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Cardioprotective effect of Perakine against myocardial ischemia-reperfusion injury of type-2 diabetic rat in Langendorff-Perfused Rat Hearts: Role of TLR4/NF-kB signalling pathway

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ORIGINAL ARTICLE

Cardioprotective effect of Perakine against myocardial ischemia-reperfusion injury of type-2 diabetic rat in Langendorff-Perfused Rat Hearts: Role of TLR4/NF-kB signalling pathway

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ABSTRACT

Myocardial ischemia-reperfusion (I/R) injury is a grave life-threatening situation, if not promptly treated. The development of natural remedies for myocardial I/R injury has witnessed dramatic growth in the last decade. Prompted by the above, in the present study, we have elucidated the pharmacological effect of Perakine (PER), an indole alkaloid in myocardial ischemia-reperfusion injury in type 2 diabetic rats. The model was established by inducing diabetes in experimental rats, followed by the development of a myocardial I/R injury model in isolated rat hearts using an improved Langendorff retrograde perfusion technique. Results of the study suggest that PER significantly lowered the infarct size and volume, and improvement in cardiac ability (LVSP, $\pm dP/dt_{max}$, and heart rate). It also significantly lowered cardiac biomarkers (CK, CK(MB), ALT, AST, and LDH) in a dose-dependent manner compared to unprotected I/R rats. The level of oxidative stress (MDA, SOD, and GSH) was also found lowered in IR rats together with a reduction in the production of pro-inflammatory cytokines (IL-1β, IL-6, IL-17, and TNF-α). The anti-inflammatory acting of PER on IR rats is believed to be linked with the reduction of TNF-α, NF-κB, and TLR4 in both RT-qPCR and western blot analysis. Our research showed that Perakine could potentially alleviate cardiac ischemia-reperfusion injury in rats by blocking the TLR4/NF-κB signaling cascade.

Keywords: Indole alkaloid, Diabetes, Infarct size, cytokines, inflammation

Introduction

Globally, ischemic heart disease (IHD) accounts for a disproportionate share of fatalities and disabilities. Coronary artery stenosis or blockage, which causes myocardial ischemia or infarction, is considered as the main pathophysiological mechanism of IHD [26]. While restoring perfusion is the gold standard for treating IHD, it does come with some drawbacks. It causes functional harm, metabolic irregularities, and changes in ultrastructure while restoring oxygen supply and cardiac perfusion. Myocardial ischemia-reperfusion damage is the umbrella term for this particular occurrence [36]. Some of the symptoms that can be observed in patients with MIRI include abnormal heart rhythms, reduced cardiac function (both systolic and diastolic), blocked blood vessels, myocardial infarction, heart failure, and no or slow blood flow to the heart. At this time, the complete elucidation of its pathogenesis remains elusive; nevertheless, certain investigations propose that diminished oxidative phosphorylation during hypoxia, heightened production of reactive oxygen species (ROS), and inadequate eradication of these ROS after reperfusion are the principal factors contributing to IR injury [11, 22, 23, 37, 47].

When it comes to curing a wide range of human illnesses, molecules derived from nature have been invaluable [6]. Many natural products contain anti-inflammatory and anti-oxidant properties that can help reduce inflammation and oxidative stress, two factors that worsen as cardiovascular disease advances, according to studies [10, 21]. Research on natural products and cardiovascular disorders has made good advancements, but there are still a lot of undiscovered natural compounds that need to have their positive benefits further investigated.

Increasing evidence links type 2 diabetes (T2D) to cardiovascular illnesses and myocardial infarction co-morbidities [12, 39]. Type 2 diabetes (T2D) enhances the vulnerability of the heart to injury caused by ischemia-reperfusion (I/R). It reduces the heart’s ability to respond to preventative measures against damage and worsens the harm caused by I/R. Additionally, T2D enlarges the area of tissue death resulting from acute myocardial infarction (AMI), leading to a higher occurrence of dangerous irregular heart rhythms and heart failure [43, 44, 46].

A long-used remedy for hypertension, snake bites, and insanity among China’s Zhuang and Yao minority regions is perakine, an indole alkaloid derived from the *Rauvolfia yunnanensis* plant, which is a member of the Rauvolfia genus and the Apocynaceae family [27, 38]. China is considered as home to nine species, four varieties, and three cultivars of around one

hundred thirty-five species of Rauvolfia found across Asia, Africa, the Americas, and Oceania. Indole alkaloids may have powerful anti-inflammatory effects, according to the research [35, 45]. Nevertheless, the bioactivity of Perakine against myocardial ischemia has not been reported in any of the studies. Given the aforementioned, the current study aimed to investigate the pharmacological impact of Perakine on diabetic rats' myocardial/ischemia injury. We have selected the diabetic rat model because to the fact that diabetes is traditionally thought to be the primary risk factor for myocardial I/R.

EXPERIMENTAL

Chemicals

All the chemicals and reagents including Perakine unless otherwise stated will be obtained from the Sigma Aldrich, USA. The Perakine (> 95% pure via HPLC; Lot. No. BP1077) was obtained from the Chengdu Biopurify Pharmaceuticals (Chengdu, China).

Induction of diabetes in the Sprague Dawley rats

The Sprague Dawley rats after obtaining from the institutional animal house were given streptozotocin (STZ, 35 mg/kg bodyweight, Sigma Aldrich, St. Louis, MO, USA) in accordance with a previously documented protocol to induce diabetes. With the exception of the rats in Group Control, all rats were administered a single intraperitoneal (*i.p.*) dosage of STZ solution mixed with citrate buffer at a concentration of 40 mg/kg body weight. In contrast, the rats in the control group were administered citrate buffer without any additional medications. During a period of 21 days, rats adhere to a diet that is heavy in fat (HFD) and are allowed to consume water without any restrictions. Glucose levels in the blood were evaluated 72 hours after the injection of STZ. Rats were deemed to have diabetes and selected for further investigation if their glucose threshold exceeded 200 mg/dL [19, 20].

Isolated heart preparation

The SD rats were rendered unconscious by administering an intraperitoneal injection of 60 mmol/L chloral hydrate at a dosage of 0.35 g/kg. The rats were anticoagulated by administering 250 U/kg of heparin intraperitoneally. The hearts were promptly removed following the thoracic surgery and placed in a cold Krebs-Henseleit buffer (118 mM NaCl, 1.2 mM KH_2PO_4, 4.7 mM KCl, 1.2 mM $CaCl_2$, 1.2 mM $MgSO_4$, 24.9 mM $NaHCO_3$ and 11.1 mM glucose, pH 7.4). The solution was also composed of 95% oxygen and 5% carbon dioxide to prevent the hearts from contracting and to minimize oxygen usage. Without delay,

the heart was placed on Langendorff's apparatus and balanced with a gas mixture containing 95% O_2 and 5% CO_2 at a temperature of 37°C. The procedure mentioned above was completed in a time frame of 2 minutes. During initial equilibration, a pressure transducer (Statham) was connected to a water-filled latex balloon that was placed into the left ventricular cavity through the left auricle. The volume of the balloon was adjusted to maintain a stable left ventricular end-diastolic pressure of 5–12 mmHg.

Treatment groups

The above diabetic rats were further distributed in Five groups (n=6) and received the following treatment.

Group I: Sham

Group II: Vehicle + IR

Group III: PER (2.5 mg/kg) + IR

Group IV: PER (5 mg/kg) + IR

Group V: PER (10 mg/kg) + IR

Hearts in the control group were perfused for a period of 90 minutes to stabilize them. The I/R group's hearts were stabilized for a duration of 30 minutes. Following this, the hearts were treated to global ischemia (no flow) for 15 minutes, followed by reperfusion for 45 minutes. The hearts in the PER treated group were maintained at a stable state for a duration of 20 minutes using K-H buffer containing the specified dose of PER. Following this, global ischemia was induced for 10 minutes, followed by reperfusion periods of 15 minutes and 45 minutes, respectively. Left ventricular (LV) function was assessed at three time points: baseline, immediately before ischemia, and 45 minutes after reperfusion. This time frame was chosen since the maximal recovery of LV function following reperfusion occurs within 45 minutes. The sham operation group did not have I/R but was given normal perfusion at the same time

Measurement of heart hemodynamic parameters

The following functional parameters were continuously monitored with a computer-based data acquisition system (PowerLab/4S with Chart 5 software, AD Instruments, Sydney, Australia): left ventricular systolic pressure (LVSP), maximum rise/down velocity of left intraventricular pressure (dp/dt_{max} and dp/dt_{min}), and heart rate (HR). The heart effluents were collected at 1 min intervals to determine the coronary flow (CF). The recovery of LVDP, dp/dt_{max}, dp/dt_{min}, and HR were expressed as the percent of 1 minute before ischemia.

Evaluation of myocardial infarct size

Following the conclusion of the experiment, the heart was extracted, cleansed with phosphate buffered saline, subjected to freezing at a temperature of –20°C for a duration of 30 minutes, and subsequently cut into 1 mm pieces perpendicular to the long axis, starting from the apex and extending towards the base. The sections were placed in a solution containing 1% triphenyltetrazolium chloride (TTC, Biodee, Beijing, China) in a buffer with a pH of 7.4. The sections were then incubated at a temperature of 37°C for a duration of 10–15 minutes. After incubation, the sections were treated with a 10% solution of formaldehyde to fix them. Finally, the sections were captured using a digital camera. The infarct areas (white) and non-infarct areas (red) were quantified using Image-Pro Plus 7.0 software (Media Cybernetics, WY, USA). The magnitude of the myocardial infarct was quantified by calculating the ratio of the infarct area (INF) to the total area of the left ventricle (LV), and then multiplying by 100%.

Estimation of the level of various cardiac injury biomarkers

The serum samples from each group were obtained from whole blood samples by centrifugation at 2,000 × g for 10 min at 4°C, and following ELISA commercial kits were used to estimate the concentration of various cardiac injury biomarkers, such as creatine kinase (CK, cat. no. A032; Nanjing Jiancheng Bioengineering Institute, Nanjing, China), CK-MB (cat. no. H197; Nanjing Jiancheng Bioengineering Institute, Nanjing, China), troponin I (cat no. E019-1; Nanjing Jiancheng Bioengineering Institute, Nanjing, China), alanine transaminase (ALT, kit no. ab105134; Abcam, Cambridge, UK), aspartate aminotransferase (AST, kit no. ab285299; Abcam, Cambridge, UK), and lactate dehydrogenase (LDH, cat. no. A020-2; Nanjing Jiancheng Bioengineering Institute, Nanjing, China). The technique was followed exactly as the manufacturer had instructed.

Estimation of lipid profile

The serum levels of various lipid indicators were measured using the previously described approach [20]. Tests for these biomarkers included measuring levels of total cholesterol (TC, Kit No. ab65390; Abcam, Cambridge, UK), triglycerides (TG, Kit No. ab65336; Abcam, Cambridge, UK), high-density lipoprotein (HDL, ELISA Kit No. ab125961; Abcam, Cambridge, UK), low-density lipoprotein (LDL, cat. no. A113-1; Nanjing Jiancheng

Bioengineering Institute, Nanjing, China), and very low-density lipoprotein (VLDL, cat. no. H249-1; Nanjing Jiancheng Bioengineering Institute, Nanjing, China).

Estimation of the level of various oxidative stress biomarkers

Blood samples were collected from the right ventricle and centrifuged at 3,000 ×g for 10 min to isolate serum. The level of superoxide dismutases (SOD; cat. no. A001-3-2), glutathione (GSH, cat no. A005-1), and malondialdehyde (MDA, cat. no. A003-1) were measured in blood serum using the commercial kits supplied from Nanjing Jiancheng Bioengineering Institute, Nanjing, China by the manufacturer's standard procedure.

Estimation of the level of various proinflammatory cytokines

ELISA kits were used to measure pro-inflammatory cytokines, such as IL-1β (cat. no. H002), IL-6 (cat. no. H007), IL-10 (cat. no. H009), IL-17 (cat. no. H014), and TNF-α (cat. no. H052) in blood serum as obtained above, following the manufacturer's procedure (Nanjing Jiancheng Bioengineering Institute, Nanjing, China).

Detection of mRNA expression of TLR4, NF-κB, and TNF-α (RT-qPCR)

About 30 mg of rat myocardial tissue was added to extract myocardial tissue RNA using Trizol. Reverse transcription was performed using a reverse transcription kit (abm; G492) on an ABI quantstudio 5 real-time PCR system (Applied Biosystems, Germany) using SYBR® Premix-Ex TagTM (Takara, Dalian, China).

Western blot analysis

The myocardial protein extracts were put into a 10% SDS PAGE before being deposited onto a PVDF membrane. Defat milk was employed for immobilizing the surface of the membrane, and the main antibody [diluted TLR4 (Abcam, USA, ab217274; 1:1,000), NF-κB (CST, Danvers, MA, USA; 8242S; 1:1,000), TNF-α (Abcam, USA, ab6671; 1:1,000), or β-actin (Affinity, Cincinnati, OH, USA; AF7018; 1:1,000) antibodies were added at a ratio of 1:1,000] was left to incubate for an entire night at 4 °C. After washing with TBST, the resulting membrane underwent incubation for 1 hour at ambient temperature with another antibody which had been HRP-conjugated (1:10,000). Employing the ECL-chemiluminescence technique, the bands that were responsive to the antibodies were detected.

Statistical analysis

For the management of the data and statistical analysis, Graphpad Prism version 8.0.1 was used. Variance analysis (one-way ANOVA) was carried out to compare various groups. Significant results were defined as $p < 0.05$

RESULTS

Effect on the cardiac status of infarct size, and infarct volume of rats

The results are displayed in Figure 1, which illustrates the influence of PER on the cardiac state of IR rats. As the infarct volume rose in IR rats, the infarct size also increased, relative to the sham group. On the other hand, animals given PER showed a dose-dependent decrease in these infarct characteristics (Fig. 1 A and E). Additionally, following IR induction, the effect of PER was assessed on the rats' hemodynamic parameters. The PER-treated rats showed markedly better LVSP, $\pm dP/dt_{max}$, and heart rate compared to the unprotected IR hearts, as demonstrated in Figures 1 B, C, D, and F. The group administered 10 mg/kg of PER showed the greatest improvement in functional recovery during early reperfusion.

aEffect on cardiac injury biomarkers

Cardiac damage indicators including CK, CK-MB, ALT, AST, and LDH were further studied to determine the impact of PER. Figure 2 shows that compared to the control group, the untreated IR rats had significantly higher levels of the biomarkers under investigation. However, after receiving PER, the levels of these biomarkers dropped dramatically and then returned to normal in the rats.

Effect on lipid profile

The next step was to examine the lipidomic status of IR rats and see how PER affected it (Fig. 3). While HDL levels were lower in IR rats, TC, TG, LDL, and VLDL were considerably higher. Nevertheless, these lipid biomarker levels were observed to be substantially restored near normal in a dose-dependent manner upon treatment of PER to IR rats. Treatment with 10 mg/kg resulted in the greatest improvement.

Effect on oxidative stress biomarkers

Quantification of PER’s antioxidant effect was analyzed by measuring oxidative stress markers. Fig. 4 shows that levels of MDA were significantly higher whereas levels of SOD

and GSH were lower. Nevertheless, these oxidative stress markers showed a dose-dependent improvement and restoration to normalcy after PER administration.

Effect on pro-inflammatory cytokines

Figure 5 shows that the unprotected IR rats had an increase in IL-1β, IL-6, IL-17, and TNF-α and a decrease in IL-10. The rats that were given PER showed a marked reversal of the altered levels of these cytokines. The 10 mg/kg group showed the greatest improvement in reversal activity.

Effect on mRNA expression of TNF-α, TLR4, and NF-κB using RT-qPCR, and western blot analysis

We used RT-qPCR and western blot analysis to explore how PER affected the protein expression of TNF-α, TLR4, and NF-κB. Figures 6 and 7 reveal that compared to the control group, the IR group had substantially higher protein expression of the investigated indicators. Nevertheless, when PER was administered, the expression of these biomarkers decreased in a dose-dependent manner compared to the unprotected IR group. Results showed the greatest improvement in the group treated with 10 mg/kg. Thus, it was hypothesized that TLR4/NF-κB inactivation was associated with the protective impact of PER on cardiac function and myocardial IR injury, given the aforementioned data.

DISCUSSION

When it comes to global causes of death, ischemic heart disease stands at the top of the list. Myocardial ischemia happens when cardiomyocytes die due to a reduction in blood flow to the heart muscle [8, 36]. However, while reperfusion of ischemic regions does reduce mortality risk, it may potentially cause complications down the road. When diabetes and other co-morbidities are prevalent in a clinical setting, ischemic heart disease is always present as well [1]. Cardiovascular disease is two to three times more likely to occur in patients with diabetes. Diabetes mellitus is a major risk factor for myocardial infarction (MI), as shown in multiple studies [41]. Heart ischemia dysfunction, including ischemia-reperfusion (IR) injury, is also more common in diabetes patients. More than half of the deaths in both children and adults with diabetes are caused by complications related to coronary artery diseases[4]. Acute myocardial infarction and coronary bypass surgery are associated with a mortality risk that is two times higher in diabetic patients compared to non-diabetic patients [33]. Several cardiac complications can arise from diabetes mellitus,

including mitochondrial dysfunction and a diminished ability of late preconditioning to reduce infarct size [18, 29]. As a result, diabetes has become a serious concern in the field of healthcare, and treating diabetic individuals who simultaneously have ischemic heart disease is a contemporary challenge. Accumulating evidence has supported the benefit of natural products in myocardial ischemia-reperfusion injury via multiple mechanisms [40]. This provided the impetus for us to develop a model of cardiac ischemia-reperfusion injury in rats with type 2 diabetes; we found that PER reduced I/R injury by blocking the TLR4/NF-kB signaling pathway.

In the current study, we have initially documented the effect of PER on the cardiac ability of I/R rats. Various studies have documented that cardiac ability is seriously compromised after the I/R injury due to its complex etiology in which many players contribute to the final damage inflicted to the myocardium [22, 23]. Our results have demonstrated that PER in the given dose significantly improved the cardiac functionality of rats.

It is thought that Creatine Kinase and Creatine Kinase-MB are the initial indicators of cardiac damage. But CK-MB fraction, which is more myocardial specific, swiftly became the gold standard, replacing CK [9, 32]. Hence, in the present study, PER considerably decreased CK and CK (MB) levels in the diabetic rats. Acute myocardial infarction (AMI) was first diagnosed using the AST and LDH enzymes; later, the ALT would be added to the panel. Research suggests that acute myocardial infarction (AMI) can be better diagnosed with the help of AST, which is secreted into the bloodstream by necrotic cardiac myocytes. The maximal skeletal muscle, kidneys, liver, heart, lungs, and erythrocytes are among the many organs that express LDH [17]. Its five isoenzymes make it up. Even though it isn't highly specialized for the heart, the LDH1 gene is present there. The patient's LDH level is more helpful in differentiating between acute and subacute myocardial infarction in patients who reach the hospital later in the course of the ailment [2, 25]. In the present study, we have shown that the levels of these biomarkers were found significantly reduced in the PER-treated rats. In connection with the above, it was speculated that PER showed a significant reduction in the cardiac injury biomarkers due to improvement in the cardiac ability of IR rats.

Circulating lipid biomarkers including cholesterol, triglycerides, HDL, and LDL are the traditional metrics for assessing CVD risk [24, 28]. A well-controlled human plasma lipid profile can reveal important information about a person's health or illness by revealing their underlying phenotype. Alshehry et al. found [3] 32 lipids in the plasma that were strongly linked to coronary artery disease and mortality in a large population-centered investigation.

The feasibility of using a plasma lipid profile to stratify the risk of unpredictable coronary artery disease, also known as CAD, was proven by Meikle et al. [30].

Results like these are making it clear that conventional lipid indicators aren't always the best way to assess cardiovascular disease risk or the underlying pathophysiology. Therefore, a successful approach to treating cardiac ischemia-reperfusion injury is to target glucolipid metabolism. Compared to unprotected IR rats, the current study demonstrated that IR rats treated with PER had considerably lower levels of TC, TG, LDL, and VLDL, and higher levels of HDL.

Myocardial infarction is not solely caused by inadequate blood flow to the heart, according to the findings of studies that provide evidence to support this hypothesis [7]. On the contrary, it is marked by secondary pathological abnormalities that are associated with one another. These changes are characterized by the presence of oxidative stress and inflammation, which, when taken together, form a complex signaling network that, in the end, results in a chain reaction of negative effects. Both the advancement of myocardial necrosis and the remodeling of the ventricles that occurs after a myocardial infarction are significantly influenced by the inflammatory response, which plays an essential part in both of these processes because of their significant impact [15, 16, 31]. Clinical studies have shown that myocardial ischemia, which causes a disruption in the equilibrium of oxygen supply and has an effect on the redox environment, is a factor that contributes to the development of myocardial infarction. A myocardial infarction is characterized by a shortage of oxygen, which results in the death of myocardial cells and sets off an inflammatory response. Thus, the inhibition of oxidative stress following myocardial IR injury has a significant protective effect on myocardial tissues and thereby also limits the generation of pro-inflammatory cytokines [14, 42]. In our present study, we have demonstrated that PER in a dose-dependent manner inhibited oxidative stress in IR rats, which further resulted in the reduction of inflammation as evidenced by the reduction of pro-inflammatory cytokines.

NF-κB has been widely known as a key modulator of inflammatory processes. In its inactive form, it can be expressed within the cytosol either alone or in a heterodimer with it [34]. When LPS is stimulated, NF-κB is activated by the phosphorylation of IκB. Through regulation of their expression, this induction controls the production and release of an array of inflammatory cytokines, which includes TNF-α, IL-6, and IL-1β. The ability of NF-κB activation to stimulate the expression of genes that cause inflammation makes it a key player in inflammation [5, 13]. Previous studies have shown that a TLR4-dependent inflammatory pathway was used to produce cytokines that are associated with inflammation, including

TNF-α, when lipopolysaccharide (LPS) activated nuclear factor kappa B (NF-κB). By analyzing NF-κB and TLR4 levels in RT-qPCR and western blot, our research has shown that PER effectively inhibits NF-κB protein activation. In addition, it causes a reduction in TNF-α expression and production, suggesting that PER has an anti-inflammatory effect in IR rats, possibly by blocking the /TLR4/ NF-κB signaling pathways.

CONCLUSIONS

The present study documented the therapeutic potential of Perakine in myocardial ischemia injury in rats. Perkine pharmacological effectiveness is mainly observed due to excellent anti-oxidant and anti-inflammatory activity via inhibition of TLR4/ NF-κB signaling pathway. However, the small sample size and the need to assess various other physiological biomarkers, such as ROS production, myocardial membrane potential, and myocardial apoptosis, may limit the results and suggest the need for further investigation.

ARTICLE INFORMATION AND DECLARATIONS

Data availability statement

The data that support the findings of this study are available from the corresponding author upon reasonable request.

Ethics statement

The authors declare that the procedures followed were under the regulations of the relevant animal research ethics committee and with those of the Code of Ethics of the World Medical Association (Declaration of Helsinki). The study was approved by the Animal Ethical Committee of Genertec Universal Medical Group Company Limited XD Group Hospital (Approval No: GUM/2023/A-42/034).

Author contributions

L.F. conceived and designed the study. J.W. performed the experiments. L.F. and J.W. acquired and analyzed the data. J.W. drafted and critically revised the article. L.F. proofread the final manuscript.

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None.

Conflict of interest

None declared.

Supplementary material

Supplementary material is available on Journal's website. It contains:

Supplementary Table 1. Details of RT-qPCR primers.

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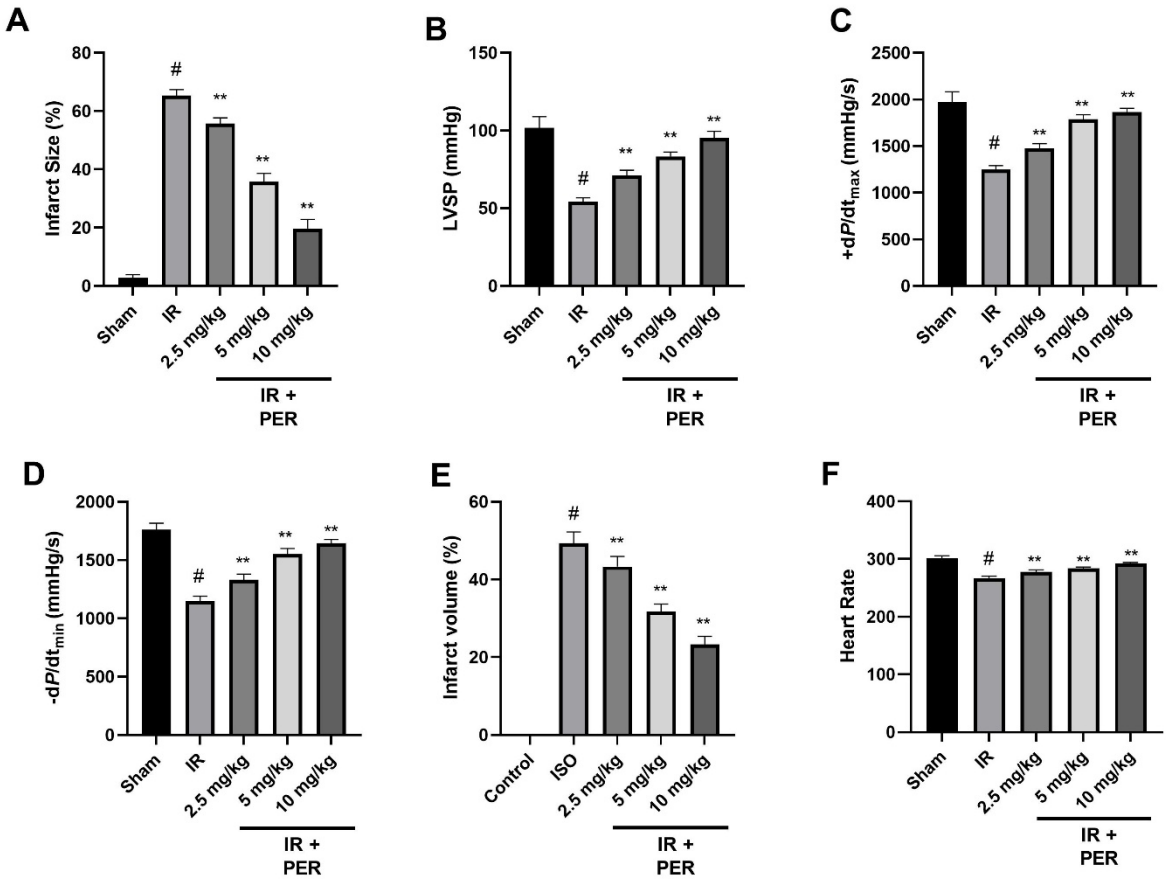

Figure 1. Effect of PER on the cardiac status of IR rats. $^{\#}P < 0.05$ *vs.* sham and $^{**}P < 0.01$ *vs.* IR group. Data are presented as means ± SEM. IR — Myocardial ischemia-reperfusion; ISO — isogenic; LVSP — left ventricular systolic pressure; PER — pharmacological effect of Perakine; SEM — scanning electron microscopy.

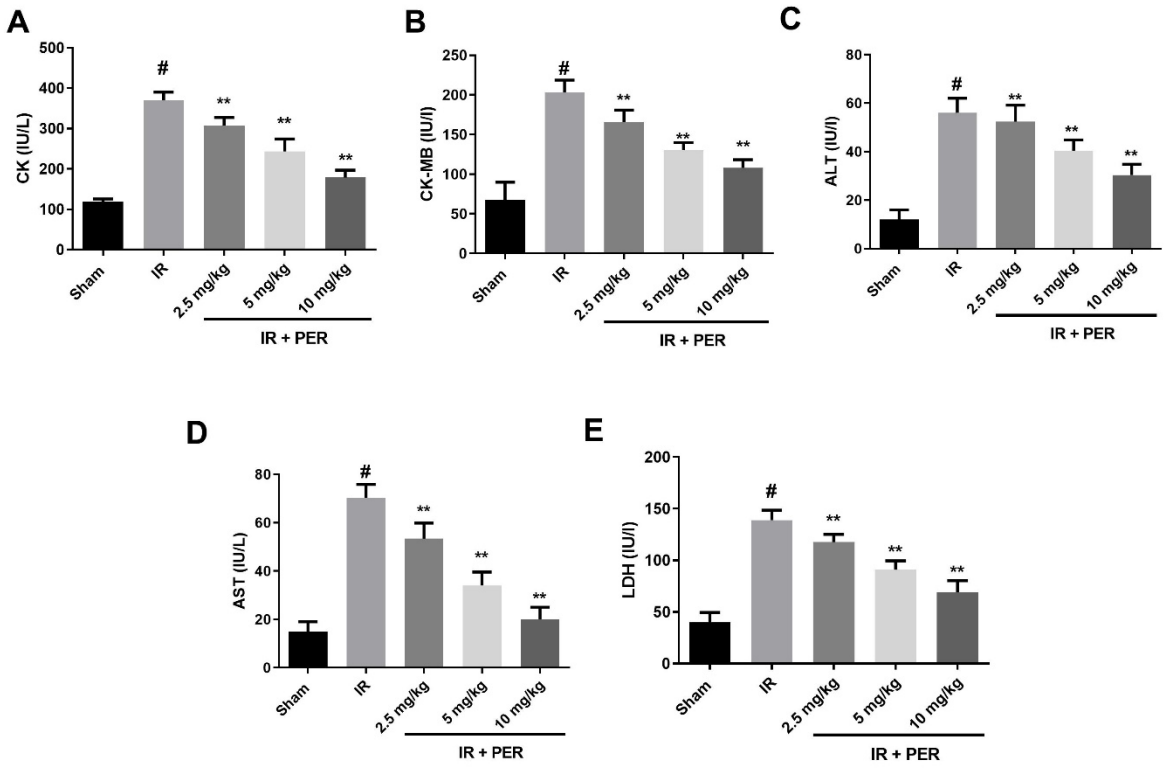

Figure 2. Effect of PER on the cardiac injury biomarkers. $^{\#}P < 0.05$ *vs.* sham and $^{**}P < 0.01$ *vs.* IR group. Data are presented as means ± SEM. ALT — alanine transaminase; AST — aspartate aminotransferase; CK — creatine kinase; IR — Myocardial ischemia-reperfusion; LDH — lactate dehydrogenase; PER — pharmacological effect of Perakine; SEM — scanning electron microscopy.

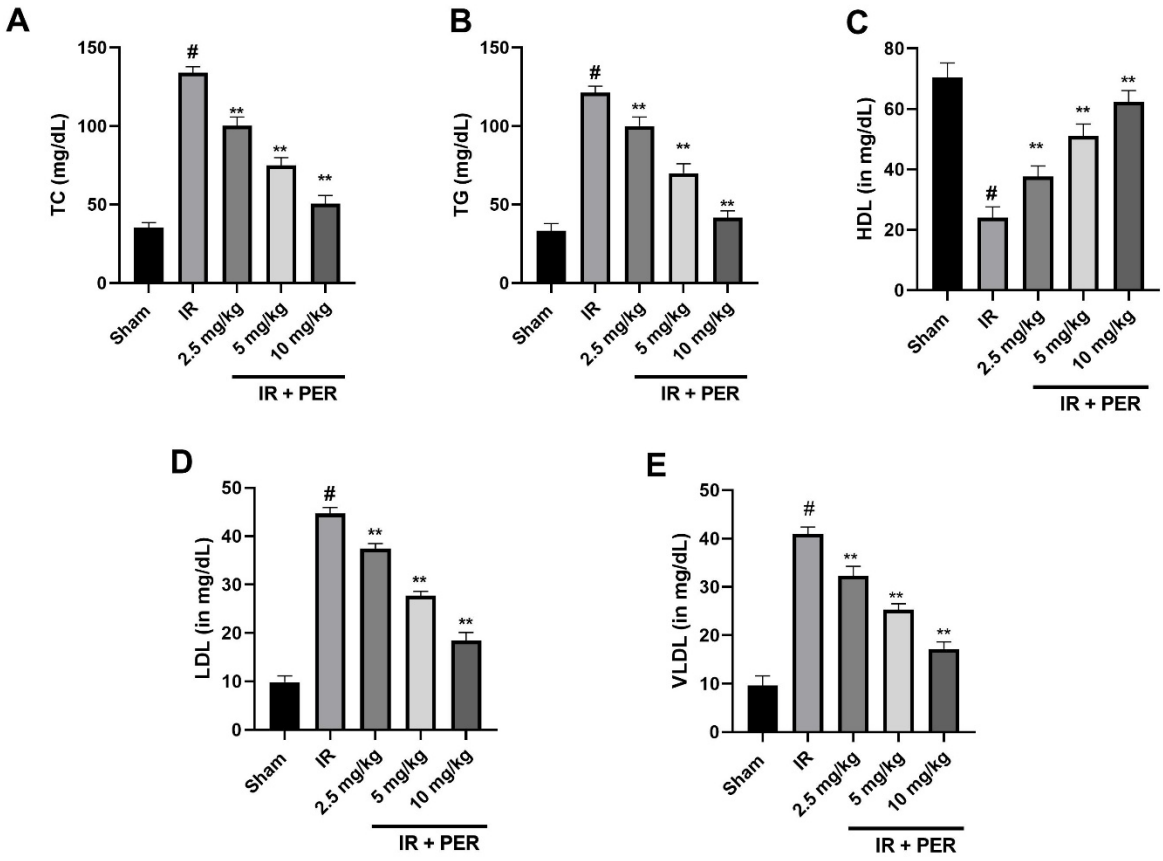

Figure 3. Effect of PER on the lipidomic of IR rats. $^{\#}P < 0.05$ *vs.* sham and $^{**}P < 0.01$ *vs.* IR group. Data are presented as means ± SEM. HDL — high-density lipoprotein; IR — Myocardial ischemia-reperfusion; LDL — low-density lipoprotein; PER — pharmacological effect of Perakine; SEM — scanning electron microscopy; TC — total cholesterol; TG — triglycerides; VLDL — very low-density lipoprotein.

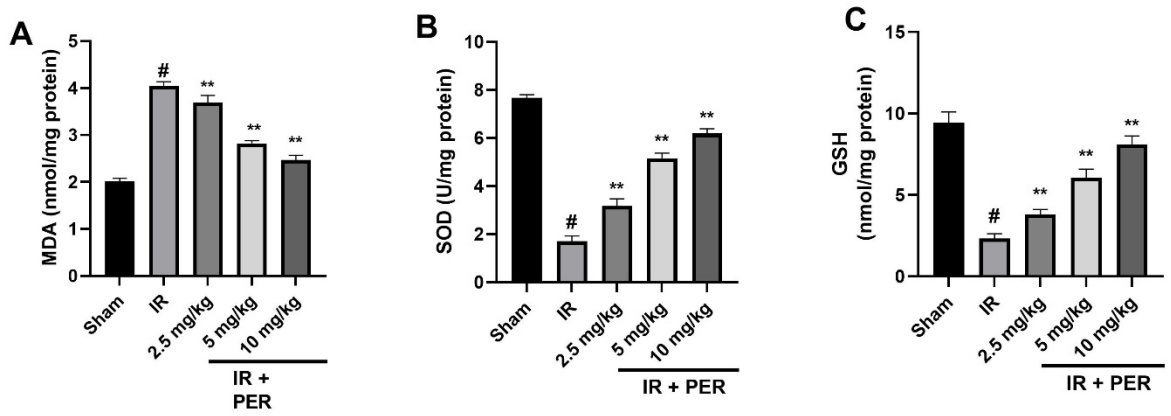

Figure 4. Effect on oxidative stress indices. $^{\#}P < 0.05$ *vs.* sham and $^{**}P < 0.01$ *vs.* IR group. Data are presented as means ± SEM. GSH — glutathione; IR — Myocardial ischemia-reperfusion; MDA — malondialdehyde; PER — pharmacological effect of Perakine; SEM — scanning electron microscopy; SOD — superoxide dismutases.

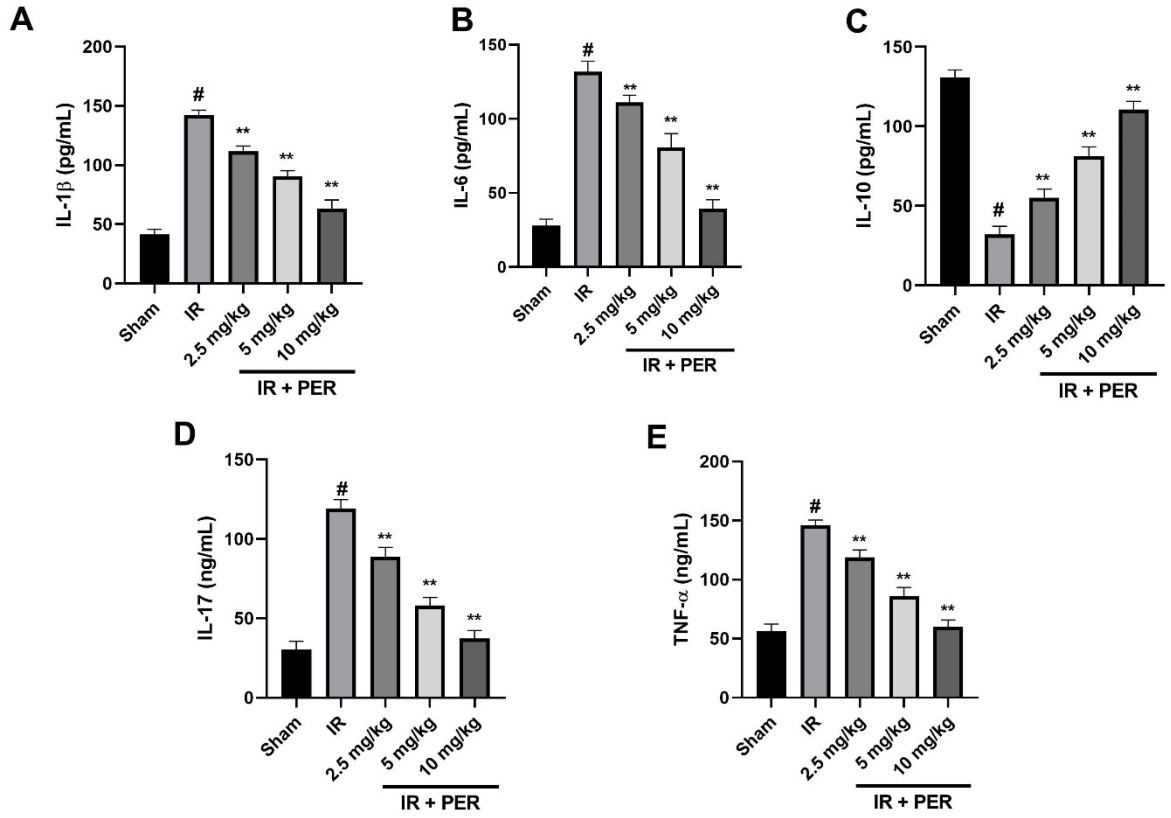

Figure 5. Effect of PER on the pro-inflammatory cytokines. #P < 0.05 *vs.* sham and **P < 0.01 *vs.* IR group. Data are presented as means ± SEM. IR — Myocardial ischemia-reperfusion; PER — pharmacological effect of Perakine; SEM — scanning electron microscopy.

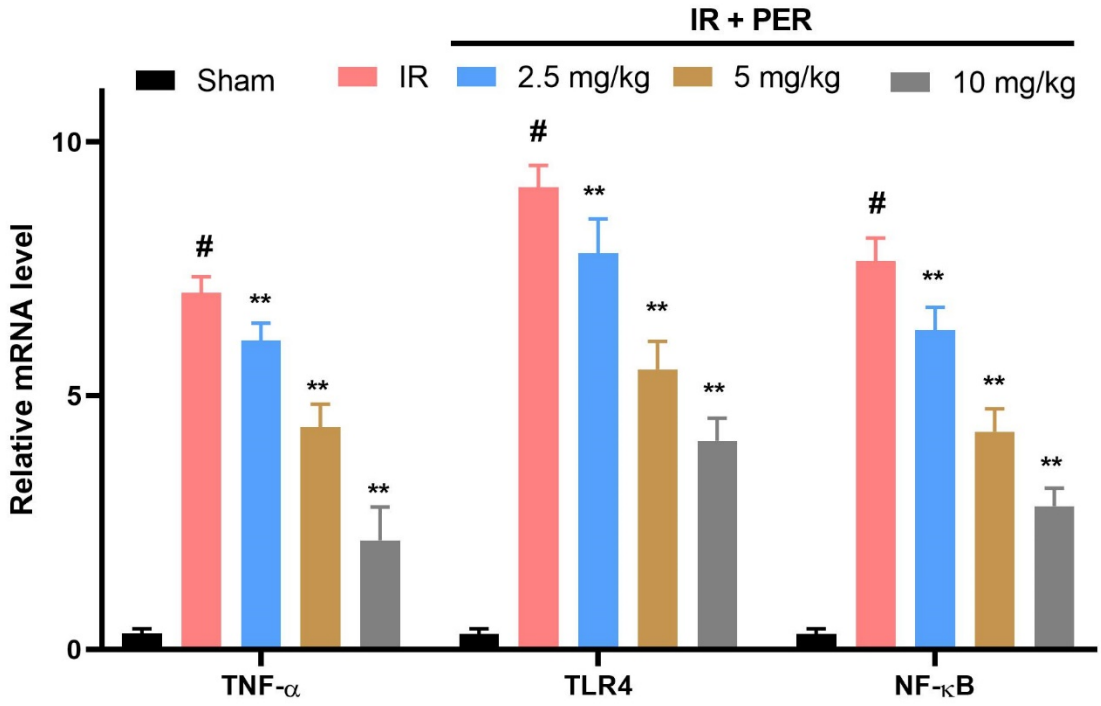

Figure 6. Effect of PER on the TNF-α, TLR4, and NF-κB using RT-qPCR. #P < 0.05 *vs.* sham and **P < 0.01 *vs.* IR group. Data are presented as means ± SEM. IR — Myocardial ischemia-reperfusion; PER — pharmacological effect of Perakine; SEM — scanning electron microscopy.

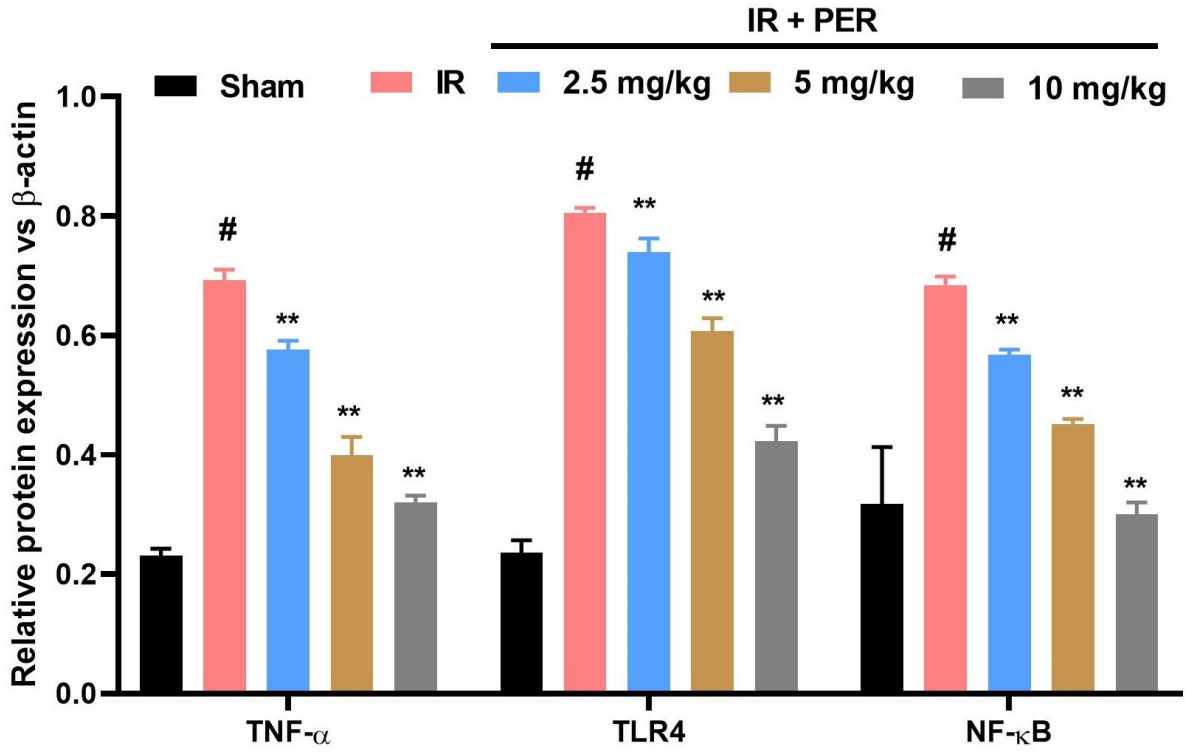

Figure 7. Effect of PER on the TNF-α, TLR4, and NF-κB using western blot analysis. $^{\#}P < 0.05$ *vs.* sham and $^{**}P < 0.01$ *vs.* IR group. Data are presented as means ± SEM. IR — Myocardial ischemia-reperfusion; PER — pharmacological effect of Perakine; SEM — scanning electron microscopy.

Supplementary Table 1. Details of RT-qPCR primers.

Primer	Sequence
TLR4 forwards	5'-ATGGCATGGCTTACACCACC-3'
TLR4 backwards	5'-GAGGCCAATTTTGTCTCCACA-3'
NF-κB forwards	5'-GCCAGACACAGATGATCGCC-3'
NF-κB backwards	5'-GTTTCGGGTAGGCACAGCAA-3'
TNF-α forwards	5'-ATGTCTCAGCCTCTTCTCATTC-3'
TNF-α backwards	5'-GCTTGTCACTCGAATTTTGAGA-3'
Actin forwards	5'-GTGCTATGTTGCTCTAGACTTCG-3'
Actin backwards	5'-ATGCCACAGGATTCCATACC-3'

NF-κB — nuclear factor kappa beta; RT-qPCR — real-time quantitative polymerase chain reaction; TLR4 — toll-like receptor 4; TNF-α — tumor necrosis factor-α.