Protective Effect of Ischemic Postconditioning Combined with Nicorandil on Myocardial Ischemia-Reperfusion Injury in Diabetic Rats

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Research Article

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Abstract

BACKGROUND

Diabetes mellitus exhibits a high sensitivity to myocardial ischemia-reperfusion injury (MIRI), which can affect the efficacy of cardioprotective interventions, and its complexity significantly reduces the therapeutic potential of existing treatment options. This study was dedicated to investigate the feasibility of shifting from monotherapy to combination therapy in diabetic MIRI.

METHODS

Rats were randomized into 10 groups: non-diabetic rats included: sham-operated (Sham), ischemia-reperfusion (I/R), ischemia postcondtioning (I-Post), nicorandil (Nic), and combination therapy (I-Post + Nic); diabetic rats included: DM Sham, DM I/R, DM I-Post, DM Nic and DM I-Post + Nic. The T2DM rats were induced by injection of low-dose streptozotocin (30 mg/kg) and supplemented with high-fat and high-sugar chow to establish a myocardial ischemia-reperfusion model. CK-MB, NO levels, ROS content, and myocardial infarct area were measured. HE staining of cardiac tissue sections, TUNEL staining of cardiac tissue sections, WGA immunofluorescence staining, and protein blotting (western blotting) were used to detect myocardial tissue PI3K, Akt, GSK3β, mToR, and eNOS protein phosphorylation levels.

RESULTS

We found that diabetes impaired the cardioprotective effects of I-Post and nicorandil against MIRI injury compared with non-diabetic I/R rats. Compared with diabetic I/R rats treated with I-Post (41.13% ± 2.17%, 454.64 ± 13.434 μm^2) or nicorandil (41.54 ± 1.45%, 439.95 ± 15.087 μm^2) alone, I-Post combined with nicorandil treatment (30.67% ± 1.38%, 344.45 ± 13.434 μm^2) significantly reduced IS/AAR ($P < 0.001$) and cardiac cell cross sectional area ($P < 0.001$), reduced the extent of myocardial tissue injury and decreased pathological scores after I/R ($P < 0.001$), inhibited myocardial apoptosis ($P < 0.001$), decreased plasma central infarct marker CK-MB levels ($P < 0.001$), and increased NO levels ($P < 0.001$). In addition, combination treatment increased the phosphorylation levels of PI3K, Akt, GSK3β, mToR, and eNOS ($P < 0.001$).

CONCLUSION

I-Post combined with nicorandil treatment maintains effective cardioprotection against diabetic MIRI by activating PI3K/Akt signaling pathway.

1. Introduction

Diabetes is an independent risk factor for cardiovascular disease (CVD), and acute myocardial infarction (AMI) is the leading cause of morbidity and mortality in diabetes patients[1, 2]. Diabetic patients have significantly higher mortality from AMI than non-diabetic patients and tend to develop more severe myocardial ischemia-reperfusion injury (MIRI) after reperfusion therapy [3], with long-term (≥ 1 year) mortality being nearly 50% higher than in non-diabetes patients[4]. Therefore, reducing MIRI in diabetes patients, preserving the maximum amount of ischemic myocardium and improving patient prognosis have become an urgent challenge for reperfusion therapy.

Ischemic preconditioning (IPC) is a protective measure that protects the myocardium from long periods of ischemia by repeatedly inducing low-level ischemia[5]. Subsequently, several studies have shown that myocardial protection against MIRI can be induced by interventions on reperfusion[6]. The damage caused by MIRI can be limited by three sets of very short ischemia (30 seconds)-reperfusion (30 seconds) cycles before reperfusion, i.e., ischemic postconditioning (I-Post) [7]. Similar to IPC, I-Post is an effective measure to preserve the myocardium from MIRI[8]. The discovery of the I-Post effect has attracted attention owing to its applicability in clinical procedures[9]. I-Post can also be induced by various interventions or by the administration of drugs and other chemical agents. These agents can attenuate the damage caused by reperfusion by administration at different times (during or after reperfusion), which is known as pharmacological conditioning (PC)[10]. Compared to IPC, I-Post and PC move the timing of mechanical or pharmacological intervention from pre-ischemia (which cannot be performed in most clinical settings) to pre-reperfusion. These conditions are similar to clinical procedures for the onset and treatment of AMI and have more immediate clinical applications. However, the modulatory role of postconditionings in diabetes has not been thoroughly investigated. Some studies suggested that the cardioprotective effect of I-Post was diminished in both type 1 and type 2 diabetes animal models, while others concluded that I-Post was ineffective in both type 1 and type 2 diabetes rat models[11]. Furthermore, previous studies have shown that diabetes impairs the cardioprotective effects of PC agents, such as cyclosporine A[12], sevoflurane[13], and remifentanil[14].Undoubtedly, the severity and complexity of diabetic myocardial ischemia-reperfusion injury affects the effectiveness of available therapeutic measures. However, most of the aforementioned current studies related to diabetic MIRI apply a type 1 diabetes model, while in actual clinical work, approximately 90% of diabetes patients have type 2 diabetes mellitus (T2DM)[15], and there are fewer studies about applying the T2DM model to study MIRI. Therefore, the establishment of a T2DM model to explore MIRI is more in line with the actual clinical requirements.

It is now believed that the way to overcome the ineffectiveness of conditioning interventions in patients with diabetes is to provide complementary therapy or combination therapy (e.g., antioxidants, antidiabetic drugs, etc.) on top of the conditioning strategy[16]. Therefore, we hypothesized that the I-Post combination PC would be superior to any of the interventions alone, and that these interventions would have a superimposed effect when the mechanism of action of any of the interventions is not identical. The combination therapy can be protective even when concomitant disease may reduce the protective effect

of one therapy. Currently, there are few studies on the effectiveness of I-Post in combination with PC. Nicorandil is widely used to treat ischemic heart disease (IHD), and some animal studies have shown that nicorandil can reduce MIRI[17], but there is still a lack of research on whether nicorandil can have a cardioprotective effect on MIRI in T2DM rats. However, some studies have shown that nicorandil attenuates apoptosis in cardiomyocytes of diabetic rats[18]; therefore, we hypothesized that I-Post combined with nicorandil has some advantage in treating MIRI in T2DM rats. In this study, we used a rat T2DM-type myocardial ischemia-reperfusion model induced by a high-fat and high-sugar diet supplemented with low doses of streptozotocin (STZ). We investigated whether the cardioprotective effect of I-Post and nicorandil on MIRI was impaired by T2DM and whether I-Post combined with nicorandil exerted a synergistic protective effect on MIRI in rats with T2DM to provide a basis for the clinical treatment of MIRI in diabetic patients.

2. Materials And Methods

2.1 Animals: 150 male Sprague-Dawley rats (200–250 g) were used for the experiments. The rats were housed in a temperature-controlled environment (21 ± 2°C) with a 12-hour light/dark cycle (lights on at 06:00) and free access to food and water. The facilities where the animals were housed followed the Association for Assessment and Accreditation of Laboratory Animal Care International (AAALAC) guidelines, which were approved at the time of the study. The study protocol was approved by the Ethics Committee of Qingdao University School of Medicine (Qingdao, China).

2.2 Type 2 diabetic rat model: The T2DM model was induced as follows: Experimental animals were fed basal chow for 1 week. After 1 week, the diabetic group was fed high-fat and high-sugar chow, and the control group was fed basal chow. At week 5, all animals were fasted for 12 h (without water), and STZ (1%) was dissolved in 0.1 mmol/L citric acid-sodium citrate buffer (pH 4.2; Solarbio, Beijing, China), fully dissolved and stored on ice away from light. A single rapid intraperitoneal injection of 30 mg/kg streptozotocin (MCE, USA) was administered to the diabetic group. An equal volume of 0.1 mM citric acid-sodium citrate buffer was intraperitoneally administered to the control group. Seven days later, blood was drawn from the tail vein to check for glucose levels of the rats in the diabetic group. Rats with blood glucose ≥ 16.7 mmol/L were fed a high-fat, high-sugar diet for another 8 weeks, while rats that did not meet the blood glucose standard were discarded. Blood glucose was retested after 8 weeks, and modeling was successful if blood glucose was still ≥ 16.7 mmol/L[19]. The success rate of model building is shown in the supplementary material (Supplementary Table I).

2.3 Myocardial ischemia-reperfusion model: The rats were weighed, fasted, and dehydrated for 12 h before surgery. Chloral hydrate (10%, 0.3 mL/100 g) was injected intraperitoneally. Each rat was fixed in the supine position and connected to a Powerlab data acquisition and analysis system. A standard II-lead ECG was recorded, and any abnormalities were excluded. The cervical trachea was incised, and a small animal ventilator (Rivard, USA) was connected for assisted breathing (tidal volume, 5 mL/100 g, frequency 60–80 breaths/min, respiratory ratio 2:1, continuous positive end-expiratory pressure). The skin was cut longitudinally 0.5 cm on the left side of the sternum, using the 3rd and 4th ribs as the upper

and lower borders. The subcutaneous tissue, pectoralis major muscle, and intercostal muscle were bluntly separated with forceps and a scalpel. Then, 6 − 0 ophthalmic sutures were passed under the left atrium and approximately 2–3 mm below the intersection of the cone of the pulmonary artery. The left anterior descending branch of the coronary artery was ligated for 30 min, after which the sutures were cut. After successful ligation, the anterior wall of the left ventricle was bruised or pale, the pulsation was reduced, and the ECG showed ST-segment elevation (≥ 0.25 mV), which is a sign of myocardial ischemia. Thirty minutes after ligation, the ligature was cut with scissors to form a reperfusion, and the ECG showed a gradual decrease in the ST segment by approximately 50% and the pale or cyanotic myocardium gradually turned red when blood flow was restored. Reperfusion was allowed to occur for 2.5 h(a safe and favorable reperfusion time for experimental results time)[20]. The rats were sacrificed at the end of reperfusion. The success rate of model building is shown in the supplementary material (Supplementary Table II).

2.4 Nicorandil administration route and dosage: In this study, Nicorandil for injection (trade name: Ricoxyl; specification: 12 mg; State Drug Administration H20120069) was used, and the dried drug powder was prepared into a solution of 100 μg/ml with 0.9% saline before the experiment and stored away from light. We selected the effective dose in the clinical study[21], a loading dose of 200 μg/kg of nicorandil was given via femoral vein 20 min before reperfusion, and a maintenance dose of 30 μg/kg/min was given for 60 min after reperfusion.

2.5 Experimental groups: A total of 10 groups were included in this study, with 15 animals in each group. as follows (Supplementary Figure I):

(1) Sham group: The sutures were threaded, but the descending artery was not ligated. The rats were sacrificed 3 h later.

(2) I/R group: Rats were treated as described above.

(3) I-Post group: After ligation, the rats were treated for myocardial ischemia (30 s ischemia/30 s reperfusion given 3 times within 3 min of the start of reperfusion). Then, reperfusion was allowed to occur for 2.5 h.

(4) Nic group: A loading dose of nicorandil (200 μg/kg) was administered 20 min before reperfusion. Then, a maintenance dose (30 μg/kg/min) was administered for 60 min during reperfusion. Afterwards, reperfusion was permitted for another 1.5 h (2.5 h total reperfusion time).

(5) I-Post + Nic group: Rats were administered a loading dose of 200 μg/kg of nicorandil 20 min before reperfusion and a maintenance dose of 30 μg/kg/min for the first 60 min of reperfusion. At the same time, the rats were post-treated for myocardial ischemia (30 s ischemia/30 s reperfusion administered 3 times within 3 min of the start of reperfusion). Reperfusion was allowed to occur for a total of 2.5 h.

(6) DM Sham group: Diabetic rats were used for this group. Sutures were threaded without ligation.

(7) DM I/R group: Diabetic rats were used for this group. I/R injury was induced as described in Myocardial ischemia-reperfusion model.

(8) DM I-Post group: Diabetic rats were treated for myocardial ischemia (30 s ischemia/30 s reperfusion given 3 times within 3 min of the start of reperfusion). Each rat was reperfused for 2.5 h.

(9) DM Nic group: Diabetic rats were administered a loading dose of 200 μg/kg nicorandil 20 min before reperfusion and a maintenance dose (30 μg/kg/min) was administered during the first 60 min of reperfusion. Then, the rats were reperfused for another 1.5 h (2.5 h total reperfusion time).

(10) DM I-Post + Nic group: Diabetic rats were administered a loading dose of 200 μg/kg of nicorandil 20 min before reperfusion and a maintenance dose of 30 μg/kg/min for the first 60 min of reperfusion. At the same time, the rats were post-treated for myocardial ischemia (30 s ischemia/30 s reperfusion administered 3 times within 3 min of the start of reperfusion). Reperfusion was allowed to occur for a total of 2.5 h.

2.6 Serum assay: After reperfusion, blood was collected from the rat abdominal aorta. The serum was obtained by centrifugation (4°C, 1000 *g*, 10 min) and frozen at -20°C until further analysis. Serum creatine kinase-MB (CK-MB) levels were measured using the rat creatine kinase isozyme MB (CK-MB) ELISA kit (Elabscience, Wuhan, China). The detection range of the kit is 31.25-2000pg/ml, the intra- and inter-batch coefficient of variation is less than 10%, and the minimum detection concentration is less than 18.75pg/ml. Serum nitric oxide (NO) levels were measured using a rat nitric oxide ELISA kit (Elabscience, Wuhan, China). The detection range of the kit is 0.16–100μmol/L, with intra- and inter-batch coefficients of variation less than 2.4% and 3.7%, respectively, and the minimum detection concentration less than 0.16μmol/L.

2.7 Reactive oxygen species (ROS) assay: At the end of reperfusion, rats were sacrificed by intraperitoneal injection of excess 10% chloral hydrate. The heart was lavaged with 100 mL 0.9% saline and stored at 4°C. Then, 50 mg of heart tissue was isolated from the below the ligation site and was assayed using a Rat Reactive Oxygen Species ELISA kit (Joln, Nanjing, China). The detection range of the kit is 1.0U/ml-80U/ml, with intra- and inter-batch coefficients of variation less than 9% and 11%, respectively, and the minimum detection concentration less than 1.0U/ml. Protein concentration (μg/μL) were determined using a BCA assay kit (Thermo, USA) to derive the mean level of ROS release (U/mg).

2.8 Triphenyl tetrazolium chloride and Evens blue staining to determine the area of myocardial infarction: After reperfusion, rats were sacrificed by intraperitoneal injection of excess 10% chloral hydrate. During this period, the rat LAD vessels were again ligated and 1 ml of 1% Evans Blue staining solution was injected rapidly from the left ventricle to show the ischemic risk area (AAR). After clipping the heart (Supplementary Figure II), the hearts were lavaged with 100 mL 0.9% saline at 4°C and placed on ice at -80°C for 10–15 min. After freezing, the hearts were cut into 2-mm thick transverse sections. The transverse sections were placed in 1% 2,3,5-triphenyltetrazolium chloride (TTC; Sigma, USA) in 0.1 mM phosphate (1X PBS) buffer (pH 7.4) at 37°C for 15–30 min. Then, the tissues were washed with 1X PBS buffer (3 times for 10 min each), and a bluish-purple color was seen in non-infarcted myocardium and no

coloration in infarcted myocardium. It was then placed in 4% paraformaldehyde at 4°C overnight. Images were analyzed using ImageJ data acquisition software (National Institutes of Health, Bethesda, MD, USA). Area measurement method: expressed as infarct area (IS) as a percentage of AAR (IS/AAR) and AAR as a percentage of total area (AAR/LV).

2.9 TUNEL assay: After reperfusion, the rats were sacrificed by intraperitoneal injection of excess 10% chloral hydrate. The hearts were then successively lavaged with 100 mL 0.9% saline and 50 mL 4% paraformaldehyde at 4°C. After lavage, a cross section approximately 2 mm thick was cut perpendicular to the sagittal plane of the heart approximately 2 mm below the ligature site. Fixed in 4% paraformaldehyde 3 days. Paraffin sections were prepared by dehydration and paraffin embedding. The prepared paraffin sections were analyzed using a TUNEL assay kit (Roche).In each slide, color images of five different fields were randomly captured and digitized. Cells that stained blue were normal cardiomyocytes and cells that stained brown were defined as TUNEL-positive cells. The apoptosis index (AI) was calculated as the number of TUNEL-positive cells/total number of cardiomyocytes × 100.

2.10 Hematoxylin-eosin (HE) staining: After taking the paraffin sections prepared in the previous step of the experiment and staining them with HE staining kit (Roche), pathological sections were evaluated by a double-blinded pathologist. Lesions consisting of interstitial edema, myofiber degeneration (i.e., myofiber swelling and myofibrillar lysis), and the formation of myocardial hypercontraction bands were graded according to their severity (0 = no lesion, 1 = mild, 2 = moderate, 3 = marked) and distribution (0 = no lesion, 1 = focal lesion, 2 = multifocal lesion, 3 = diffuse lesion). The mean score for each variable was calculated for each heart, and the group mean score was calculated[22].

2.11 Wheat germ agglutinin (WGA) immunofluorescence staining: Plaster sections were made by taking the previous step. The sections were blotted dry with absorbent paper, the heart sections were circled with a histochemical pen, and WGA staining solution (iFluor 488 wheat germ agglutinin conjugate, ATT-Bioquest, USA) diluted 200 times in PBS was added dropwise to the circles, and then incubated for 30 min at 37℃ in a constant temperature chamber protected from light. After incubation, the slides were washed 3 times with PBS (5 min/time) and fluorescence quenching agent was added dropwise for 5 min. After washing, the slides were washed 3 times with PBS (5 min/time), anti-fluorescence quenching sealer was added dropwise in the circle, and the slides were covered with coverslips and sealed with nail polish and stored in a wet box at 4℃. The digital section scanning system of the Department of Pathology, The Affiliated Hospital of Qingdao University was used to take pictures for observation and analyze the cross-sectional area of cells and the degree of tissue lesions.

2.12 Western blotting: Rats were sacrificed by intraperitoneal injection of excess 10% chloral hydrate. Then, the heart was lavaged with 100 mL 0.9% saline at 4°C. Heart tissue from the anterior wall of the left ventricle was isolated from the AAR and used for western blotting. The extracted heart tissue was homogenized with RIPA lysis buffer (Elabscience, Wuhan, China). The tissue was then centrifuged at 15,000 × *g* for 10 min at 4°C. The supernatant was collected, and the protein concentration was determined using a BCA assay kit (Thermo, USA). The samples were separated using 10% SDS-PAGE gels (10 μg/well). The protein bands were transferred onto nitrocellulose membranes (Merck Millipore, USA). The membranes were then blocked in 5% skim milk for 2 h and incubated overnight at 4°C with the following primary antibodies: p-PI3K (#4228), PI3K (#4257); p-GSK3β (#5558), GSK3β (#12456); p-Akt

(#4060), Akt (#4691); p-mToR (#5536), mToR (#2983); p-eNOS (#9574), eNOS (#32027); GAPDH (#5174) (all rabbit, 1:1000, Cell Signaling Technology, USA). horseradish peroxidase (HRP)-labeled goat anti-rabbit IgG (1;10000, Absin, Shanghai, China) was used as the secondary antibody. Target bands were detected using chemiluminescent ECL (Merck Millipore, USA) and visualized using an Amersham Imager 600 (GE Healthcare, Little Chalfont, UK). The images were analyzed using ImageJ data acquisition software.
2.12 Statistical analysis: The monitoring data were statistically analyzed using GraphPad Prism9 (La Jolla, CA, USA) and Image J. All data were expressed as mean ± standard error of mean (SEM). Differences between multiple groups were analyzed using one-way ANOVA and Dunnett's multiple comparisons test was performed for two-way comparisons. Statistical significance was set at $P < 0.05$.

3. Results

3.1 Confirming the phenotype of type 2 diabetic rats

As shown in Fig. 1, we induced type 2 diabetes mellitus using a single intraperitoneal injection of STZ combined with a high-fat, high-sugar diet. After 8 weeks, the body weight of DM rats in each group decreased by approximately 35% compared to that of rats without STZ injection ($^{***}P < 0.001$, Fig. 1A). Blood glucose in the DM rats increased approximately 2.9-fold ($^{***}P < 0.001$, Fig. 1B). DM rats showed significant wasting, yellowing and darkening of the fur, and even blindness due to diabetic retinopathy compared to non-DM rats (Fig. 1C.D)

3.2 Diabetes attenuates the cardioprotective effect of I-Post on MIRI

As shown in Fig. 2, we first assessed the extent of myocardial injury in diabetic I/R rats. TTC and Evens blue staining visualized the area of myocardial injury, and we found that the infarct area was 20% larger in diabetic rats than in non-diabetic rats (50.96% ± 1.59% vs. 33.65% ± 1.44%, $▲▲▲P < 0.001$, Fig. 2A). TUNEL staining detected the degree of myocardial apoptotic index (AI), which was significantly increased in diabetic I/R rats ($▲▲▲P < 0.001$, Fig. 2B).CK-MB and reactive oxygen species (ROS) are an important indicator of the degree of myocardial injury, and we observed that plasma CK-MB levels and myocardial tissue ROS levels in diabetic rats after I/R compared with non-diabetic rats were significantly higher ($▲▲▲P < 0.001$, Fig. 2C,D), which may reflect the higher sensitivity of diabetic patients to MIRI (23). Nitric oxide (NO), an important endogenous vasodilator in humans, we found that plasma levels of NO were significantly lower in diabetic rats than in non-diabetic rats ($▲▲▲P < 0.001$, Fig. 2E). Furthermore, we also observed by HE staining those diabetic rats had more severe histomorphological lesions than non-diabetic rats, including extensive myocardial cell edema, massive inflammatory cell infiltration into the myocardial interstitial space, disorganized myocardial fiber structure, and diffuse foci of necrosis (Fig. 3A). The cross-sectional area of cardiac myocytes in diabetic rats after I/R was found to be

significantly increased by WGA staining (585.68 ± 26.119 vs 465.83 ± 22.251, ▲▲▲$P < 0.001$, Fig. 3B). All of these indicated an increase in myocardial necrosis in diabetic rats after I/R.

Compared with I/R, I-Post therapy significantly reduced IS/AAR by approximately 13% in non-diabetic rats, but failed to achieve the expected therapeutic effect in diabetic rats (20.62% ± 1.42% vs. 33.65% ± 1.44%, †††$P < 0.001$, Fig. 2A). Although myocardial IS/AAR was reduced by approximately 9% in diabetic rats after I-Post (41.13% ± 2.17% vs. 50.96% ± 1.59%, ‡‡‡$P < 0.001$, Fig. 2C), myocardial IS/AAR was still 20% higher than in non-diabetic rats under the same conditions, which may indicate that diabetes impairs the cardioprotective effect of I-Post (▲▲▲$P < 0.0011$, Fig. 2A). Similar to the TTC/Evens blue staining results, although I-Post treatment reduced plasma CK-MB levels and myocardial tissue ROS levels in diabetic rats exposed to I/R (‡‡‡$P < 0.001$, Fig. 2D) and increased plasma NO levels in diabetic rats exposed to I/R (‡‡$P < 0.01$, Fig. 2D), CK-MB and ROS remained higher than in non-diabetic rats (▲▲▲$P < 0.001$, Fig. 2D) Plasma NO remained at reduced levels (▲▲$P < 0.01$, Fig. 2D). We also analyzed the number of necrotic cardiomyocytes (Fig. 2B) and systematically assessed myocardial histomorphology using pathology scoring criteria (Fig. 3A) as well as the cross-sectional area of diabetic cardiomyocytes after I-Post treatment as determined by WGA staining, and although I-Post therapy reduced cardiomyocyte area in diabetic I/R rats (454.64 ± 22.589 μm^2 vs. 585.68 ± 26.119 μm^2, ‡‡‡$P < 0.001$, Fig. 3B), it was still greater than in non-diabetic rats with the same treatment (454.64 ± 22.589 μm^2 vs. 371.17 ± 16.939 μm^2, ▲$P < 0.05$, Fig. 3B).

3.3 Diabetes weakens the cardioprotective effect of nicorandil on MIRI

Nicorandil, a treatment for ischemic heart disease, remains cardioprotective in both hypercholesterolemic and diabetic rats[18, 23]. We diabetic rats during I/R with nicorandil to investigate whether nicorandil alone has a beneficial cardioprotective effect in diabetic rats.

As shown in Fig. 2, we found that nicorandil treatment resulted in a significant reduction in myocardial IS/AAR (19.52% ± 2.17% vs. 33.65% ± 1.44%, †††$P < 0.001$, Fig. 2A) and AI (†††$P < 0.001$, Fig. 2B) in non-diabetic rats compared with MIRI rats, but also failed to achieve the expected therapeutic effect in diabetic rats. Although myocardial infarct area was reduced by approximately 9% in nicorandil-treated diabetic I/R rats (41.54 ± 1.45% vs. 50.96 ± 1.59%, ‡‡‡$P < 0.0011$, Figure A) and reduced the number of apoptotic cardiomyocytes (‡‡‡$P < 0.0011$, Fig. 2B), IS/AAR and AI remained higher in diabetic rats than in non-diabetic rats receiving the same treatment (▲▲▲$P < 0.001$, Fig. 2A, B). Plasma CK-MB and myocardial tissue ROS levels decreased in diabetic I/R rats treated with nicorandil (both †††$P < 0.001$, Fig. 2C, D), but remained higher than in non-diabetic I/R rats treated with nicorandil (both▲▲▲$P < 0.001$, Fig. 2C, D). And their pathological injury scores (‡‡‡$P < 0.001$, Fig. 3A) as well as cardiomyocyte cross-sectional area (439.95 ± 15.087 μm^2 vs. 585.68 ± 26.119 μm^2, ‡‡‡$P < 0.001$, Fig. 3B) showed that although nicorandil

reduced the degree of cardiac injury in diabetic I/R rats to some extent ($^{▲▲▲}P < 0.001$, Fig. 3A), it was still less effective than in non-diabetic rats, and cardiomyocytes were still swollen (439.95 ± 15.087 μm^2 vs 356.73 ± 12.355 μm^2, $^{▲▲▲}P < 0.001$, Fig. 3B).

Furthermore, we unexpectedly found that plasma NO levels were not significantly different in diabetic I/R rats after treatment with nicorandil compared with non-diabetic I/R rats, which may indicate that diabetes did not affect the nitrate effect of nicorandil ($^{ns}P > 0.05$, Fig. 3D).

3.4 Protective effect of I-Post combined with nicorandil in diabetic rats with myocardial infarction

As described previously, diabetes impaired the cardioprotective effect of I-Post or nicorandil on MIRI, suggesting that one therapeutic measure alone does not provide a good therapeutic effect and that combination therapy may be a potential treatment modality for such diseases. Therefore, we applied the therapeutic measure of I-Post combined with nicorandil to explore its rationality and feasibility.

As shown in Fig. 4, we observed that the IS/AAR was reduced by approximately 20% in diabetic rats treated with I-Post combined with nicorandil (30.67% ± 1.38% vs. 50.96% ± 1.58%, $^{‡‡‡}P < 0.001$, Fig. 2A), which was more effective in reducing IS/AAR than in diabetic rats treated with I-Post (30.67% ± 1.38% vs. 41.13% ± 2.17%, $^{###}P < 0.001$, Fig. 2A) or nicorandil (30.67% ± 1.38% vs. 41.54 ± 1.45%, $^{###}P < 0.001$, Fig. 2A) alone. Moreover, the plasma levels of CK-MB and myocardial tissue levels of ROS in diabetic I/R rats after I-Post combined with nicorandil treatment were significantly reduced (all $^{‡‡‡}P < 0.001$, Fig. 2D), which was a significant improvement compared I-Post (all $^{###}P < 0.001$, Fig. 2D) or nicorandil (all $^{###}P < 0.001$, Fig. 2D) alone. In addition, the combination treatment significantly reduced plasma NO levels in diabetic I/R rats ($^{‡‡‡}P < 0.001$, Fig. 2D), but there was no significant difference in plasma NO levels between the two groups when compared with diabetic I/R rats treated with nicorandil alone ($^{ns}P > 0.05$, Fig. 2D).

WGA staining and AI were similar to these results. I-Post combined with nicorandil resulted in a significant reduction in apoptotic cells ($^{‡‡‡}P < 0.001$, Fig. 2B), a decrease in cardiomyocyte edema, and a significant reduction in cardiomyocyte cross-sectional area (344.45 ± 13.434 μm^2 vs 585.68 ± 26.119 μm^2, $^{‡‡‡}P < 0.001$, Fig. 3B). Compared to diabetic I/R rats treated with I-Post (454.64 ± 13.434 μm^2 vs 344.45 ± 13.434 μm^2, $^{###}P < 0.001$, Fig. 3B) or nicorandil (439.95 ± 15.087 μm^2 vs 344.45 ± 13.434 μm^2, $^{##}P < 0.01$, Fig. 3B) alone, the benefits of combination treatment were higher. In addition, myocardial fibers were more regularly aligned, and no localized necrotic lesions were observed. the pathological scores of I-Post combined with nicorandil were lower than those of I-Post ($^{##}P < 0.01$, Fig. 3A) or nicorandil ($^{##}P < 0.01$, Fig. 3A) alone.

Although the IS/AAR, AI, the cross sectional area of myocardial cells, the plasma levels of CK-MB and myocardial tissue levels of ROS in diabetic rats was still larger than that in non-diabetic rats after the

combined treatment (all $^{▲}P < 0.01$, Fig. 2A, C, D; Fig. 3B), the IS/AAR was greatly reduced by the combination treatment. And, the cardiac histopathology and the plasma levels of NO were not significantly different between the two groups ($^{ns}P > 0.05$, Fig. 2E; Fig. 3A). These results suggest that I-Post combined with nicorandil showed better cardioprotective effect s in MIRI treatment.

3.5 Diabetes affects the cardioprotective effect of I-Post on MIRI in diabetic rats by weakening PI3K/Akt signaling pathway

RISK signaling pathway is a key pathway to protect the heart from MIRI, and previous studies have also shown that I-Post can exert cardioprotective effects by activating PI3K/Akt signaling pathway[8]. The activation of PI3K/Akt signaling pathway leads to the activation of downstream mammalian rapamycin (mTOR), glycogen synthase kinase 3β (GSK3β) and endothelial nitric oxide synthase (eNOS) to reduce reperfusion-induced cell death with potent cardioprotective effects.

Our study confirmed this finding by western blotting. Compared with non-diabetic I/R rats, the expression of phosphorylated PI3K (p-PI3K), phosphorylated Akt (p-Akt), phosphorylated mTOR (p-mTOR), phosphorylated GSK3β (p-GSK3β) and phosphorylated eNOS (p-eNOS) was significantly increased in I-Post-treated rats (all $^{†††}P < 0.001$, Fig. 5), and their MIRI impairment was significantly reduced. Although the expression of p-PI3K, p-Akt, p-GSK3, p-mToR and p-eNOS was significantly increased in diabetic I/R rats after I-Post treatment (all $^{‡‡‡}P < 0.001$, Fig. 4), these key proteins in the PI3K/Akt signaling pathway failed to achieve the same level of phosphorylated expression in diabetic I/R rats as in non-diabetic rats ($^{▲▲▲}P < 0.001$, Fig. 4). This may be the main reason why diabetes impairs the cardioprotective effects of I-Post in I/R rats.

3.6 Diabetes affects the cardioprotective effect of nicorandil on MIRI in diabetic rats by weakening PI3K/Akt signaling pathway

Nicorandil also has a cardioprotective effect against MIRI by activating the PI3K/Akt signaling pathway and reducing reperfusion-induced cell death[18]. This finding was confirmed by our study. However, the expression of p-PI3K, p-Akt, p-GSK3, p-mToR and p-eNOS was increased in diabetic I/R rats after nicorandil treatment (all $^{‡‡‡}P < 0.001$, Fig. 4), but these key proteins of the PI3K/Akt signaling pathway failed to reach the same phosphorylated expression in diabetic I/R rats as at the level of non-diabetic rats ($^{▲▲▲}P < 0.01$, Fig. 4). Similar to I-Post, which may be the main reason that diabetes jeopardizes the cardioprotective effect of nicorandil in I/R rats.

3.7 I-Post combined with nicorandil in diabetic I/R rats can have a superimposed effect on activation of PI3K/Akt signaling pathway

Diabetic I/R rats treated with I-Post in combination with nicorandil had significantly increased expression levels of p-PI3K, p-Akt and its downstream effectors p-GSK3b, p-mToR and p-eNOS in myocardial tissue (all $^{‡‡‡}P < 0.001$, Fig. 4). And the phosphorylation levels of all these proteins were higher than those of diabetic I/R rats with I-Post (all $^{\#\#}P < 0.01$, Fig. 4) or nicorandil (all $^{\#\#\#}P < 0.001$, Fig. 4) alone. However, the phosphorylation levels of key proteins in the RISK pathway remained lower than those of non-diabetic rats after the same treatment (all $^{▲▲}P < 0.01$, Fig. 4).

3.8 Activation of PI3K/Akt signaling pathway in diabetic I/R rats by I-Post combined with nicorandil is inhibited by PI3K phosphorylation inhibitor by wortmannin

We found that I-Post combined with nicorandil treatment may have a superimposed effect on the activation of PI3K/Akt signaling pathway, which has a better cardioprotective effect on reducing MIRI in diabetic I/R rats. To verify the mechanism of its cardioprotective effect, we applied PI3K phosphorylation inhibitor wortmannin before treatment with I-Post combined with nicorandil in diabetic I/R rats, observed the extent of myocardial injury and analyzed the expression of related protein phosphorylation in PI3K/Akt signaling pathway. After TTC and Evens Blue staining, it was found that the application of wortmannin impeded the protective effect of I-Post combined with nicorandil on the myocardium of diabetic I/R rats, (30.67% ± 1.38% vs. 49.08 ± 1.47%, $^{***}P < 0.001$, Fig. 5), and that wortmannin impeded the phosphorylation of PI3K and p-Akt expression was significantly reduced (all $^{***}P < 0.001$, Fig. 5), which affected the activation of downstream target proteins such as eNOS, GSK3b and mToR (all $^{***}P < 0.001$, Fig. 5). Confirmed that the therapeutic measures of I-Post combined with nicorandil act through PI3K/Akt signaling pathway

4. Discussion

Diabetes mellitus, a metabolic disease characterized by hyperglycemia and endogenous insulin hypersecretion or resistance, is an independent risk factor for cardiovascular disease, and a large number of diabetic patients die suddenly due to AMI. Although timely reperfusion therapy is important to save diabetic AMI patients, diabetic patients exhibit a high susceptibility to MIRI, which leads to loss of endogenous cardioprotective mechanisms and mitochondrial dysfunction[16]. In addition, the physiological balance of NO is altered in the hyperglycemic state caused by diabetes[24] and leads to apoptosis triggered by massive production of ROS[25, 26]. However, it remains inconclusive whether I-Post protects the diabetic heart exposed to I/R.

Recent large clinical trials such as the POSTEMI (ST elevation post-myocardial infarction management) and DANAMI-3-iPOST(Third Danish study of optimal acute treatment of patients with ST-segment elevation myocardial infarction–ischemic postconditioning) studies[27, 28], showed that I-Post does not reduce the size of myocardial infarction, nor does it reduce mortality and heart failure hospitalization rates in patients with ST-segment elevation myocardial infarction undergoing emergency PCI. Possible reasons for this are the more complex clinical situation of the patients, their age and concomitant diseases such as hypertension, diabetes mellitus and hyperlipidemia, which may affect the cardioprotective effect of I-Post. Although effective in animal studies, patients encountered in clinical work often have multiple co-morbidities and therefore have limited impact in humans. Another important reason may be that repeated balloon dilatation-reperfusion during clinical interventions with I-Post may cause thrombus dislodgement and lead to coronary microvascular embolization, thus affecting the efficacy of reperfusion therapy. Our study also found that diabetic I/R rats treated with I-Post, although their myocardial infarct size and degree of myocardial injury improved somewhat, still did not reach the desired therapeutic level compared with non-diabetic I/R rats after the same treatment. This suggests that diabetes may have somewhat diminished the cardioprotective effect of I-Post on MIRI. Combined with large-scale clinical studies, we suggest that the complexity and severity of diabetes combined with AMI diminishes the cardioprotective effect of I-Post and that a single treatment may not achieve the desired therapeutic effect. Moreover, clinical studies have shown that the complexity of severe disease highly diminishes the therapeutic potential of existing treatment options. As a result, current efforts to treat these diseases have gradually shifted from a focus on monotherapy to combination or multiple therapies[29]. Therefore, we speculate that the addition of antidiabetic as well as microcirculatory vasodilators may provide better cardioprotection in diabetic I/R rats when good therapeutic results cannot be achieved with the I-Post regimen alone.

Nicorandil, as a drug with improved microvascular circulation[30], causes vasodilation through two pathways: first, it has a nitrate-like effect, providing NO; second, it selectively opens ATP-sensitive potassium channels (K_{ATP}) in cell membranes and mitochondria[31]. In addition, nicorandil has the efficacy to reduce apoptosis in cardiomyocytes of rats with diabetic cardiomyopathy. Clinical studies have found that intracoronary injection of nicorandil prior to PCI for ST-segment elevation myocardial infarction (STEMI) significantly improved myocardial perfusion and reduced arrhythmias during PCI in patients with STEMI[32], and it was effective in reducing the incidence of adverse cardiovascular events and improving cardiac function in patients undergoing elective PCI[33]. Moreover, nicorandil is approved as a first-line drug for the treatment of CHD and as a long-term treatment for chronic stable angina in Japan and Europe[34].However, whether nicorandil can develop good MIRI in T2DM rats is still lacking such studies. In our study, in which we applied nicorandil for the first time in diabetic I/R rats, we found that the nitrate effect of nicorandil was not affected by diabetes. However, although the expression of phosphorylated proteins related to PI3K/Akt signaling pathway in myocardial tissue of nicorandil-treated diabetic I/R rats was significantly increased, myocardial tissue ROS content was significantly decreased, and myocardial apoptotic cells were significantly reduced, the degree of myocardial injury was still higher than that of nicorandil-treated non-diabetic I/R rats, which may suggest that nicorandil has a beneficial

effect on the treatment of diabetic MIRI has some advantages, but this protective effect is still relatively weak. There is no doubt that drugs with anti-diabetic-induced cardiomyocyte apoptosis and microcirculatory vasodilatation cannot afford to accord favorable therapeutic effects in diabetic I/R rats.

Previous studies have shown that Akt phosphorylation can activate its downstream target mToR and inhibit endoplasmic reticulum stress, thereby reducing ROS release from myocardial tissue and decreasing the degree of myocardial tissue injury[35].Meanwhile, increased Akt phosphorylation stimulates GSK3β phosphorylation, inhibits the opening of the mitochondrial permeability translocation pore (mPTP), and reduces apoptosis in cardiac myocytes[36]. In addition, eNOS, a key target protein downstream of Akt, is a key enzyme for the induction of NO production[37], and adequate NO levels are critical for the regulation of blood flow and vasodilation[38], which play an important role in myocardial protection[39, 40]. However, our study found that these signaling pathways and key proteins, which are closely related to cardioprotective effects, did not reach ideal phosphorylation levels in I-Post or nicorandil-treated diabetic I/R rats, which may be the reason why diabetes impairs the cardioprotective effects of I-Post and nicorandil. This resulted in the extent of myocardial damage in I-Post and nicorandil-treated diabetic I/R rats remained severe.

There is no doubt that the complexity and severity of diabetes combined with AMI diminishes the myocardial protective effect of I-Post as well as nicorandil in MIRI and that the available single treatment options do not provide the desired therapeutic effect. Combining multiple therapeutic measures to combat MIRI in diabetic patients may be an innovative concept. To investigate the feasibility of combination therapy, this study was the first to apply I-Post in combination with nicorandil in I/R rats with T2DM. Our study found that the treatment regimen of I-Post combined with nicorandil had significant advantages in reducing myocardial infarct size and limiting myocardial cell injury compared with diabetic I/R rats with single application of I-Post and nicorandil, while there were no significant differences in the degree of myocardial histopathological injury and intravascular NO levels compared with non-diabetic rats. The reason for this may be that, on the one hand, since both treatments have the effect of activating the RISK signaling pathway, when the two treatments are used in combination, they can have a superimposed effect and can better activate endogenous cardioprotective mechanisms. The reason may be that, on the one hand, since both treatments have the effect of activating the RISK signaling pathway, when the two treatments are combined, they can have a superimposed effect and can better activate the endogenous cardioprotective mechanisms. Although diabetes affects the phosphorylation levels of some key proteins associated with endogenous cardioprotective effects, the superimposed effect can lead to better cardioprotection. On the other hand, the myocardial protection mechanisms differed between the two treatments. Although eNOS phosphorylation was lower in nicorandil-treated diabetic I/R rats than in non-diabetic rats, nicorandil not only led to endogenous NO production by promoting eNOS activation, but also increased NO levels through the reaction of its nitrate moiety with sulfhydryl groups in vascular smooth muscle cells, resulting in no significant difference in plasma NO levels between non-diabetic and diabetic rats. When diabetes reduces the myocardial protective effect of I-Post, nicorandil can directly produce NO, reduce the aggregation and infiltration of inflammatory cells and platelets, improve coronary

blood flow, and protect myocardial endothelial cells, thus protecting the heart from MIRI. Therefore, we believe that the complexity of acute myocardial infarction in diabetic patients combined with MIRI highly diminishes the therapeutic potential of existing treatment options and that combining multiple therapeutic measures to combat MIRI in diabetic patients may be an innovative concept.

However, our study still has some limitations. Firstly, nicorandil, as a drug with dual pharmacological effects, was not explored in depth for its role as ATP sensitive potassium channel activation. And we did not evaluate in depth the response curve of the most appropriate dose of nicorandil, choosing only the effective dose for clinical studies to assess its protective effect. In addition, whether increasing the drug dose of nicorandil alone can provide better cardioprotection in diabetic I/R rats remains to be investigated. On the other hand, because the mechanism of MIRI is very complex, our study only analyzed the expression of some signaling proteins, and a series of questions about the signaling pathways and regulatory mechanisms of how I-Post combined with nicorandil exerts its protective effect on diabetic MIRI need to be further investigated. Finally, although our study found that I-Post combined with nicorandil was more advantageous in reducing diabetic MIRI compared to either treatment alone, we also found that the degree of cardiac damage was still severe compared to non-diabetic rats. Possible reasons for such a reduction are that apoptosis is highly involved in organ complications in diabetic patients, and myocardial apoptosis in the diabetic state may lead to greater cardiac dysfunction[12, 41], and that the impaired endogenous cardioprotective mechanisms induced by diabetes itself limit the therapeutic effect. Thus, the degree of myocardial damage remains higher in diabetic I/R rats than in non-diabetic I/R rats after combination treatment, however, the combination still offers some advantages in terms of increasing plasma NO levels and maintaining normal morphology of cardiac cells and tissues. Therefore, the combined application of several different cardioprotective measures to reduce MIRI in diabetic patients has a better cardioprotective effect. In addition, research on diabetic MIRI may not be limited to the cardiovascular field; an integrated, multidisciplinary approach is the future trend in the treatment of complex and severe disease.

5. Conclusion

Our study found that diabetes impairs the cardioprotective effects of I-Post and nicorandil on MIRI, and that treatment with I-Post combined with nicorandil reduced the degree of cardiac damage in diabetic rats better than either treatment alone.

Declarations

Ethics approval and consent to participate

This project fully protects the rights and interests of mice and has passed the requirements of the Laboratory Animal Welfare Ethics Committee of Qingdao University.

Consent for publication

Not applicable.

Conflict of interest

The authors confirm that there are no conflicts of interest

Authors' contributions

Bing Chen and Chi Zhou were responsible for the development of the animal model of diabetes mellitus. Zongyi Xia performed the myocardial ischemia-reperfusion model, TUNEL and HE staining, and western blot protein blotting experiments. Jinyang Ren, Xunjin Yao, and Qi Wan analyzed the data and provided guidance on writing the paper. Zongyi Xia was the major contributor to the writing of the manuscript and the Elisa experiments. All authors read and approved the final manuscript.

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Data and materials availability statement

All data and material generated or analyzed during this study are included in this published article

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Figures

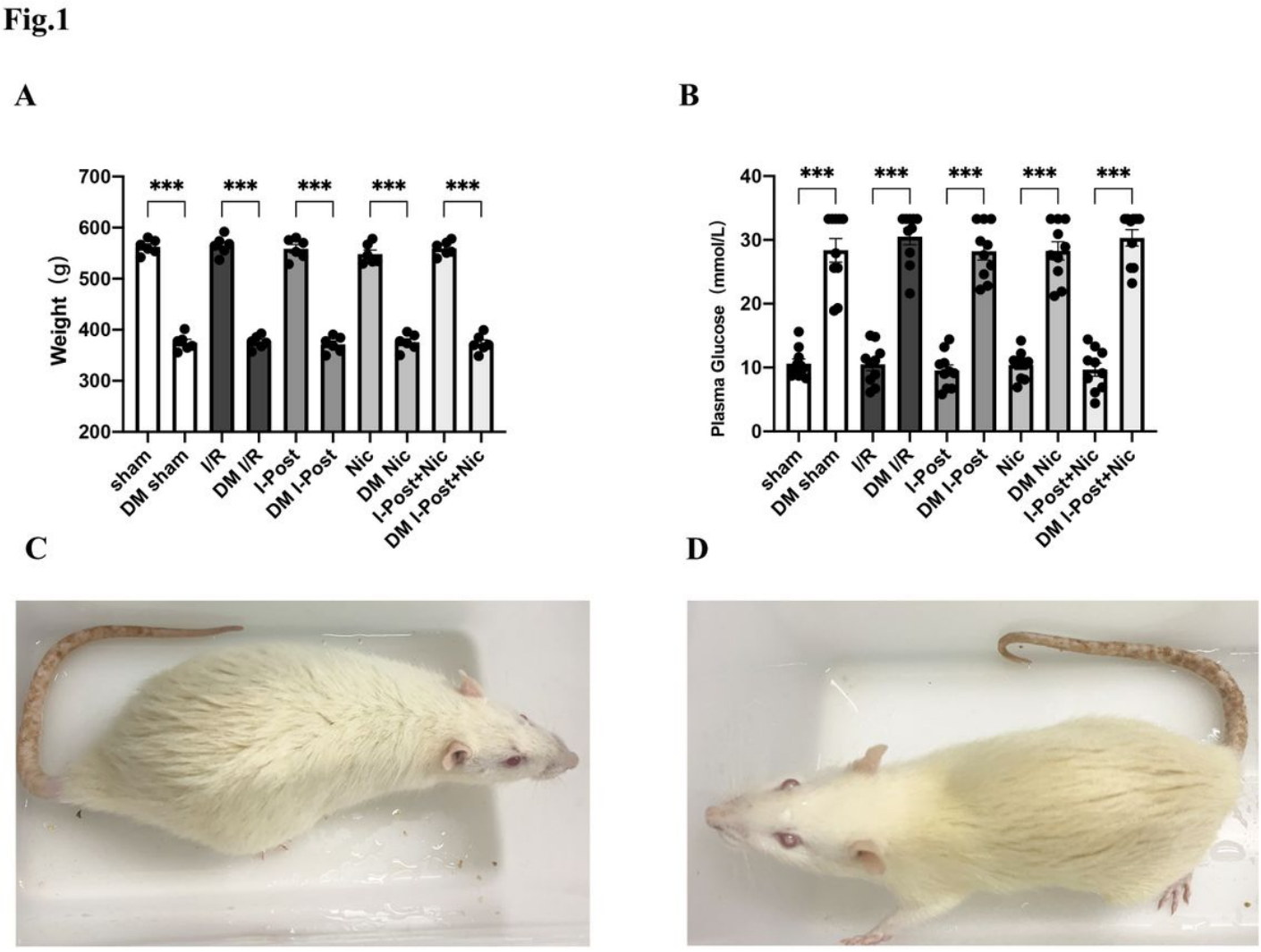

Figure 1

Body weight and blood glucose of rats in each experimental group. (A) Plasma glucose (n=10), (B) body weight (n=6). (C) A representative non-diabetic rat. (D) A representative diabetic rat. $^{***}P < 0.001$. Data are expressed as mean ± standard error of the mean (SEM) and were analyzed by one-way ANOVA and Dunnett's multiple comparisons test.

Fig.2

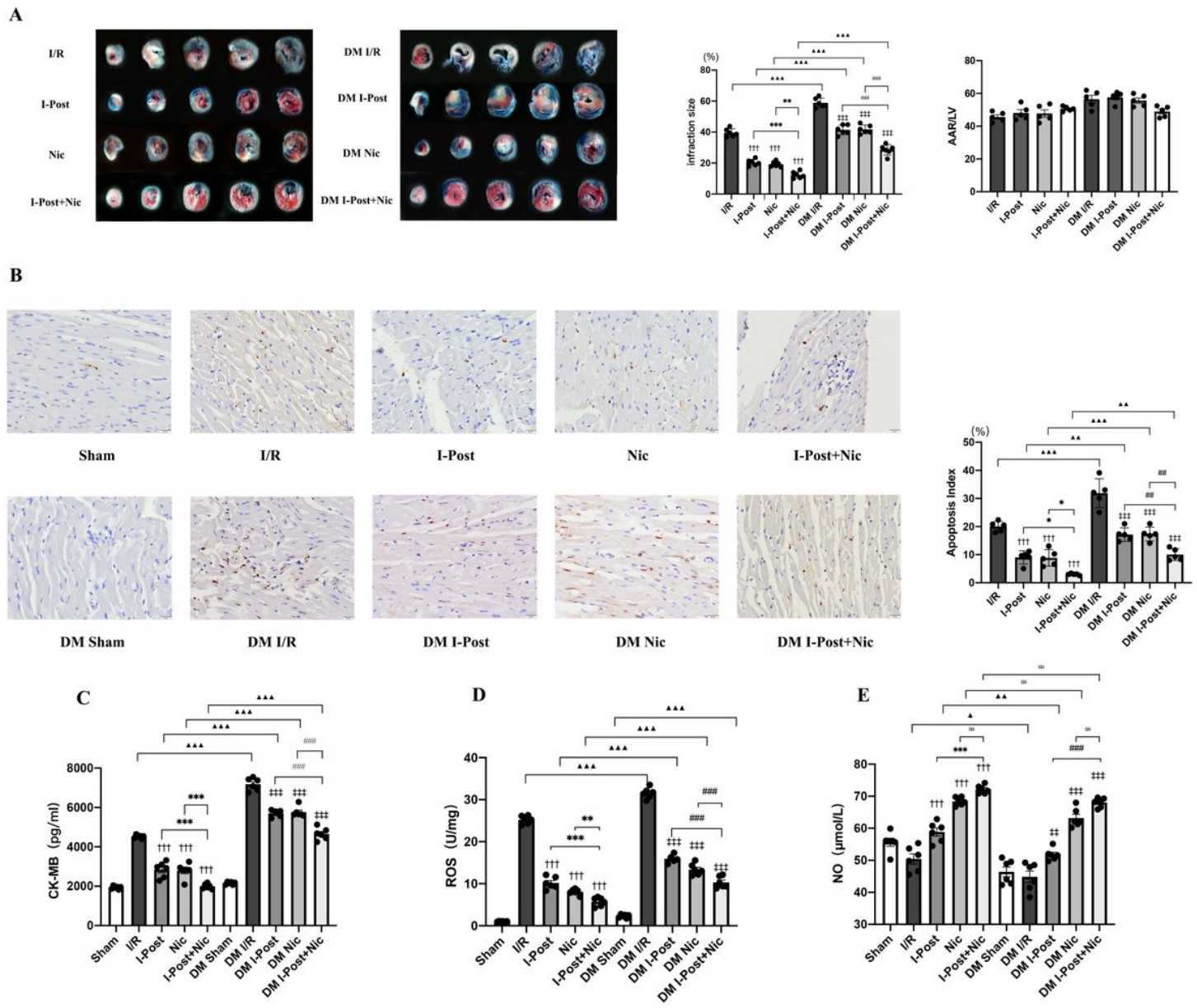

Figure 2

Diabetes impairs the cardioprotective effects of I-Post and nicorandil, and the cardioprotective effect of the combination treatment on MIRI in diabetic rats. (A) Representative sections of TTC/Evens Blue stained heart tissue subjected to 30 min myocardial ischemia followed by 2.5 h of reperfusion (n=5). (B) Representative TUNEL-stained sections of the heart (n=5). Brown nuclei indicate TUNEL-positive nuclei (marked by red arrow). (C) CK-MB plasma levels in rats treated with I-Post combined with nicorandil (n=6). (D) ROS levels in myocardial tissue in rats treated with I-Post combined with nicorandil (n=6). (E) NO plasma levels in rats treated with I-Post combined with nicorandil (n=6). Compared with I/R: $^{\dagger\dagger\dagger}P < 0.001$; Compared with DM I/R: $^{\ddagger\ddagger\ddagger}P < 0.001$; Compared with I-Post+Nic: $^{***}P < 0.001$, $^{**}P < 0.01$, $^{ns}P > 0.05$; Compared with DM I-Post+Nic: $^{\#\#\#}P < 0.001$, $^{\#\#}P < 0.01$, $^{ns}P > 0.05$; Comparison between non-diabetic and diabetic groups applying the same treatment$^{\blacktriangle\blacktriangle\blacktriangle}P < 0.001$, $^{\blacktriangle\blacktriangle}P < 0.01$, $^{\blacktriangle}P < 0.05$, $^{ns}P > 0.05$. Data

represent the mean±SEM. Data were analyzed using one-way ANOVA and Dunnett's multiple comparisons test. Sham, sham surgery; I/R, ischemia-reperfusion; I-Post, ischemia postconditioning; Nic, nicorandil; I-Post+Nic, ischemia postconditioning combined with nicorandil; DM, diabetes mellitus.

Fig.3

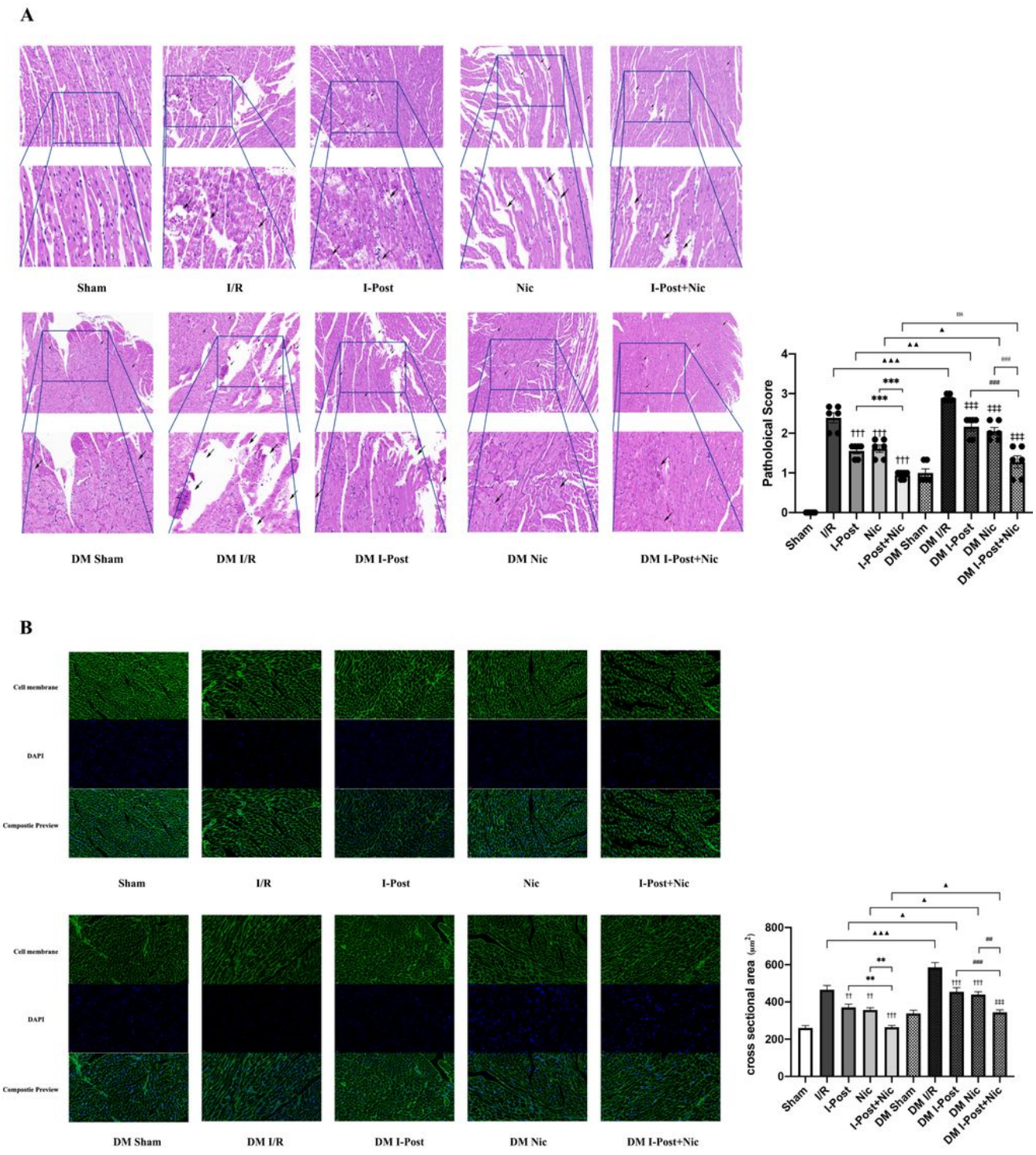

Figure 3

Alterations in myocardial tissue structure in diabetic rats after I-Post, nicorandil and combined treatment. (A) Representative cross-sectional HE-stained pathological section of the heart (n=6). The black arrows indicate cellular edema, swelling of myocardial muscle fibers, and myogenic fiber lysis. (B) Representative cross-sectional WGA staining of the heart, and quantitative data on the cross-sectional area of cardiomyocytes (n=6). Compared with I/R: $^{\dagger\dagger\dagger}P < 0.001$; Compared with DM I/R: $^{\ddagger\ddagger\ddagger}P < 0.001$; Compared with I-Post+Nic: $^{***}P < 0.001$, $^{**}P < 0.01$; Compared with DM I-Post+Nic: $^{\#\#\#}P < 0.001$, $^{\#\#}P < 0.01$; Comparison between non-diabetic and diabetic groups applying the same treatment $^{\blacktriangle\blacktriangle\blacktriangle}P < 0.001$, $^{\blacktriangle\blacktriangle}P < 0.01$, $^{\blacktriangle}P < 0.05$, $^{ns}P > 0.05$. Data represent the mean±SEM. Data were analyzed using one-way ANOVA and Dunnett's multiple comparisons test. Sham, sham surgery; I/R, ischemia-reperfusion; Nic, nicorandil; DM, diabetes mellitus.

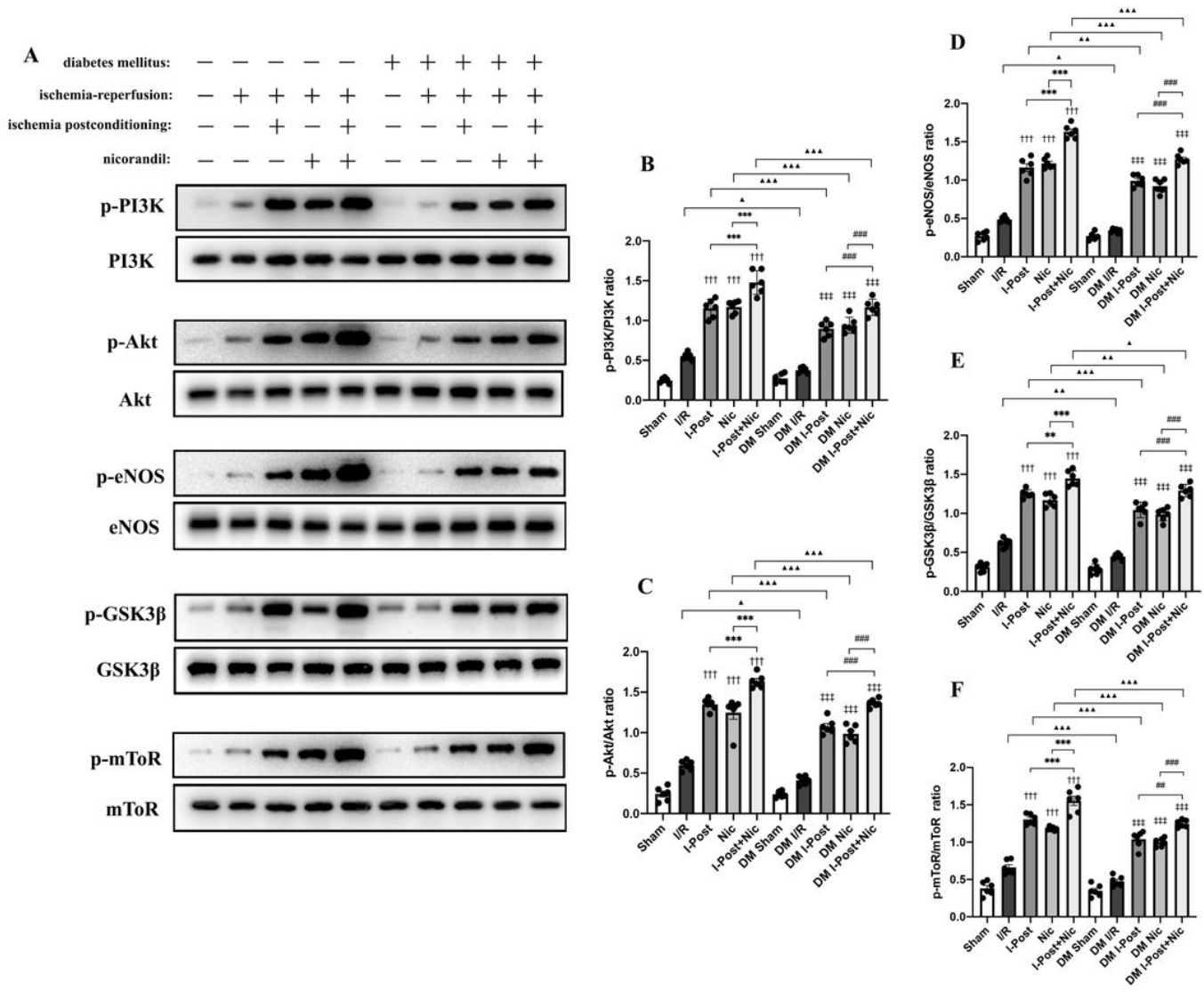

Figure 4

Alteration of PI3K/Akt signaling pathway in diabetic rats after I-Post, nicorandil and combination treatment (A) Representative western blots showing total expression and the phosphorylation state. (B) Comparison of p-PI3K expression levels in myocardial tissue of rats treated with I-Post combined with nicorandil (n=6). (C) Comparison of p-Akt expression levels in myocardial tissue of rats treated with I-Post combined with nicorandil (n=6). (D) Comparison of p-eNOS expression levels in myocardial tissue of rats treated with I-Post combined with nicorandil (n=6). (E) Comparison of p-GSK3β expression levels in myocardial tissue of rats treated with I-Post combined with I-Post combined with nicorandil (n=6). (F) Comparison of p-mTOR expression levels in myocardial tissue of rats treated with I-Post combined with nicorandil (n=6). Compared with I/R: $^{\dagger\dagger\dagger}P < 0.001$; Compared with DM I/R: $^{\ddagger\ddagger\ddagger}P < 0.001$; Compared with I-Post+Nic: $^{***}P < 0.001$, $^{**}P < 0.01$; Compared with DM I-Post+Nic: $^{\#\#\#}P < 0.001$, $^{\#\#}P < 0.01$; Comparison between non-diabetic and diabetic groups applying the same treatment $^{▲▲▲}P < 0.001$, $^{▲▲}P < 0.01$, $^{▲}P < 0.05$. Data represent the mean±SEM. Data were analyzed using one-way ANOVA and Dunnett's multiple comparisons test. Sham, sham surgery; I/R, ischemia-reperfusion; I-Post, ischemia postconditioning Nic, nicorandil; DM, diabetes mellitus.

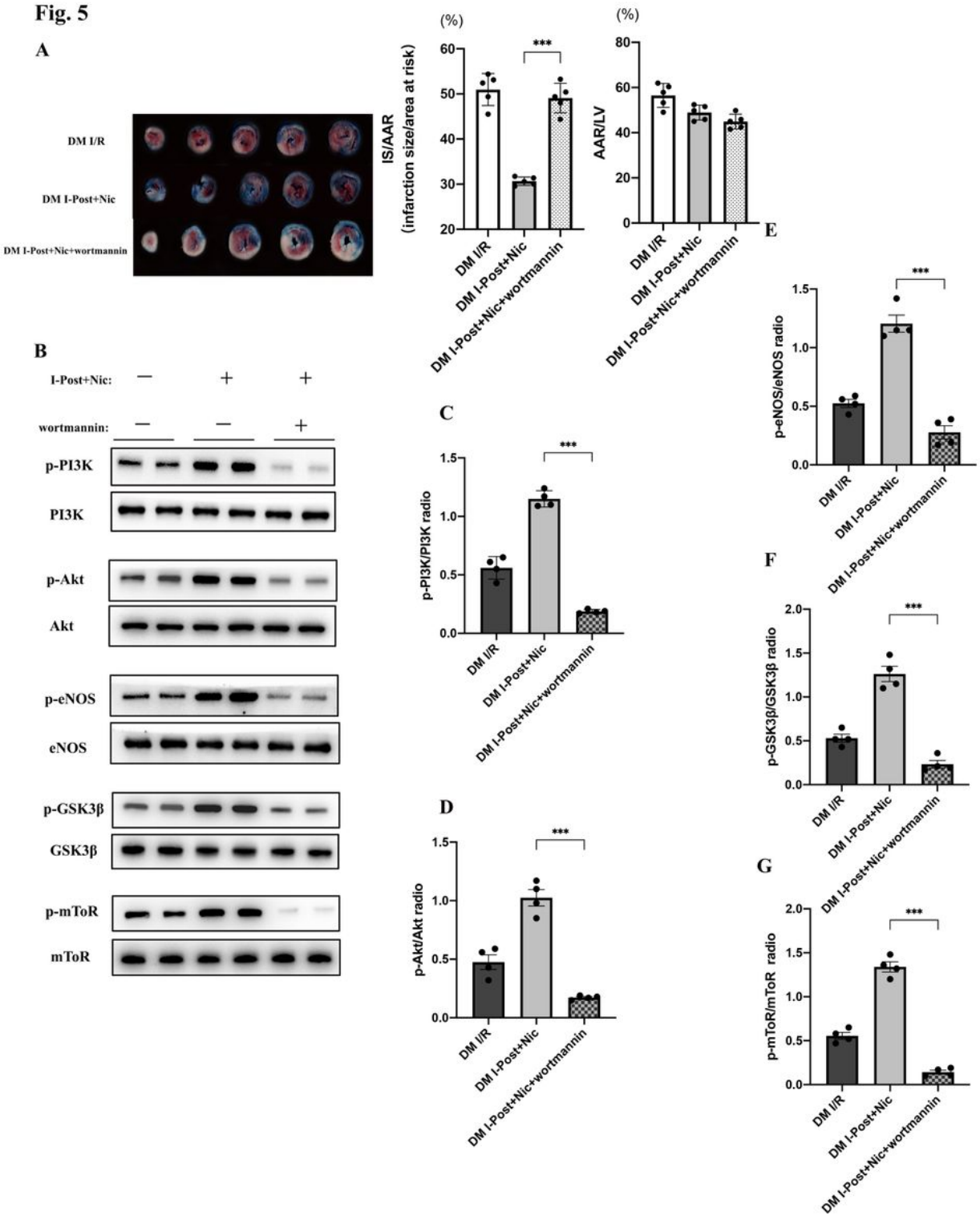

Figure 5

Activation of PI3K/Akt signaling pathway by I-Post combined with nicorandil is hindered by PI3K phosphorylation inhibitor wortmannin (A) Representative sections of TTC/Evens Blue stained heart tissue subjected to 30 min myocardial ischemia followed by 2.5 h of reperfusion (n=5). (B) Representative western blots showing total expression and the phosphorylation state. (C) Comparison of p-PI3K expression levels in myocardial tissue of rats treated with (n=4). (D) Comparison of p-Akt expression

levels in myocardial tissue of rats treated with I-Post combined with nicorandil (n=4). (E) Comparison of p-eNOS expression levels in myocardial tissue of rats treated with I-Post combined with nicorandil (n=4). (F) Comparison of p-GSK3β expression levels in myocardial tissue of rats treated with I-Post combined with I-Post combined with nicorandil (n=4). (G) Comparison of p-mTOR expression levels in myocardial tissue of rats treated with I-Post combined with nicorandil (n=4). $^{***}P < 0.001$, $^{**}P < 0.01$, $^{*}P < 0.05$. Data represent the mean±SEM. Data were analyzed using one-way ANOVA and Dunnett's multiple comparisons test. I/R, ischemia-reperfusion; I-Post, ischemia postconditioning; Nic, nicorandil; wortmannin, PI3K phosphorylation inhibitor; DM, diabetes mellitus.

Supplementary Files

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- Supplementary1.docx