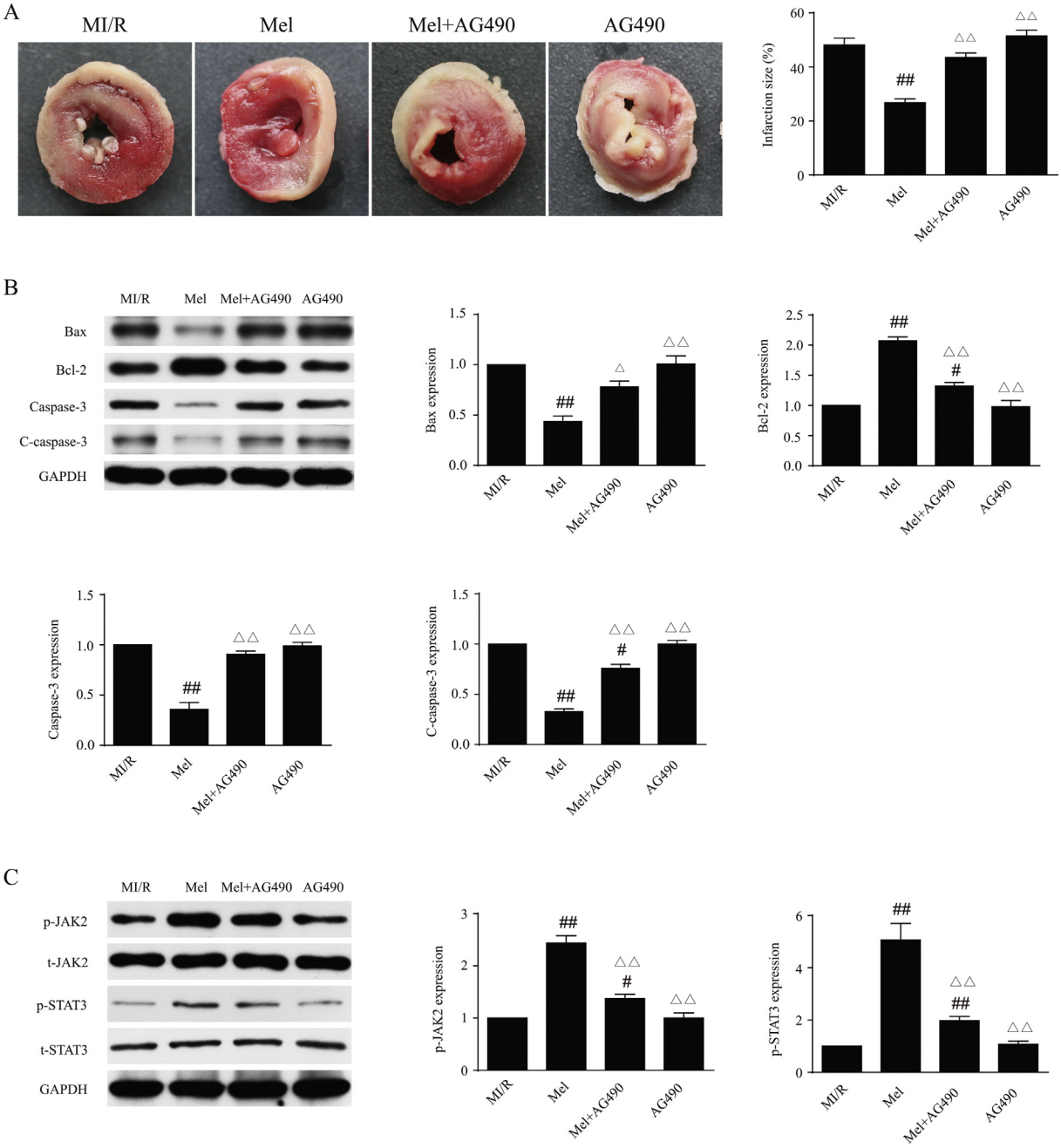

Fig. 7. Effect of AG490 treatment on myocardial apoptosis and activity of JAK2/STAT3 in ischemia/reperfusion injured hearts.
(A) TTC staining. The myocardial infarct area was expressed as the percentage of infarct relative to the total area. (B, C) Expression levels of Bax, Bcl-2, caspase-3, C-caspase-3, p-JAK2, and p-STAT3. Data are shown as the mean ± SEM. #, ## indicate $P < 0.05$, $P < 0.01$ compared to the MI/R group. Δ, ΔΔ indicate $P < 0.05$, $P < 0.01$ compared to the Mel group.

3.4. JAK2/STAT3 signalling pathway is essential to alleviate myocardial oedema as well as the anti-inflammatory effects of melatonin following MI/R

We confirmed the essential role of the JAK2/STAT3 signalling pathway to mediate the cardioprotective effects of melatonin *in vivo*. AG490, a specific inhibitor of JAK2, can compete for binding with receptor tyrosine kinases and block the JAK2/STAT3 signalling pathway. As shown in Fig. 5A–B, the administration of AG490 abolished the protective effects of melatonin, as confirmed from the significant increase in myocardial water content and wet/dry ratio ($P < 0.01$ for water content, $P < 0.05$ for wet/dry ratio). Inflammation aggravation was more evident in the hearts from AG490 group than in those from the Mel group in HE staining (Fig. 5C) compared to the Mel group. Moreover, the expression levels of MMP-9 and IL-6 were up-regulated by AG490 treatment ($P < 0.01$ for MMP-9, $P < 0.05$ for IL-6) (Fig. 5D). Interestingly, we found that the reduction in the expression level of TNF-α was weakly impaired by AG490 ($P > 0.05$), indicating that other signalling pathways may be involved in the expression regulation of TNF-α in this process. These data indicate that the alleviation in myocardial oedema and anti-inflammatory activity of melatonin following MI/R occurred *via* the activation of the JAK2/STAT3 signalling pathway.

3.5. JAK2/STAT3 signalling pathway is essential for the alleviation of the oxidative stress of melatonin following MI/R

We continued to investigate whether the JAK2/STAT3 signalling pathway was involved in the antioxidant stress by melatonin pre-treatment. As shown in Fig. 6A, the decrease in the level of MDA following melatonin treatment was markedly blocked by AG490 administration ($P < 0.01$). Notably, we also observed attenuation in the melatonin-mediated increase in the activities of SOD and GPX after AG490 treatment ($P < 0.01$) (Fig. 6B–C). Additionally, compare to the Mel group, the expression levels of NQO1 and Nrf2 in the hearts from the Mel + AG490 group were significantly reduced ($P < 0.05$) (Fig. 6D).

Furthermore, mitochondrial integrity was maintained by melatonin but was abolished by AG490 treatment, as evident from the reduced expression level of Cyto-C in Fig. 6E ($P < 0.01$). These data suggest that the antioxidant stress of melatonin following MI/R is mediated through the activation of the JAK2/STAT3 signalling pathway.

3.6. JAK2/STAT3 signalling pathway is essential for the anti-apoptotic effects of melatonin following MI/R

We further explored the anti-apoptotic effects of melatonin and investigated whether these effects involve the JAK2/STAT3 signalling pathway following MI/R injury. As shown in Fig. 7A, the decrease in myocardial infarction after melatonin pre-treatment was impaired in the hearts from the Mel + AG490 group ($P < 0.01$). The anti-apoptotic effects of melatonin were abolished by AG490, as demonstrated from the increased expression levels of the pro-apoptotic factors Bax, caspase-3, and C-caspase-3 and the decreased expression level of the anti-apoptotic factor Bcl-2 ($P < 0.01$ for caspase-3, C-caspase-3, and Bcl-2, and $P < 0.05$ for Bax) (Fig. 7B). The protective effects were attenuated through a decrease in the expression levels of p-JAK2 and p-STAT3 (Fig. 7C). These results suggest that the anti-apoptotic activity of melatonin is mediated through the activation of the JAK2/STAT3 signalling pathway.

4. Discussion

In the present study, we first explored the protective effects of melatonin in a DCD heart model after *ex vivo* perfusion, and found that melatonin effectively protected the DCD heart from MI/R through the activation of the JAK2/STAT3 signalling pathway. Melatonin pre-treatment conferred cardioprotective effects during reperfusion of rat hearts, as demonstrated from the reduction in myocardial oedema and inflammation, alleviation in oxidative stress, and decrease in myocardial apoptosis of the heart tissues. These cardioprotective effects of melatonin were mediated, at least in part, through the activation of the JAK2/STAT3 signalling pathway, as the treatment with a specific STAT3 inhibitor remarkably abolished these protective effects of melatonin on MI/R injury.

The applicability of DCD hearts has been widely tested over the past few years, owing to the shortage of donor hearts. However, repairing DCD hearts after MI/R injury is difficult. Excessive reactive oxygen species (ROS) are generated by MI/R that may cause lipid peroxidation, ultimately leading to myocardial injury. The concomitant excessive ROS production observed during reperfusion causes excessive damage to the heart, contributing to the expansion of infarct size and deterioration of cardiac function. Therefore, pharmacological intervention targeting MI/R is thought to be the most effective method to improve the prognosis of DCD hearts after transplantation. To expand the donor pool, *ex vivo* donor organ perfusion is recommended for solid organ transplantation, particularly DCD heart transplantation, over the past few years. Unlike static cold storage, EVHP continuously perfuses the donor heart with normothermic blood, nutrition, and oxygen, and may minimise cold ischemia injury. In EVHP system, the donor heart can be assessed and repaired when it is beating *ex vivo* in a normothermic state. An increasing number of platforms have focused on organ preservation of the liver, kidney, lung, and heart [12–15]. Numerous clinic trials have demonstrated that the limitations of cold ischemia preservation can be overcome by *ex vivo* donor organ perfusion [9,16]. In the present study, the blood for reperfusion was collected from a normal rat rather than a DCD rat because of its better consistency and capacity to carry oxygen. We established a model that mimics the clinical conditions by harvesting the DCD after asphyxiation and preserving the heart by *ex vivo* perfusion before transplantation. The protective effects of pharmacological intervention in the model provide a reference value for clinical practice.

Melatonin is produced and released into the circulatory system from the pineal gland and extrapineal sites, including the skin, bone marrow, retina, gut, ovaries, thymus, heart, liver, and muscle [17,18]. However, few melatonins are released from the extrapineal sites under physiological conditions. A decrease in the elevation in the nocturnal serum melatonin level has been reported in patients with acute myocardial infarction [19], indicative of the protective role of melatonin in ischemia/reperfusion. In fact, growing evidence supports melatonin as a potential therapeutic drug in ischemia/reperfusion, owing to its multiple properties [20,21]. Melatonin has been used to protect against ischemia/reperfusion injury in the brain [22], liver [23], and heart partly because of its anti-inflammatory capacity [24]. The melatonin-mediated alleviation in inflammation during MI/R has been previously reported [25,26]. The expression of cytokines such as interleukins and TNF-α was inhibited through the decrease in the binding of nuclear factor kappa B (NF-κB) to the DNA upon melatonin administration. The expression levels of chemokines, adhesion molecules, cyclooxygenase, and inducible nitric oxide synthase were also reduced after melatonin treatment [27]. In the present study, MMP-9, IL-6, and TNF-α levels increased in the MI/R group as compared with the Sham group but decreased in the Mel group. Additionally, the pre-treatment with melatonin alleviated myocardial oedema possibly through inflammation mitigation.

As an antioxidant, melatonin is a powerful free radical scavenger. Free radicals generated during the MI/R process could be eliminated by melatonin through the 5′-AMP-activated protein kinase/sarcoplasmic/endoplasmic reticulum calcium ATPase 2a (AMPK/SERCA2a) pathway [28]. In the present study, the oxidative stress was aggravated through the upregulation in the expression level of MDA, reduction in the activities of SOD and GPX, downregulation in the expression levels of Nrf2 and NQO1, and increase in Cyto-C expression level during MI/R. However, these effects were alleviated by melatonin pre-treatment.

Additionally, melatonin has been shown to reduce apoptosis [29]. Melatonin pre-treatment reduced the apoptotic ratio, decreased the level of the pro-apoptotic factor Bim, and increased the level of the anti-apoptotic factor Bcl-2 [30]. In the present study, the levels of pro-apoptotic factors Bax, caspase-3, and C-caspase-3 increased and Bcl-2 level decreased in MI/R hearts. However, apoptosis during MI/R was attenuated by melatonin pre-treatment as observed in the Mel group.

The cardioprotective mechanism of melatonin during MI/R was thought to involve the JAK2/STAT3 signalling pathway. The JAK2/STAT3 signalling pathway plays a vital role in signal transduction as the target of cytokines, growth factors in cells, and the cytoplasm. A previous study has shown that the beneficial effects of melatonin are attributed to the activation of the JAK2/STAT3 signalling pathway [31]. Heusch et al. revealed the activation of the JAK2/STAT3 signalling pathway in a pig model of MI/R [32]. Qiao and colleagues confirmed that the expression of p-STAT3 increased during MI/R both *in vivo* MI/R and *in vitro* in a hypoxia/reoxygenation model, and this up-regulation was abolished by a specific JAK2 inhibitor [33]. Boengler et al. showed that the cardioprotective effects of melatonin disappeared in a STAT3-deficient mouse model, suggesting that STAT-3 activation plays a crucial role in protecting the heart from MI/R damage and that the anti-inflammatory effects of melatonin, which mediated through the

phosphorylation of STAT-3, were blocked after AG490 administration [34]. JAK2/STAT-3 signalling pathway activation by melatonin during MI/R was also demonstrated by Yang and colleagues that showed improvement in cardiac function, reduction in myocardial infarct size, decrease in apoptotic cardiomyocyte number, downregulation in Bax level, and alleviation in oxidative stress damage after melatonin treatment. However, these protective effects of melatonin were abolished by AG490 or JAK2 small-interfering RNA (siRNA).

At the subcellular level, the mitochondrion is considered as the major site of melatonin synthesis and metabolism [35]. Melatonin treatment preserves mitochondrial integrity and maintains the mitochondrial potential through the increase in the activity of the oxidative phosphorylation complex and reduction in the level of oxidative stress and lipid peroxidation [24,36,37]. In addition, melatonin protects mitochondrial functions through its involvement in the mitochondrial biogenesis, dynamics, and mitophagy [24,38]. The pro-survival mechanism of the JAK2/STAT-3 pathway is thought to be attributed to the maintenance of mitochondrial function, particularly MPTP. STAT-3 may be located in the matrix of mitochondria [39], and is imported to the mitochondria through a TOM20-dependent pathway [40]. Melatonin treatment was shown to protect the heart from MI/R through the activation of the JAK2/STAT-3 signalling pathway and inhibition of MPTP opening [41].

The cardioprotective effects of melatonin were mediated by the JAK2/STAT3 signalling pathway; however, we do not deny the possibility of the involvement of other mechanisms and pathways. Most cardioprotective effects of melatonin involved melatonin receptors. Luzindole, a melatonin receptor antagonist, abolished the cardioprotective effects of melatonin in an isolated perfused working rat heart [42]. In addition, the expression of STAT3, protein kinase B (PKB/Akt), extracellular signal-regulated kinase (ERK)-42/44, and glycogen synthase kinase (GSK)-3β increased during MI/R after 3 or 6 weeks of melatonin treatment [43]. Yu and colleagues showed that the cardioprotective effects of melatonin pre-treatment and the upregulated SIRT1 expression were abolished by luzindole or the selective SIRT1 inhibitor EX527, indicating that melatonin pre-treatment activated the SIRT1 signalling pathway during MI/R [44]. SIRT3, a member of the Sirtuin family, was known to be activated during MI/R upon melatonin pre-treatment, and the administration of the selective SIRT3 inhibitor 3-TYP or an SIRT3-targeted siRNA resulted in the inhibition of the SIRT3 signalling pathway and blockade of the cardioprotective effects [45]. Furthermore, AMPK-peroxisome proliferator-activated receptor gamma coactivator 1-alpha (PGC1α)-SIRT3 was activated in diabetic rats during MI/R, suggesting that SIRT3 may be a downstream molecule in the process of cardioprotection mediated by melatonin [24]. Recent studies have revealed the involvement of the Notch signalling pathway in mediating the cardioprotective effects of melatonin during MI/R [46,47].

5. Conclusions

In conclusion, our data reveal that melatonin pre-treatment protects the heart from MI/R in a DCD heart model, alleviates inflammation, attenuates mitochondrial oxidative damage, and reduces myocardial apoptosis. The cardioprotective effects seem to be largely associated with the activation of the JAK2/STAT3 signalling pathway. These findings suggest that the therapeutic use of melatonin as a promising strategy to repair DCD hearts as well as to treat ischemic heart disease.

Disclosure

The authors declare that there are no conflicts of interest.

References

[1] E.M. Hsich, Matching the market for heart transplantation, Circ. Heart Fail. 9 (4) (2016) e2679.
[2] J.R. Trivedi, E. Schumer, M. Black, et al., Risk factors of waiting list mortality for patients awaiting heart transplant, J. Heart Lung Transplant. 35 (4S) (2016) 574.
[3] Dun-Xian Tan, Li-Dun Chen, Burkhard Poeggeler, et al., Melatonin: a potent, endogenous hydroxyl radical scavenger, Endocr. J. 1 (1993) 57–60.
[4] D. Ozdemir, N. Uysal, K. Tugyan, et al., The effect of melatonin on endotoxemia-induced intestinal apoptosis and oxidative stress in infant rats, Intensive Care Med. 33 (3) (2007) 511–516.
[5] Y. Deng, C. Jiao, C. Mi, et al., Melatonin inhibits manganese-induced motor dysfunction and neuronal loss in mice: involvement of oxidative stress and dopaminergic neurodegeneration, Mol. Neurobiol. 51 (1) (2015) 68–88.
[6] D. Zephy, J. Ahmad, Type 2 diabetes mellitus: role of melatonin and oxidative stress, Diab. Metab. Syndr. 9 (2) (2015) 127–131.
[7] S. Guo, C. Gao, W. Xiao, et al., Matrine protects cardiomyocytes from ischemia/reperfusion injury by regulating HSP70 expression via activation of the JAK2/STAT3 pathway, SHOCK 50 (6) (2018) 664–670.
[8] Z. Wang, J. Yu, J. Wu, et al., Scutellarin protects cardiomyocyte ischemia-reperfusion injury by reducing apoptosis and oxidative stress, Life Sci. 157 (2016) 200–207.
[9] A. Ardehali, F. Esmailian, M. Deng, et al., Ex-vivo perfusion of donor hearts for human heart transplantation (PROCEED II): a prospective, open-label, multicentre, randomised non-inferiority trial, Lancet 385 (9987) (2015) 2577–2584.
[10] G. Lindner, B. Zapletal, C. Schwarz, et al., Acute hyponatremia after cardioplegia by histidine-tryptophane-ketoglutarate — a retrospective study, J. Cardiothorac. Surg. 7 (1) (2012).
[11] F. Liu, S.M. Kang, Heterotopic heart transplantation in mice, J. Vis. Exp. (6) (2007).
[12] M. Cypel, J.C. Yeung, M. Liu, et al., Normothermic ex vivo lung perfusion in clinical lung transplantation, N. Engl. J. Med. 364 (15) (2011) 1431–1440.
[13] I. Jochmans, C. Moers, J.M. Smits, et al., Machine perfusion versus cold storage for the preservation of kidneys donated after cardiac death, Ann. Surg. 252 (5) (2010) 756–764.
[14] B.G. Bruinsma, H. Yeh, S. Özer, et al., Subnormothermic machine perfusion for ex vivo preservation and recovery of the human liver for transplantation, Am. J. Transplant. 14 (6) (2014) 1400–1409.
[15] P. Leprince, A.F. Popov, A.R. Simon, et al., Ex vivo perfusion of the heart with the use of the organ care system, Eur. J. Cardiothorac. Surg. 49 (5) (2016) 1318–1320.
[16] J.L. Chan, J.A. Kobashigawa, H.J. Reich, et al., Intermediate outcomes with ex-vivo allograft perfusion for heart transplantation, J. Heart Lung Transplant. 36 (3) (2017) 258–263.
[17] C. Venegas, J.A. García, G. Escames, et al., Extrapineal melatonin: analysis of its subcellular distribution and daily fluctuations, J. Pineal Res. 52 (2) (2012) 217–227.
[18] D. Acuna-Castroviejo, G. Escames, C. Venegas, et al., Extrapineal melatonin: sources, regulation, and potential functions, Cell. Mol. Life Sci. 71 (16) (2014) 2997–3025.
[19] A. Dominguez-Rodriguez, P. Abreu-Gonzalez, M.J. Garcia, et al., Decreased nocturnal melatonin levels during acute myocardial infarction, J. Pineal Res. 33 (4) (2002) 248–252.
[20] A. Lochner, E. Marais, B. Huisamen, Melatonin and cardioprotection against ischaemia/reperfusion injury: what's new? A review, J. Pineal Res. 65 (2018) e12490, , https://doi.org/10.1111/jpi.12490.
[21] Z. Jiki, S. Lecour, F. Nduhirabandi, Cardiovascular benefits of dietary melatonin: a myth or a reality? Front. Physiol. 9 (2018) 528.
[22] M. Cervantes, G. Moral, G. Letechipa-Vallejo, Melatonin and ischemia-reperfusion injury of the brain, J. Pineal Res. 45 (1) (2008) 1–7.
[23] M.A. Zaoualí, R.J. Reiter, S. Padrissa-Altés, et al., Melatonin protects steatotic and nonsteatotic liver grafts against cold ischemia and reperfusion injury, J. Pineal Res. 50 (1) (2010) (no-no).
[24] L. Yu, B. Gong, W. Duan, et al., Melatonin ameliorates myocardial ischemia/reperfusion injury in type 1 diabetic rats by preserving mitochondrial function: role of AMPK-PGC-1alpha-SIRT3 signaling, Sci. Rep. 7 (2017) 41337.
[25] J.L. Mauriz, P.S. Collado, C. Veneroso, et al., A review of the molecular aspects of melatonin's anti-inflammatory actions: recent insights and new perspectives, J. Pineal Res. 54 (1) (2013) 1–14.
[26] R.J. Reiter, J.R. Calvo, M. Karbownik, et al., Melatonin and its relation to the immune system and inflammation, Ann. N. Y. Acad. Sci. 917 (1) (2000) 376–386.
[27] Y. Guo, J. Sun, T. Li, et al., Melatonin ameliorates restraint stress-induced oxidative stress and apoptosis in testicular cells via NF-κB/iNOS and Nrf2/HO-1 signaling pathway, Sci. Rep. 7 (1) (2017).
[28] J. Cui, Z. Li, S. Zhuang, et al., Melatonin alleviates inflammation-induced apoptosis in human umbilical vein endothelial cells via suppression of Ca^{2+}-XO-ROS-Drp1-mitochondrial fission axis by activation of AMPK/SERCA2a pathway, Cell Stress Chaperones 23 (2) (2018) 281–293.
[29] W. Zhang, B. He, Y. Wu, et al., Melatonin protects against sepsis-induced cardiac dysfunction by regulating apoptosis and autophagy via activation of SIRT1 in mice, Life Sci. 217 (2019) 8–15.
[30] Y. Dong, C. Fan, W. Hu, et al., Melatonin attenuated early brain injury induced by subarachnoid hemorrhage via regulating NLRP3 inflammasome and apoptosis signaling, J. Pineal Res. 60 (3) (2016) 253–262.
[31] Y. Yang, W. Duan, Z. Jin, et al., JAK2/STAT3 activation by melatonin attenuates the mitochondrial oxidative damage induced by myocardial ischemia/reperfusion injury, J. Pineal Res. 55 (3) (2013) 275–286.
[32] G. Heusch, J. Musiolik, N. Gedik, et al., Mitochondrial STAT3 activation and cardioprotection by ischemic postconditioning in pigs with regional myocardial ischemia/reperfusion, Circ. Res. 109 (11) (2011) 1302–1308.
[33] S. Qiao, X. Mao, Y. Wang, et al., Remifentanil preconditioning reduces postischemic

myocardial infarction and improves left ventricular performance via activation of the Janus activated kinase-2/signal transducers and activators of transcription-3 signal pathway and subsequent inhibition of glycogen synthase kinase-3β in rats, Crit. Care Med. 44 (3) (2016) e131–e145.
[34] K. Boengler, A. Buechert, Y. Heinen, et al., Cardioprotection by ischemic post-conditioning is lost in aged and STAT3-deficient mice, Circ. Res. 102 (1) (2008) 131–135.
[35] L.C. Manchester, A. Coto-Montes, J.A. Boga, et al., Melatonin: an ancient molecule that makes oxygen metabolically tolerable, J. Pineal Res. 59 (4) (2015) 403–419.
[36] D. Mukherjee, A.K. Ghosh, M. Dutta, et al., Mechanisms of isoproterenol-induced cardiac mitochondrial damage: protective actions of melatonin, J. Pineal Res. 58 (3) (2015) 275–290.
[37] S.M. Nair, R.M.A. Rahman, A.N. Clarkson, et al., Melatonin treatment following stroke induction modulates L-arginine metabolism, J. Pineal Res. 51 (3) (2011) 313–323.
[38] B. Jayaraman, A.M. Smith, J.D. Fernandes, et al., Oligomeric viral proteins: small in size, large in presence, Crit. Rev. Biochem. Mol. Biol. 51 (5) (2016) 379–394.
[39] G. Heusch, Molecular basis of cardioprotection: signal transduction in ischemic pre-, post-, and remote conditioning, Circ. Res. 116 (4) (2015) 674–699.
[40] K. Boengler, D. Hilfiker-Kleiner, G. Heusch, et al., Inhibition of permeability transition pore opening by mitochondrial STAT3 and its role in myocardial ischemia/reperfusion, Basic Res. Cardiol. 105 (6) (2010) 771–785.
[41] C.R. White, S. Giordano, G.M. Anantharamaiah, High-density lipoprotein, mitochondrial dysfunction and cell survival mechanisms, Chem. Phys. Lipids 199 (2016) 161–169.
[42] A. Lochner, S. Genade, A. Davids, et al., Short- and long-term effects of melatonin on myocardial post-ischemic recovery, J. Pineal Res. 40 (1) (2006) 56–63.
[43] F. Nduhirabandi, B. Huisamen, H. Strijdom, et al., Short-term melatonin consumption protects the heart of obese rats independent of body weight change and visceral adiposity, J. Pineal Res. 57 (3) (2014) 317–332.
[44] L. Yu, Y. Sun, L. Cheng, et al., Melatonin receptor-mediated protection against myocardial ischemia/reperfusion injury: role of SIRT1, J. Pineal Res. 57 (2) (2014) 228–238.
[45] M. Zhai, B. Li, W. Duan, et al., Melatonin ameliorates myocardial ischemia reperfusion injury through SIRT3-dependent regulation of oxidative stress and apoptosis, J. Pineal Res. 63 (2) (2017) e12419.
[46] L. Yu, H. Liang, Z. Lu, et al., Membrane receptor-dependent Notch1/Hes1 activation by melatonin protects against myocardial ischemia-reperfusion injury: in vivo and in vitro studies, J. Pineal Res. 59 (4) (2015) 420–433.
[47] H. Pei, Q. Yu, Q. Xue, et al., Notch1 cardioprotection in myocardial ischemia/reperfusion involves reduction of oxidative/nitrative stress, Basic Res. Cardiol. 108 (5) (2013).