

Metformin alleviates lung ischemia-reperfusion injury via the SIRT1 pathway following lung transplantation in diabetic rats

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Abstract. Diabetes mellitus (DM) exacerbates lung ischemia-reperfusion (IR) injury and leads to poor survival in lung transplantation recipients. Metformin protects a number of tissues from IR injury. The present study aimed to investigate the effect of metformin on diabetic lung IR injury and the potential mechanisms. Rats with type 2 DM were exposed to metformin with or without administration of EX527, an inhibitor of the silent information regulator 1 (SIRT1) pathway, following lung transplantation. Lung function, alveolar-capillary permeability, inflammatory response, oxidative stress, cell apoptosis, mitochondrial function, mitochondrial biogenesis key proteins and the SIRT1 signaling pathway were assessed. The effect of metformin on diabetic lung IR injury was evaluated by ELISA, oxidative stress assays, immunofluorescence, flow cytometry, TUNEL assay and western blotting. The results demonstrated that DM was associated with a significant increase in the IR-induced alveolar-capillary permeability, inflammatory response, oxidative stress and cell apoptosis. Furthermore, DM was associated with a significant decrease in mitochondrial function and biogenesis, SIRT1 expression and lung function. Metformin treatment markedly attenuated diabetic lung IR injury by alleviating the inflammatory response, oxidative stress and cell apoptosis, preserving mitochondrial function, and promoting mitochondrial biogenesis. However, EX527 inhibited the protective effect of metformin. In conclusion,

metformin alleviated the inflammatory response, oxidative stress and cell apoptosis, preserved mitochondrial function, and promoted mitochondrial biogenesis via the activation of the SIRT1 pathway in diabetic lung IR injury.

Introduction

Lung ischemia-reperfusion (IR) injury is a major complication following lung transplantation and remains one of the major causes for lung transplantation failure (1). The prevalence of diabetes mellitus (DM) and prediabetes in patients awaiting lung transplantation is high, reaching up to 41% (2). DM is a risk factor for 1- and 5-year mortality following lung transplantation (3). Mitochondrial function is closely related to IR injury and DM (4,5). Furthermore, in our previous study, mitochondrial dysfunction was demonstrated to be one of the key factors in diabetic lung IR injury (6). Therefore, the protection of mitochondrial function serves an important role in diabetic lung IR injury therapy.

Mitochondrial biogenesis and mitophagy are two critical factors associated with mitochondrial homeostasis (7). Our previous study indicated that there was impaired mitophagy in diabetic lung IR injury, which was associated with mitochondrial dysfunction (8). The impairment of mitochondrial biogenesis may be a major contributor to the IR injury in DM. Peroxisome-proliferator-activated receptor γ coactivator-1 α (PGC-1 α) serves as the master regulator of mitochondrial biogenesis, activating the expression of multiple genes associated with mitochondrial biogenesis. Therefore, increasing the expression of PGC-1 α can enhance mitochondrial biogenesis, thereby increasing both the quantity and quality of mitochondria. Increasing mitochondrial biogenesis elevated ATP production, inhibited mitochondrial apoptosis and preserved mitochondrial function in diabetic cardiac IR injury (9). However, whether mitochondrial biogenesis is reduced in diabetic lung IR injury remains unclear.

Metformin, an anti-DM drug, exerts protective effects against IR injury in multiple organs, such as the ovaries and retina, through several mechanisms (10,11), and preserving mitochondrial function is one of the primary mechanisms (12,13). In a previous study, metformin maintained

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mitochondrial homeostasis by promoting mitophagy, inhibiting apoptosis and alleviating cerebral IR injury aggravated by hyperglycemia (14). In addition, metformin has been reported to stimulate mitochondrial biogenesis and mitigate high glucose-induced oxidative stress in cardiomyocytes (15). Metformin also mediates partial neuro-protection effects by enhancing mitochondrial biogenesis in diabetic mice (16). Furthermore, mitophagy and mitochondrial biogenesis are decreased in patients with type 2 DM and metformin reverses these effects (17). Metformin also increases placental mitochondrial biogenesis and inhibits the aberrant epigenetic alterations occurring in gestational diabetes, conferring protective effects on the offspring in humans and mice (18). Nevertheless, to the best of our knowledge, the protective effect of metformin on diabetic lung IR injury by increasing mitochondrial biogenesis has not been reported.

Therefore, the present study aimed to investigate the effect of metformin on diabetic lung IR injury and the potential mechanisms by which metformin increases mitochondrial biogenesis.

Materials and methods

Animals. A total of 82 adult male Sprague-Dawley rats, 8-9 weeks old and weighing 200-250 g, were purchased from the Animal Center of the Second Affiliated Hospital of Harbin Medical University (Harbin, China). All experimental rats were housed in a clean feeding environment with a 12 h light/dark cycle at a temperature of $24\pm 2^{\circ}\text{C}$ with a humidity of $60\pm 10\%$. The rats were fed with a fresh high-fat diet or standard laboratory chow and provided with clean water *ad libitum*. The animal health and behavior were monitored once a day. The animal experiment procedures were approved by the Institutional Animal Care and Use Committee of Second Affiliated Hospital of Harbin Medical University (approval no. SYDW2021-088; Harbin, China).

Type 2 diabetes rat model. The DM rat model was established by the administration of a high-fat diet (2.5% cholesterol, 5% sesame oil, 15% lard, 20% sucrose and 57.5% normal chow) for 6 weeks followed by intraperitoneal injection of streptozotocin (35 mg/kg; MilliporeSigma) dissolved in citric acid sodium citrate buffer (pH 4.5). After 72 h, blood samples were obtained via tail vein puncture, and blood glucose levels were measured using a glucometer. Based on previous literature references, rats with fasting blood glucose ≥ 11.1 mmol/l were considered to be the type 2 diabetic rats (8,19). The rats fed standard laboratory chow were used as nondiabetic controls.

Lung transplantation. Lung transplantation was performed as previously described (20). The donor rats were anesthetized with 8% sevoflurane, intubated and then mechanically ventilated (Model 683; Harvard Apparatus) with a tidal volume of 10 ml/kg, a rate of 40-60 breaths/min and a positive end-expiratory pressure of 2 cm H_2O . After intravenous injection of heparin (500 U/kg), median sternotomy was performed. The lungs were flushed through the main pulmonary artery with 20 ml saline at 4°C with a constant pressure of 20 cm H_2O . The heart and lungs were harvested and the left hilum

was dissected, and cuffs were fixed in the artery, vein and bronchus. The donor lungs were preserved in saline at 4°C for 60 min.

The recipient rats were anesthetized and ventilated in the same way as the donor rats. Left thoracotomy at the fifth intercostal space was performed and the left hilum was exposed. The left pulmonary artery, bronchus and vein of recipient rats were anastomosed with a donor lung using the cuff technique. The chest was sutured closed. After spontaneous respiration was restored, the recipient rats were extubated. Each recipient rat was housed individually in a clean cage. The animal health and behavior were monitored at 1, 3, 6, 12 and 24 h after surgery. The recipient rats received 0.125% ropivacaine by local infiltration anesthesia for analgesia every 12 h. At 24 h after reperfusion, the recipient rats were anesthetized with 8% sevoflurane by inhalation and then sacrificed by exsanguination under deep anesthesia. The peripheral blood samples and lung grafts were collected. Rat death was confirmed as follows: i) Cardiac arrest and absence of spontaneous respiration; and ii) no nervous reflex.

Experimental groups. The rats were randomly divided into six groups ($n=8$) as follows: Control + sham group (Con + Sham), control + IR group (Con + IR), DM + sham group (DM + Sham), DM + IR group (DM + IR), DM + IR + metformin group (DM + IR + M) and DM + IR + metformin + EX527 group (DM + IR + M + E). Rats in the sham group were only subjected to left thoracotomy without transplantation. In all of the IR groups, the donor rats were nondiabetic rats, and recipient rats were diabetic rats except in the Con + IR group. Metformin (Selleck Chemicals) was injected intravenously at a dose of 200 mg/kg immediately after reperfusion. EX527 (Selleck Chemicals) was intraperitoneally injected at a dose of 5 mg/kg/day for 3 days before surgery and once 20 min before reperfusion. The dose of metformin and EX527 was selected on the basis of our previous studies (8,20).

Arterial blood gas analysis. At 24 h following reperfusion, an arterial blood sample was obtained through the femoral artery from the recipients for blood gas analysis (Rapid Lab 348; Bayer). The ratio of the partial pressure of O_2 to the fraction of inspired O_2 ($\text{PaO}_2/\text{FiO}_2$) was calculated. The oxygenation index is a key indicator of pulmonary function.

Histopathological analysis. The middle segment of the lung graft was fixed in 4% paraformaldehyde at 4°C for 72 h and immersed in 70, 80, 90 and 95% ethanol, 100% ethanol I and 100% ethanol II solutions for dehydration, each for 30 min. Next, lung tissue was transferred into xylene I and xylene II solutions for transparency treatment, each for 90 min, placed into a pre-warmed embedding cassette and embedded in paraffin. Tissue sections (4 μm thickness) were cut and stained with hematoxylin and eosin for 30 min at room temperature. The degree of lung injury was evaluated by light microscopy (Nikon Corporation) with a histologic study score using the following criteria: i) Neutrophil infiltration; ii) airway epithelial cell damage; iii) interstitial edema; iv) hyaline membrane formation; and v) hemorrhage. Each criterion was scored on a semi-quantitative scale of 0-4 as follows: Normal, 0; minimal change, 1; mild change, 2; moderate change, 3; and severe

change, 4 (21). Therefore, each field of view was given five scores, and the sum of these scores constituted the final lung injury score.

Lung wet-to-dry (W/D) weight ratio. The upper segment of the lung graft was used to calculate the lung W/D weight ratio to evaluate lung edema. The wet weight was measured immediately after harvest and then the specimen was placed in an 80°C oven for 72 h to measure the dry weight. The W/D weight ratio was calculated.

Total protein concentration in bronchoalveolar lavage fluid (BALF). Bronchoalveolar lavage of the left lung was performed after the right hilum was clamped. The left lung was slowly instilled three times with 2 ml cold sterile saline, and the returned fluid was centrifuged at 206 x g for 10 min at 4°C. The total protein concentrations in BALF were measured using a BCA protein assay kit (Beyotime Institute of Biotechnology) according to the manufacturer's instructions.

Immunofluorescence analysis. The lung graft tissue was fixed in 4% paraformaldehyde at 4°C for 72 h and embedded in paraffin and processed into 4- μ m thick paraffin sections. The sections were sequentially immersed in xylene I and xylene II for 15 min each to remove paraffin and then placed in 100, 95, 90, 80 and 70% ethanol, and distilled water, each for 5 min. The sections were immersed in citrate buffer (pH 6.0) and microwaved at high power until boiling, then cooled to room temperature. Subsequently, the sections were washed three times with PBS, 5 min each and incubated with 10% goat serum (cat. no. C0265; Beyotime Institute of Biotechnology) for 30 min at 37°C to block non-specific binding. Subsequently, the sections were incubated with rabbit anti-vascular endothelial (VE)-cadherin polyclonal antibody (1:80; cat. no. 120264; Absin Bioscience Inc.) at 4°C overnight and then incubated with Alexa Fluor® 594-conjugated goat anti-rabbit IgG fluorescent secondary antibodies (1:50; cat. no. ZF-0516; Zhongshan Golden Bridge Biotechnology Co., Ltd.) for 30 min at 37°C. The nuclei were counterstained with DAPI at room temperature for 5 min. Image assessment was performed using a fluorescence microscope (IX71; Olympus Corporation). The expression levels of VE-cadherin were determined using ImageJ version 1.61 software (National Institutes of Health).

ELISA. The levels of IL-1 β , IL-6 and TNF- α in the serum were measured with ELISA kits, including the rat IL-1 β ELISA research kit (cat. no. JM-01454R1; Jiangsu Jingmei Biotechnology Co., Ltd.), the rat IL-6 ELISA research kit (cat. no. JM-01597R1; Jiangsu Jingmei Biotechnology Co., Ltd.), and the rat TNF- α ELISA research kit (cat. no. JM-01587R1; Jiangsu Jingmei Biotechnology Co., Ltd.), according to the manufacturer's instructions.

Oxidative stress assay. The lower part of the lung graft was homogenized for myeloperoxidase (MPO) activity detection using a commercially available assay kit (cat. no. A044-1-1; Nanjing Jiancheng Bioengineering Institute). In the lung homogenate supernatants from the lung graft,

malondialdehyde (MDA; cat. no. A003-1-2; Nanjing Jiancheng Bioengineering Institute), glutathione (GSH) activity (cat. no. A006-2-1; Nanjing Jiancheng Bioengineering Institute) and glutathione peroxidase (GSH-px) activity (cat. no. A005-1; Nanjing Jiancheng Bioengineering Institute) kits were used to measure the MDA level, GSH activity and GSH-px activity in transplanted lung tissues. All experiments were performed according to the manufacturer's instructions.

Apoptosis assay. A TUNEL apoptosis assay kit (Beyotime Institute of Biotechnology) was used to detect apoptotic cells in transplanted lung tissues according to the manufacturer's instructions. Briefly, the lung graft was fixed in 4% paraformaldehyde at 4°C for 72 h and embedded in paraffin. The lung tissue sections were prepared and incubated with protease K at 37°C for 30 min. Subsequently, the sections were rinsed with PBS and incubated with detection solution at 37°C for 60 min in the dark. Nuclei were counterstained with DAPI (2 μ g/ml) for 10 min at room temperature. After staining, the sections were mounted using an antifade mounting medium (cat. no. P0126; Beyotime Institute of Biotechnology). Four random fields of view per section were selected for blind analysis using a fluorescence microscope (IX71; Olympus Corporation). The apoptosis index was calculated as the ratio of the number of apoptotic nuclei to the total number of nuclei counted.

ATP content. ATP content in transplanted lung tissues was measured using the enhanced ATP assay kit (Beyotime Institute of Biotechnology) according to the manufacturer's instructions. Briefly, ~20 mg lung tissues were homogenized in 200 μ l lysates and centrifuged at 12,000 x g for 5 min at 4°C. Subsequently, the supernatant was added to 100 μ l ATP assay buffer and the ATP concentrations were calculated from the standard curve data.

Mitochondrial membrane potential. The mitochondrial membrane potential was determined with JC-1 using the enhanced mitochondrial membrane potential assay kit (Beyotime Institute of Biotechnology) according to the manufacturer's instructions. Briefly, single-cell suspensions were prepared from the lung graft tissue, as previously described (22). Subsequently, the suspensions were added to JC-1 staining working solution and incubated at 37°C for 20 min and then centrifuged at 600 x g for 5 min at 4°C to obtain the cells. The cells were resuspended in JC-1 staining buffer solution. The aggregated JC-1 showed red fluorescence, while monomeric JC-1 showed green fluorescence. The fluorescence was analyzed using CXP analysis 2.0 software (Beckman Coulter, Inc.) provided with the Cytomics FC500 flow cytometer (Beckman Coulter, Inc.). The mitochondrial membrane potential was expressed as the ratio of aggregated JC-1 to monomeric JC-1.

Transmission electron microscopy. Lung graft tissue (~1 mm³) was fixed with 2.5% glutaraldehyde at 4°C overnight and embedded in epoxy resin at room temperature overnight. Ultrathin tissue sections (70 nm thickness) were cut and stained with uranium and lead at room temperature for 15 min, and observed under a transmission electron microscope (Hitachi

High-Technologies Corporation). ImageJ version 1.61 software (National Institutes of Health) was used to identify and mark normal and damaged mitochondria.

Western blotting. Lung graft tissues were homogenized in RIPA lysis buffer (cat. no. P0013C; Beyotime Institute of Biotechnology) and the protein concentrations of samples were determined using an enhanced BCA protein assay kit (Beyotime Institute of Biotechnology). Protein samples (30 μ g/lane) were separated by SDS-PAGE using a 4% stacking gel and 10% separating gel, and subsequently transferred to PVDF membranes. After being blocked with 5% fat-free milk for 2 h at room temperature, membranes were incubated with rabbit polyclonal anti-VE-cadherin (1:1,000; cat. no. DF7514; Affinity Biosciences), rabbit polyclonal anti-Bax (1:1,000; cat. no. AF0120; Affinity Biosciences), mouse monoclonal anti-Bcl-2 (1:1,000; cat. no. BF9103; Affinity Biosciences), rabbit polyclonal anti-caspase-3 (1:1,000; cat. no. AF6311; Affinity Biosciences), rabbit polyclonal anti-cleaved caspase-3 (1:1,000; cat. no. AF7022; Affinity Biosciences), rabbit polyclonal anti-silent information regulator 1 (SIRT1; 1:1,000; cat. no. DF6033; Affinity Biosciences), rabbit polyclonal anti-peroxisome-proliferator-activated receptor γ coactivator-1 α (PGC-1 α ; 1:1,000; cat. no. AF5395; Affinity Biosciences), rabbit polyclonal anti-nuclear respiratory factor 1 (NRF-1; 1:1,000; cat. no. AF5298; Affinity Biosciences), rabbit polyclonal anti-mitochondrial transcription factor A (TFAM; 1:500; cat. no. AF0531; Affinity Biosciences) and rabbit polyclonal anti- β -actin (1:5,000; cat. no. AF7018; Affinity Biosciences) antibodies at 4°C overnight. Subsequently, the membranes were incubated with HRP-labeled goat anti-rabbit IgG secondary antibody (1:1,000; cat. no. A0208; Beyotime Institute of Biotechnology) or HRP-labeled goat anti-mouse IgG secondary antibody (1:1,000; cat. no. A0216; Beyotime Institute of Biotechnology) at room temperature for 2 h. The blots were visualized using an enhanced chemiluminescence reagent (Cytiva). Protein band intensity was semi-quantified with ImageJ version 1.61 software (National Institutes of Health) and normalized to β -actin.

Statistical analysis. GraphPad Prism 9.0 (Dotmatics) was used for statistical analyses. Data are presented as the mean $\pm$ standard deviation. All experiments were repeated at least three times. Analysis of variance among groups was performed by one-way analysis of variance, followed by the Tukey test for multiple comparisons. $P < 0.05$ was considered to indicate a statistically significant difference.

Results

General data. A total of 8 rats were included in each sham group and 8 pairs of rats were included in each lung transplant group. In the Con + IR group, 1 pair of rats was excluded due to death of the rat resulting from failure of pulmonary vein anastomosis, with 8 pairs left. Therefore, 82 rats were used and sacrificed in the present study. The total duration of the surgical experiment (from the beginning of anesthesia in the donor rats to the end of anesthesia in the recipient rats) was 3 h. All rats were sacrificed by exsanguination under general anesthesia in the present study.

Metformin alleviates diabetic lung IR injury via the SIRT1 pathway. The arterial blood gas analysis showed that the oxygenation index (PaO₂/FiO₂) in the Con + Sham group was significantly higher compared with that in the DM + Sham group ($P < 0.05$). The index in the Con + IR group was significantly higher compared with that in the DM + IR group ($P < 0.05$). The index in the DM + IR + M group was significantly higher compared with that in the DM + IR group ($P < 0.05$). The index in the DM + IR + M + E group was significantly lower compared with that in the DM + IR + M group ($P < 0.05$; Fig. 1A).

Histopathological examination indicated that the left lung tissues of the Con + Sham group were almost normal. A few inflammatory cells and thickened basal membranes were observed in the DM + Sham group. More neutrophil infiltration, interstitial edema and hemorrhage were present in the Con + IR group. These histological alterations were more notable in the DM + IR group, with significantly higher lung injury scores ($P < 0.05$, compared with the Con + IR group). These changes were ameliorated in the DM + IR + M group, with significantly lower lung injury scores ($P < 0.05$, compared with the DM + IR group). This improvement was reduced in the DM + IR + M + E group ($P < 0.05$, compared with the DM + IR + M group; Fig. 1B and C).

Western blotting revealed that the SIRT1 levels exhibited similar trends as the oxygenation index (Fig. 1D and E).

Metformin improves diabetic lung IR-induced alveolar-capillary permeability via the SIRT1 pathway. The W/D weight ratio, an indicator of lung edema, was significantly lower in the Con + Sham group compared with the DM + Sham group ($P < 0.05$). The ratio in the Con + IR group was significantly lower compared with that in the DM + IR group ($P < 0.05$). The ratio in the DM + IR + M group was significantly lower compared with that in the DM + IR group ($P < 0.05$). The ratio in the DM + IR + M + E group was significantly higher compared with that in the DM + IR + M group ($P < 0.05$; Fig. 2A). Furthermore, a similar pattern of the total protein concentrations in BALF was detected in these groups (Fig. 2B). Immunofluorescence and western blot analyses indicated that VE-cadherin levels exhibited an opposite trend compared with the W/D weight ratio (Fig. 2C-F).

Metformin ameliorates diabetic lung IR-induced inflammatory responses and oxidative stress via the SIRT1 pathway. The levels of IL-1 β , IL-6 and TNF- α in the serum in the Con + Sham group were significantly lower compared with those in the DM + Sham group ($P < 0.05$). These inflammatory factor levels in the Con + IR group were significantly lower compared with those in the DM + IR group ($P < 0.05$). These inflammatory factor levels in the DM + IR + M group were significantly lower compared with those in the DM + IR group ($P < 0.05$). These inflammatory factor levels in the DM + IR + M + E group were significantly higher compared with those in the DM + IR + M group ($P < 0.05$; Fig. 3A-C). In addition, MPO activity and MDA levels in lung grafts exhibited the same changes as the inflammatory factor levels (Fig. 3D and E). GSH activity and GSH-px activity exhibited opposite changes compared with the inflammatory factor levels (Fig. 3F and G).

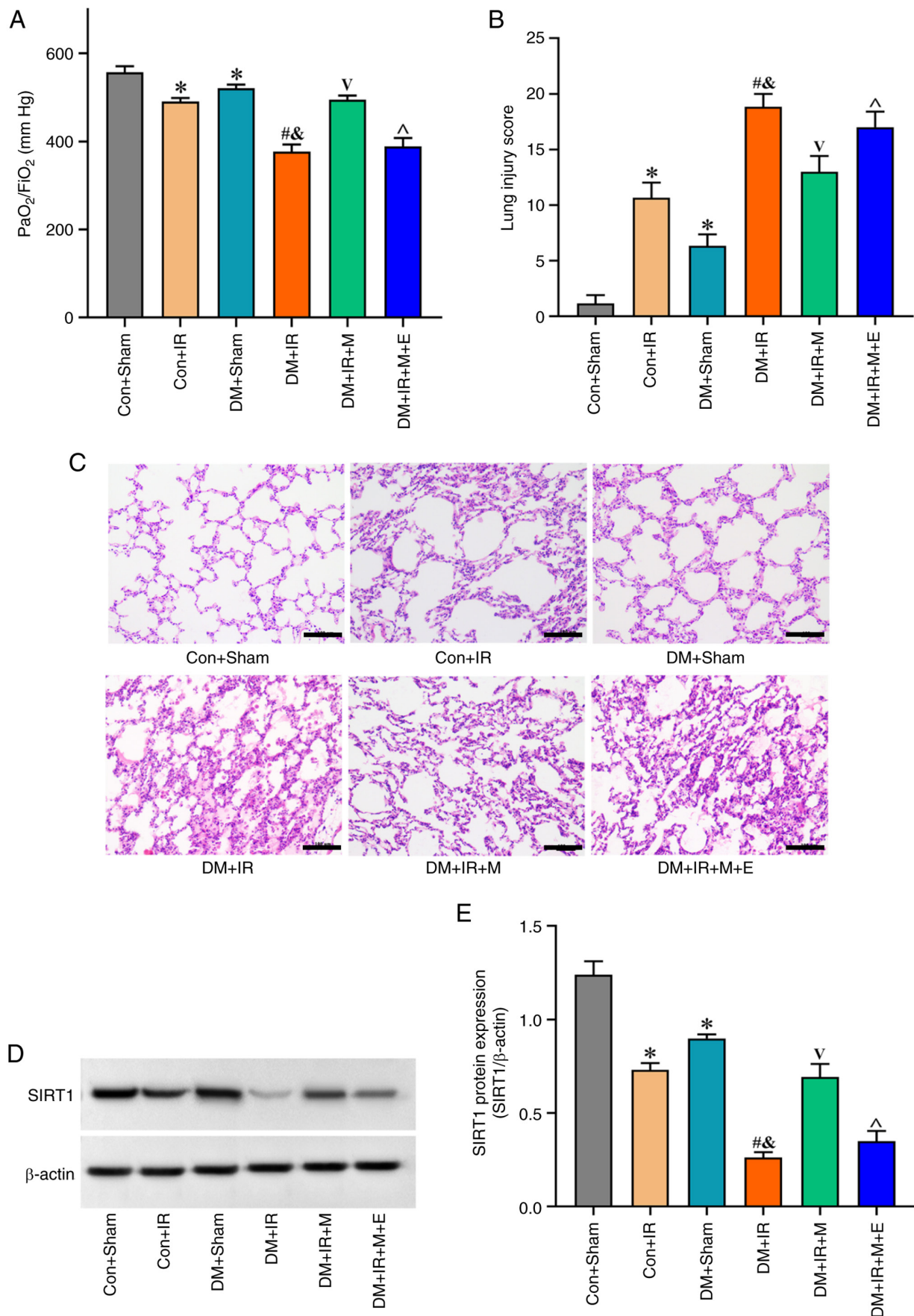


Figure 1. Metformin alleviates diabetic lung IR injury via the SIRT1 pathway. (A) PaO₂/FiO₂ ratio (n=8). (B) Lung injury score (n=6). (C) Representative hematoxylin and eosin staining images (magnification, x200; scale bar, 100 μm). (D) Representative western blot image of SIRT1. (E) SIRT1 expression (n=3). *P<0.05 vs. Con + Sham group; #P<0.05 vs. Con + IR group; &P<0.05 vs. DM + Sham group; ^vP<0.05 vs. DM + IR group; [△]P<0.05 vs. DM + IR + M group. IR, ischemia-reperfusion; SIRT1, silent information regulator 1; PaO₂/FiO₂, ratio of the partial pressure of O₂ to the fraction of inspired O₂; Con, control; DM, diabetes mellitus; M, metformin; E, EX527.

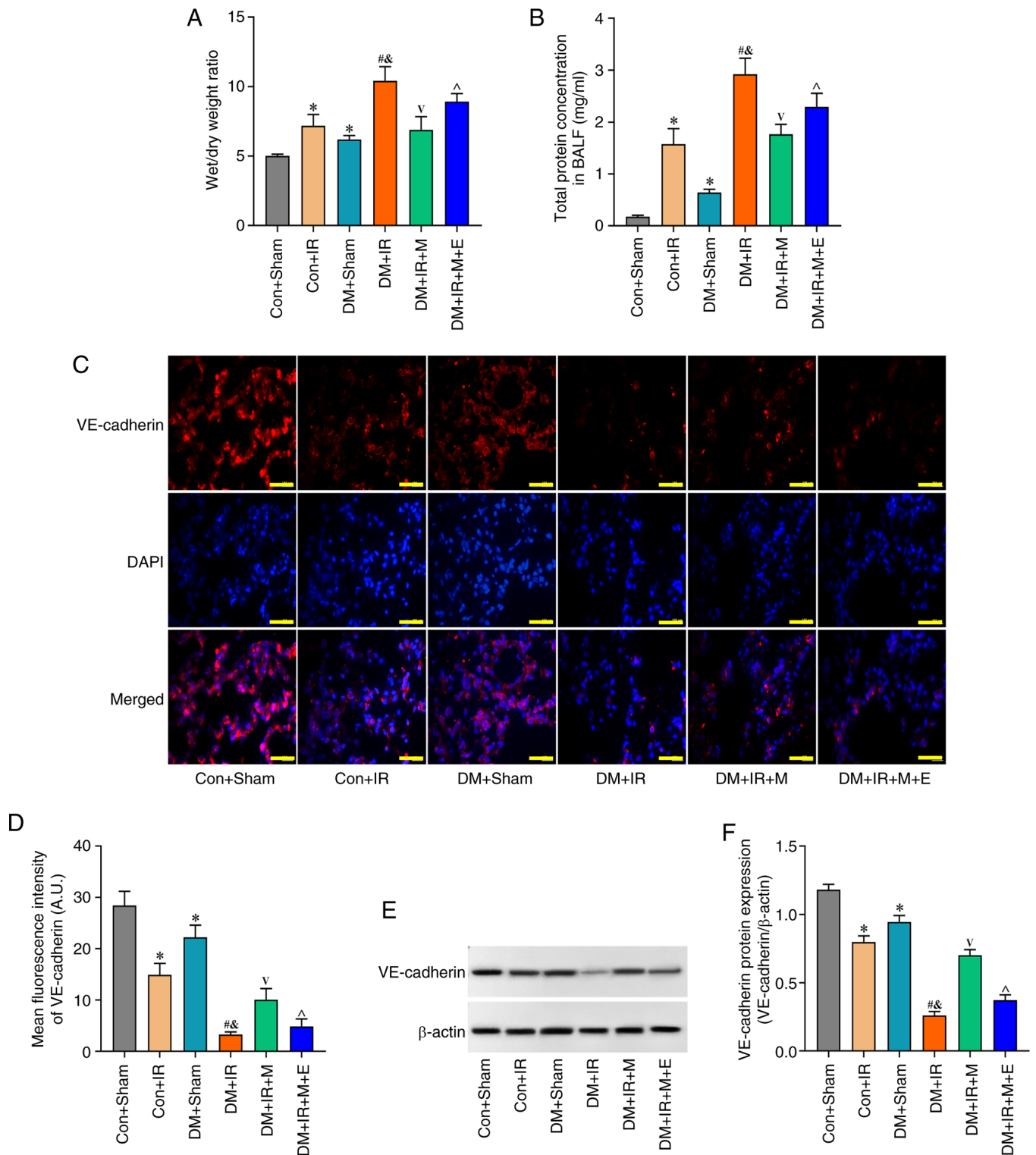


Figure 2. Metformin improves diabetic lung IR-induced alveolar-capillary permeability via the silent information regulator 1 pathway. (A) Wet/dry ratio (n=8). (B) Total protein concentration in BALF (n=5). (C) Representative immunofluorescence staining images of VE-cadherin (magnification, x400; scale bar, 100 μm). (D) VE-cadherin mean fluorescence intensity (n=4). (E) Representative western blotting image of VE-cadherin. (F) VE-cadherin expression (n=3). *P<0.05 vs. Con + Sham group; [#]P<0.05 vs. Con + IR group; [&]P<0.05 vs. DM + Sham group; ^γP<0.05 vs. DM + IR group; ^ΔP<0.05 vs. DM + IR + M group. IR, ischemia-reperfusion; BALF, bronchoalveolar lavage fluid; VE-cadherin, vascular endothelial cadherin; Con, control; DM, diabetes mellitus; M, metformin; E, EX527; A.U., arbitrary units.

Metformin decreases diabetic lung IR-induced cell apoptosis via the SIRT1 pathway. The apoptotic index in the Con + Sham group was significantly lower compared with that in the DM + Sham group (P<0.05). The index was significantly

lower in the Con + IR group compared with the DM + IR group (P<0.05). The index was significantly lower in the DM + IR + M group compared with the DM + IR group (P<0.05). The index in the DM + IR + M + E group was

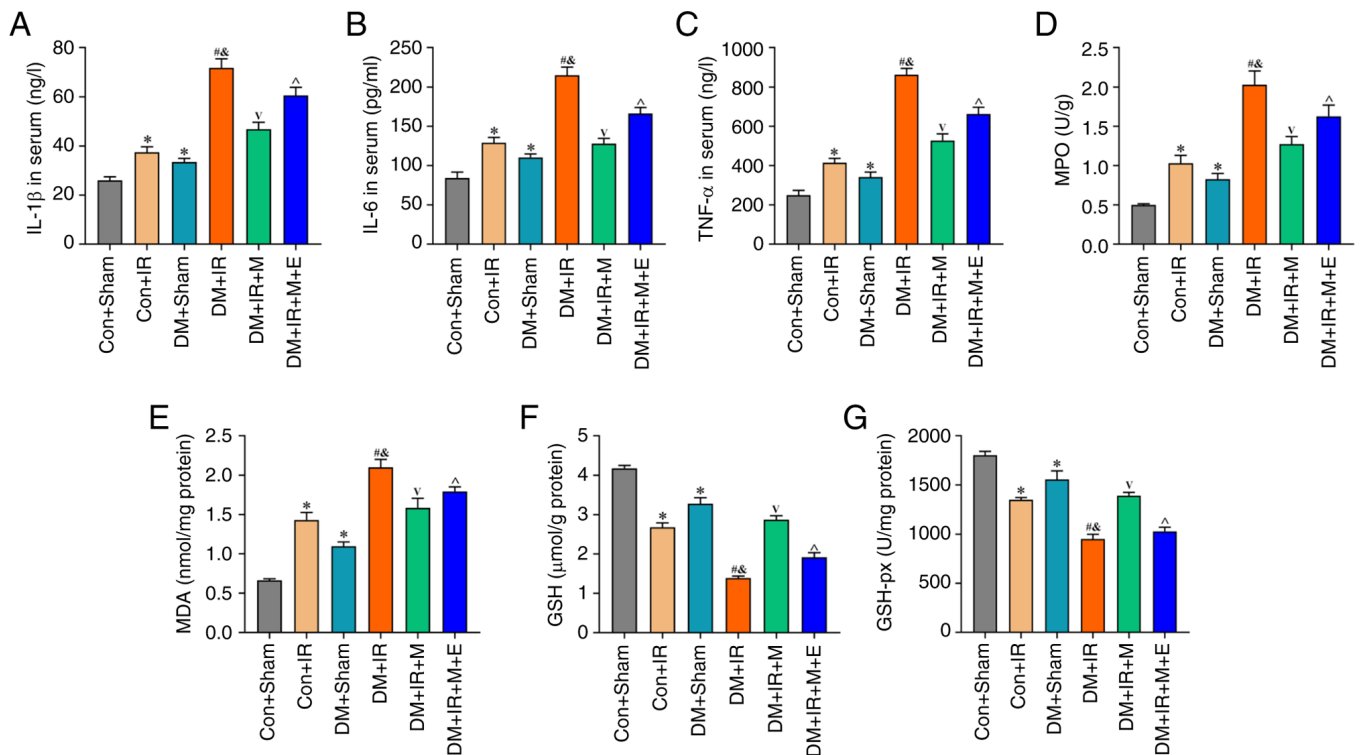


Figure 3. Metformin ameliorates diabetic lung IR-induced inflammatory responses and oxidative stress via the silent information regulator 1 pathway. (A) IL-1 β concentration in the serum (n=8). (B) IL-6 concentration in the serum (n=8). (C) TNF- α concentration in the serum (n=8). (D) MPO activity (n=8). (E) MDA level (n=8). (F) GSH activity (n=8). (G) GSH-px activity (n=8). *P<0.05 vs. Con + Sham group; #P<0.05 vs. Con + IR group; &P<0.05 vs. DM + Sham group; ^P<0.05 vs. DM + IR group; vP<0.05 vs. DM + IR + M group. IR, ischemia-reperfusion; MPO, myeloperoxidase; MDA, malondialdehyde; GSH, glutathione; GSH-px, glutathione peroxidase; Con, control; DM, diabetes mellitus; M, metformin; E, EX527.

significantly higher compared with that in the DM + IR + M group (P<0.05; Fig. 4A and B). Western blot analyses showed that the ratio of cleaved caspase-3/caspase-3 and Bax levels showed similar trends as the apoptotic index (Fig. 4C-F), and the levels of the anti-apoptotic factor Bcl-2 exhibited opposite trends compared with the apoptotic index (Fig. 4G and H).

Metformin reduces diabetic lung IR-induced mitochondrial dysfunction via the SIRT1 pathway. The ATP level in the Con + Sham group was significantly higher compared with that in the DM + Sham group (P<0.05). The ATP level was significantly higher in the Con + IR group compared with the DM + IR group (P<0.05). The ATP level was significantly higher in the DM + IR + M group compared with the DM + IR group (P<0.05). The ATP level in the DM + IR + M + E group was significantly lower compared with that in the DM + IR + M group (P<0.05) (Fig. 5A). The mitochondrial membrane potential exhibited the same changes as ATP levels (Fig. 5B and C).

At the ultrastructural level, mitochondrial morphology was demonstrated to be almost normal with an intact membrane and clearly discernable cristae in the Con + Sham group; however, several mitochondria displayed a swollen appearance with loss of discernable cristae in the DM + Sham group, and more changes were observed in the Con + IR group, including mitochondrial swelling, membrane rupture and cristae loss. These morphological alterations were most notable in the DM + IR group; however, these mitochondrial injury changes

were mitigated in the DM + IR + M group, whereas this improvement was decreased in the DM + IR + M + E group (Fig. 5D).

Metformin promotes mitochondrial biogenesis via the SIRT1 pathway in the diabetic rat lung subjected to IR injury. Western blotting revealed that the PGC-1 α level in the Con + Sham group was significantly higher compared with that in the DM + Sham group (P<0.05). The PGC-1 α level was significantly higher in the Con + IR group compared with the DM + IR group (P<0.05). The PGC-1 α level was significantly higher in the DM + IR + M group compared with the DM + IR group (P<0.05). The PGC-1 α level in the DM + IR + M + E group was significantly lower compared with that in the DM + IR + M group (P<0.05) (Fig. 6A and B). The NRF-1 and TFAM levels exhibited similar trends as the PGC-1 α levels (Fig. 6C-F).

Discussion

The major findings of the present study were as follows: i) DM exaggerated lung IR injury, which was manifested by increases in inflammatory reactions, oxidative stress, apoptosis and decreased mitochondrial biogenesis, accompanied by SIRT1 downregulation; and ii) metformin alleviated inflammatory reactions, oxidative stress and apoptosis, promoted mitochondrial biogenesis via the SIRT1 pathway, and protected lung function in diabetic lung transplantation recipient rats (Fig. 7).

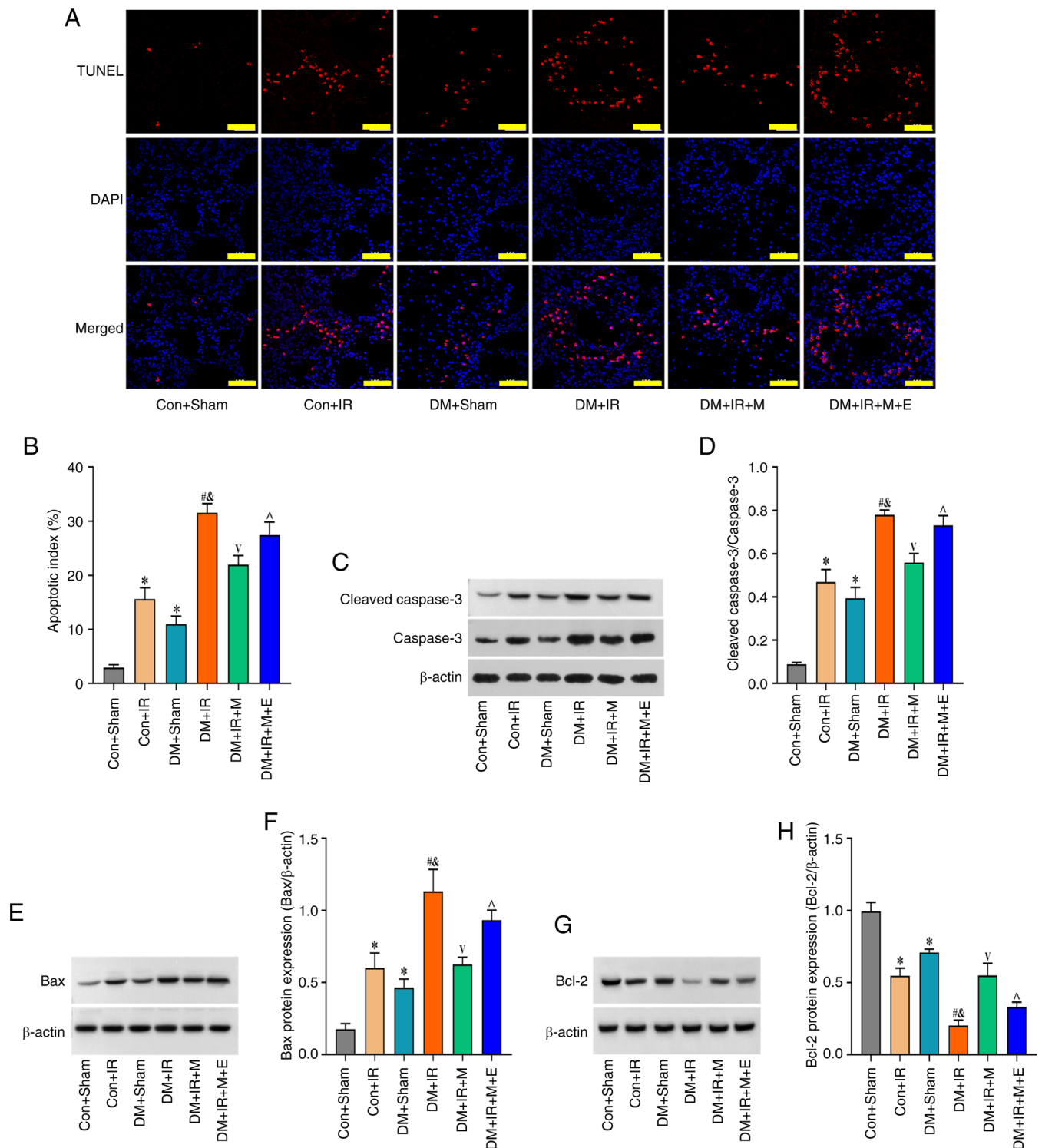


Figure 4. Metformin decreases diabetic lung IR-induced cell apoptosis via the silent information regulator 1 pathway. (A) Representative cell apoptosis images (magnification, x400; scale bar, 100 μ m). (B) Apoptosis index (n=4). (C) Representative western blotting image of cleaved caspase-3 and caspase-3. (D) Cleaved caspase-3/caspase-3 (n=3). (E) Representative western blotting image of Bax. (F) Bax expression (n=3). (G) Representative western blotting image of Bcl-2. (H) Bcl-2 expression (n=3). *P<0.05 vs. Con + Sham group; [#]P<0.05 vs. Con + IR group; [&]P<0.05 vs. DM + Sham group; ^vP<0.05 vs. DM + IR group; [^]P<0.05 vs. DM + IR + M group. IR, ischemia-reperfusion; Con, control; DM, diabetes mellitus; M, metformin; E, EX527.

A number of studies have reported that the prognosis of patients with DM is poor following lung transplantation (23-25). A previous study has reported that type 2 DM was associated with mitochondrial dysfunction, which led to increased production of reactive oxygen species (ROS), causing oxidative stress (26). ROS accumulation caused

by diabetes can also activate nuclear transcription factors, triggering an increase in inflammatory factor levels, alveoli capillary permeability and mitochondria-mediated cellular apoptosis (27,28). In the present study, DM further aggravated the lung IR-induced inflammatory response, oxidative stress, apoptosis and mitochondrial dysfunction

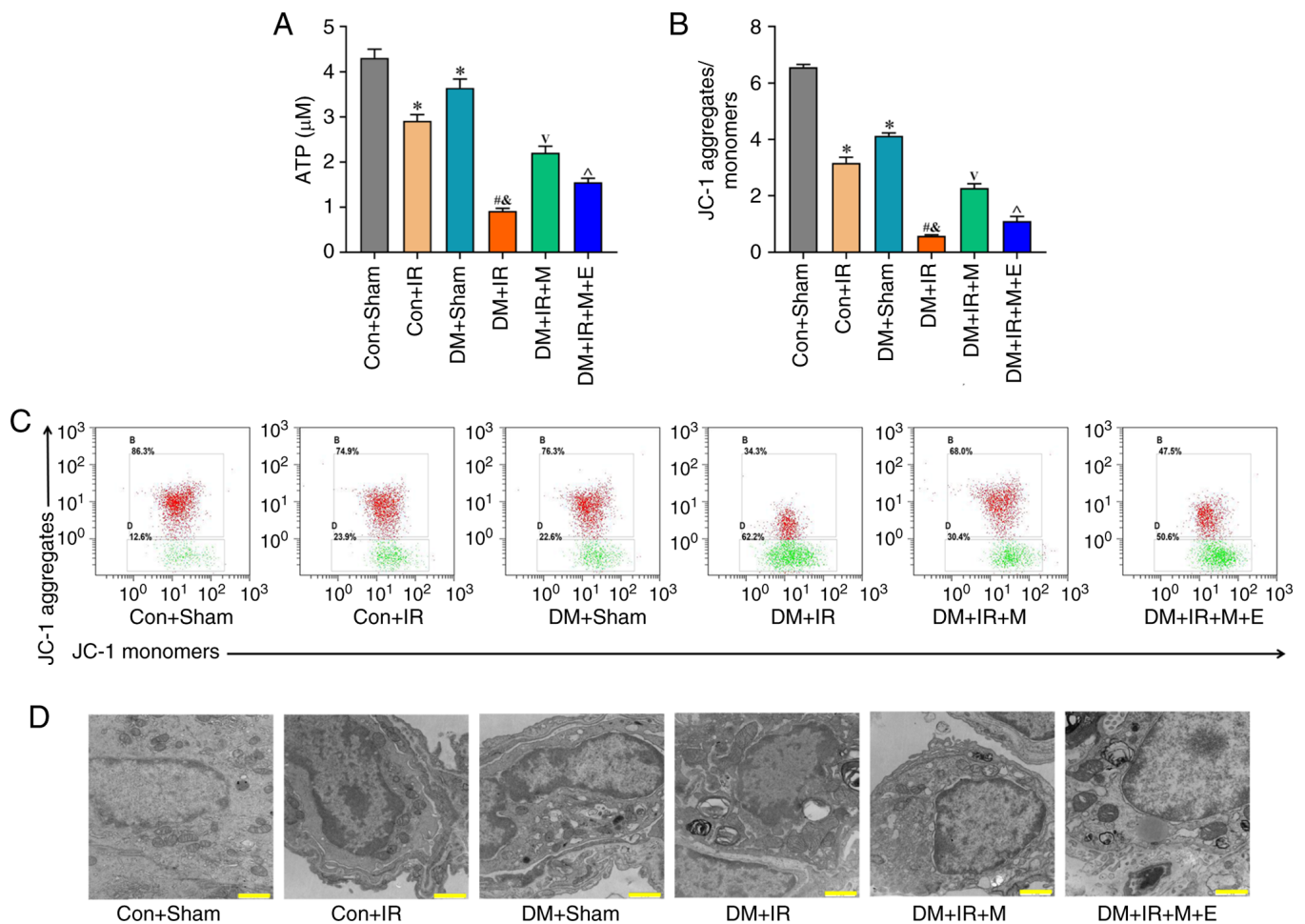


Figure 5. Metformin reduces diabetic lung IR-induced mitochondrial dysfunction via the silent information regulator 1 pathway. (A) ATP level (n=8). (B) Mitochondrial membrane potential (n=3). (C) Representative flow cytometry plots. (D) Mitochondrial ultrastructure representative images of alveolar type II epithelial cells (magnification, x20,000; scale bar, 1 μm). *P<0.05 vs. Con + Sham group; #P<0.05 vs. Con + IR group; &P<0.05 vs. DM + Sham group; ^γP<0.05 vs. DM + IR group; ^ΔP<0.05 vs. DM + IR + M group. IR, ischemia-reperfusion; Con, control; DM, diabetes mellitus; M, metformin; E, EX527.

in particular, which was consistent with the findings of previous studies (8,19,21).

SIRT1, a NAD⁺-dependent histone deacetylase, exerts a protective effect on IR injuries of a number of organs (29,30). SIRT1 activation reduces oxidative stress and maintains mitochondrial function, while SIRT1 inhibition abolishes the beneficial effects (31). A number of studies have reported that SIRT1 levels are reduced in DM (19,32,33), perhaps due to decreased NAD⁺ levels caused by hyperglycemia. Furthermore, impaired SIRT1 signaling is involved in the pathologic process of diabetic complications (34). IR has also been reported to be associated with reduced NAD⁺ levels, which consequently contributed to SIRT1 downregulation (33). In agreement with these studies, the present study demonstrated that SIRT1 signaling was downregulated in the DM model and further decreased in the diabetic lung IR injury model. Therefore, upregulating SIRT1 may be a promising therapeutic approach against diabetic lung IR injury.

The present study also demonstrated that metformin administration effectively ameliorated lung IR injury by reducing inflammatory reactions, oxidative stress and apoptosis, and preserving the alveolar-capillary barrier

by activating SIRT1 in a rat DM model. A retrospective clinical study demonstrated that, among patients with DM who experienced sudden cardiac arrest, those prescribed metformin exhibited lower 24-h peak serum troponin levels compared with those not prescribed metformin (35). In a rat model of myocardial infarction, metformin was reported to markedly reduce infarct size (36), relieve mitochondrial damage and increase the ejection fraction (37). Metformin is a direct SIRT1-activating compound (38). A previous study has also reported that metformin notably inhibited the NF-κB signaling pathway and increased the expression levels of SIRT1 in lipopolysaccharide-stimulated lung tissues, and inhibition of SIRT1 reversed the protective effects (39). Metformin can inhibit oxidative stress and promote autophagy, thereby alleviating the development process of diabetic kidney disease by activating SIRT1 (40). Metformin has also been reported to attenuate IR-induced myocardial apoptosis via the activation of SIRT1, thus exerting a cardioprotective effect (41). Furthermore, our previous study demonstrated that metformin possessed anti-inflammatory and antioxidant properties, and maintained alveolar-capillary barrier integrity (20). These data all supported the findings of the present study. Overall, the

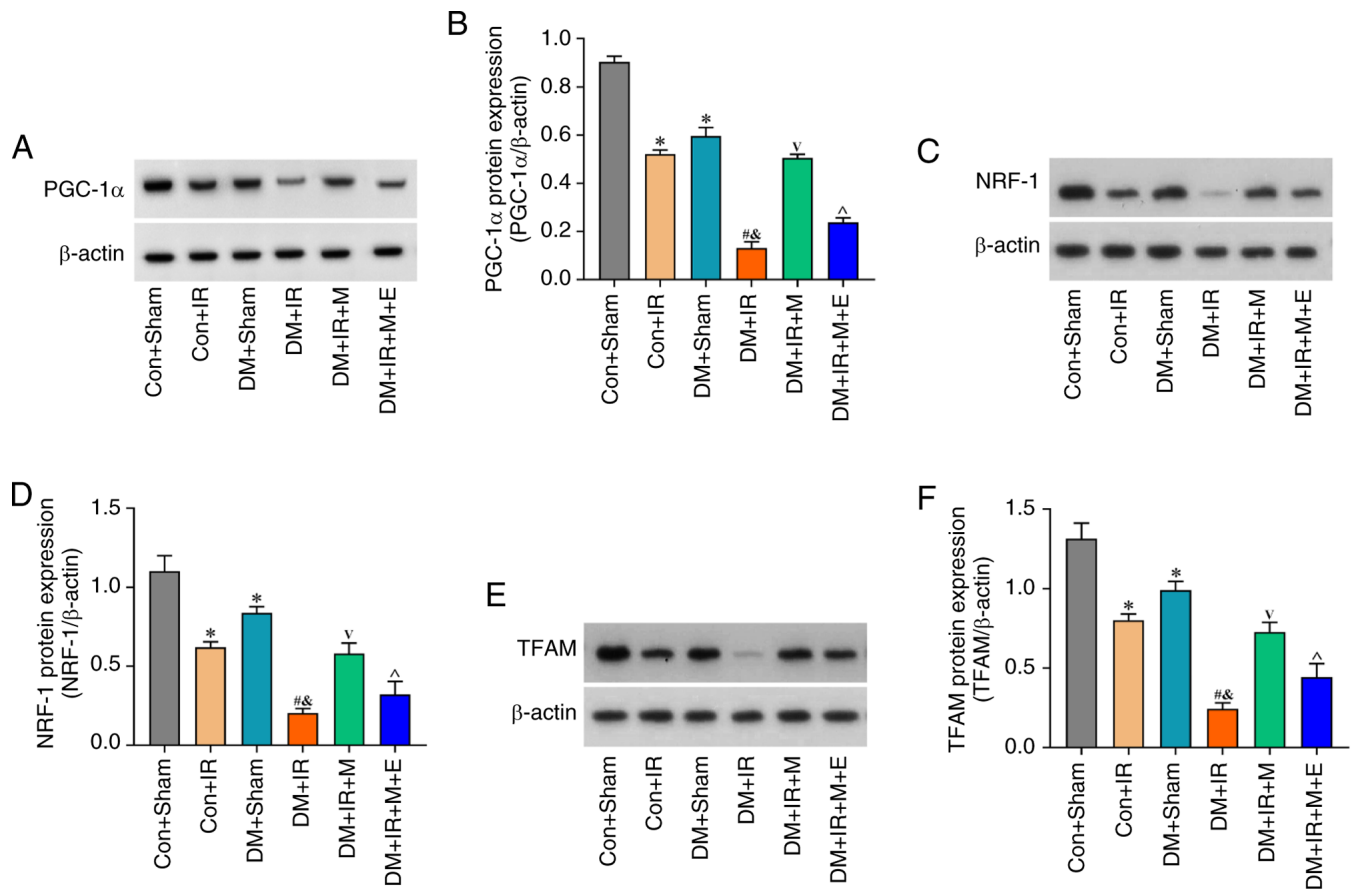


Figure 6. Metformin promotes mitochondrial biogenesis via the silent information regulator 1 pathway in the diabetic rat lung subjected to IR injury. (A) Representative western blotting image of PGC-1 α . (B) PGC-1 α expression (n=3). (C) Representative western blotting image of NRF-1. (D) NRF-1 expression (n=4). (E) Representative western blotting image of TFAM. (F) TFAM expression (n=4). *P<0.05 vs. Con + Sham group; #P<0.05 vs. Con + IR group; &P<0.05 vs. DM + Sham group; ^P<0.05 vs. DM + IR group; ^P<0.05 vs. DM + IR + M group. IR, ischemia-reperfusion; PGC-1 α , peroxisome-proliferator-activated receptor γ coactivator-1 α ; NRF-1, nuclear respiratory factor 1; TFAM, mitochondrial transcription factor A; Con, control; DM, diabetes mellitus; M, metformin; E, EX527.

data suggested that metformin protected against diabetic lung IR injury and that SIRT1 served a critical role in this process.

Mitochondrial dysfunction has been reported to be a critical mechanism in diabetic lung IR injury (8), which manifests as decreased ATP content, reduced mitochondrial membrane potential, increased mitochondrial apoptosis and impaired mitochondrial integrity (6,42). These findings are consistent with the results of the present study. PGC-1 α , the main regulator of mitochondrial biogenesis, activates the transcription factor NRF-1, which promotes TFAM expression that drives transcription and replication of mitochondrial DNA (mtDNA) (43). In a previous study, the mtDNA copy numbers and PGC-1 α levels were decreased in myocardial IR injury (44). SIRT1 activation also increased PGC-1 α expression and mitochondrial content in cardiomyocytes in cardiac IR injury models (45). It has been reported that the enhancement of mitochondrial biogenesis can mitigate ischemic brain injury and improve neuronal damage by increasing the number and function of mitochondria (46,47). The present study demonstrated that metformin increased mitochondrial biogenesis. This was indicated by the increased expression levels of PGC-1 α , NRF-1 and TFAM, thus improving mitochondrial function in the diabetic lung IR injury model, whilst

the inhibition of SIRT1 reversed these benefits. In addition, a previous study reported that resveratrol enhanced mitochondrial biogenesis in skeletal muscle and improved exercise tolerance via SIRT1 activation (48). Additionally, SIRT1 activation stimulated mitochondrial biogenesis and decreased renal IR-induced nitrosative stress and inflammation (49). Therefore, these studies indicate that SIRT1 is involved in mitochondrial biogenesis, and the present study suggested that SIRT1-mediated mitochondrial biogenesis serves a key role in the protective effects of metformin against diabetic lung IR injury.

The present study has several limitations. First, although the results demonstrated that metformin alleviated diabetic lung IR injury, future studies are required to determine the most appropriate dose of metformin. Second, a high-fat diet-fed streptozotocin-induced type 2 diabetic rat model was used in the present study, but whether this model is representative of DM in humans requires further detailed investigations. Third, SIRT1 can directly interact with several mitochondrion-related proteins except PGC-1 α (50), thus future experiments are needed to investigate more signaling pathways. Fourth, whether similar results could be obtained with female rats needs to be explored. Fifth, a DM + IR + EX527 group was not set up in the present study. Previous

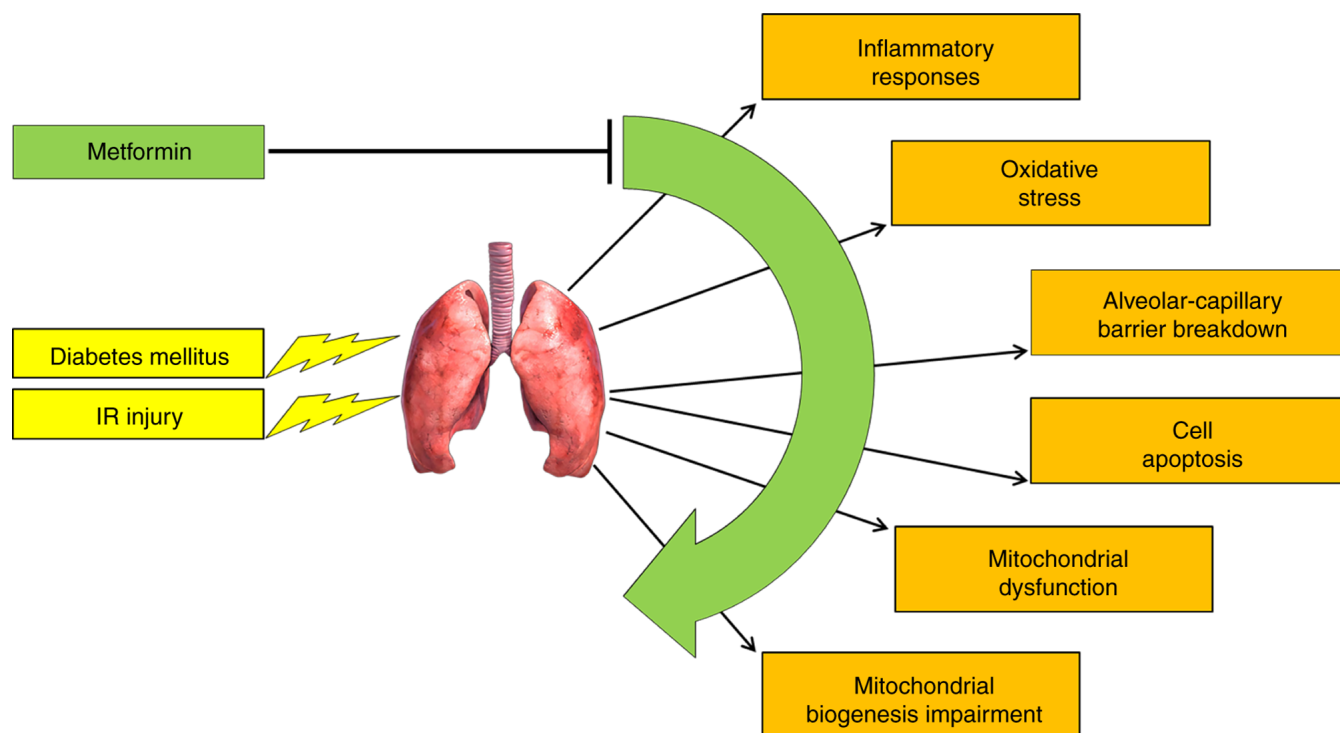


Figure 7. Schematic illustration showing the effect of metformin on IR injury of transplanted lungs in type 2 diabetic recipient rats. Metformin alleviated inflammatory responses, oxidative stress, apoptosis and mitochondrial dysfunction, while enhancing mitochondrial biogenesis, maintaining alveolar-capillary barrier integrity and improving lung function in diabetic lung transplantation recipient rats. IR, ischemia-reperfusion.

studies have shown that EX527 itself did not alter the cell apoptosis and microglial activation in the hippocampus of septic mice and did not influence the infarct size and neurological functions in a rat model of middle cerebral artery occlusion (51,52). Although these studies demonstrated that EX527 alone did not influence the results, the absence of this group in the present study is still a limitation as its effect could not be verified. In a future study, the DM + IR + EX527 group will be set up. Sixth, dynamic observation subgroups at different time points were not set up in present study. In the future, the dynamic changes of diabetic lung IR injury and the effects of metformin on diabetic lung IR injury at different time points will be explored. Finally, the AMP-activated protein kinase (AMPK) and NF- κ B signaling pathway were not examined in the present study. AMPK is an important regulator of diverse cellular pathways. Our previous study demonstrated that phosphorylation of AMPK was decreased in DM rats compared with normal rats under the same conditions, and metformin further increased the diabetic lung IR-induced upregulation of AMPK activity (20). Additionally, the NF- κ B pathway is a prototypical pro-inflammatory signaling pathway and serves an important role in inflammation reactions. A future study will explore the AMPK and NF- κ B signaling pathway in diabetic lung IR injury.

In conclusion, the present study demonstrated that type 2 DM further increased the lung IR-induced inflammatory reaction, oxidative stress, apoptosis and mitochondrial dysfunction, and decreased mitochondrial biogenesis and SIRT1 expression. Metformin exerted protective effects against lung IR injury by attenuating inflammation reactions, oxidative stress

and cell apoptosis, and particularly improving mitochondrial biogenesis through the SIRT1 pathway in rats with type 2 DM. Furthermore, SIRT1 may serve as a potential therapeutic target in diabetic lung IR injury.

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Availability of data and materials

The data generated in the present study may be requested from the corresponding author.

Authors' contributions

HW and THL contributed equally to the work. HW and THL conceived and designed the study, performed experiments, prepared figures, and drafted and revised the manuscript. LJZ and WY carried out the animal experiments and acquired the data. CM and SHL analyzed and interpreted the data. XZZ analyzed and interpreted the data, and helped to correct the manuscript. WZL conceived and designed the study, and critically revised the manuscript for important intellectual content. HW and WZL confirm the authenticity of all the raw data. All authors have read and approved the final version of the manuscript.

Ethics approval and consent to participate

The present study was approved by the Institutional Animal Care and Use Committee of Second Affiliated Hospital of Harbin Medical University (grant no. SYDW2021-088; Harbin, China). All animal experiments were performed in accordance with the Animal Research: Reporting of *In Vivo* Experiments guidelines.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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