MATERIALS AND METHODS

Simulated DM + MI in cultured cells and AA treatement

To simulate DM+MI in vitro, H9C2 cells were treated with high glucose (HG) and oxygen deprivation (OGD) as previously described (Paillard M, et al. 2013). For the treatment procedure, H9C2 cells were first cultured in DMEM with high glucose (33 mM) and Mannitol for 72 hours for HG cell model. Then, in order to simulate hypoxia, the cell culture medium was replaced with a Tyrode's solution without glucose, which contained the following components: 130 mM NaCl, 5 mM KCl, 10 mM HEPES, 1 mM $MgCl_2$ and 1.8 mM $CaCl_2$, at pH 7.4/37°C and the cell plate was moved into a controlled hypoxic chamber for 3 hours to establish the OGD cell model. In HG+OGD+AA group, different concentrations of AA (1, 5, or 25 μM) were supplemented in the medium at the same time.

Conventional X-ray coronary angiography - X-ray angiography was performed on pigs before and immediately after coronary ligation using standard transfemoral Judkins technology. After administering 10 mL of iopromide (Clarograf; Juste, Madrid, Spain) into the left coronary artery, the pig heart is dynamically scanned by the C-arm X-ray machine (9800-12, Beijing Tongyong Medical Equipment Co., Ltd, China) at a rate of 3 frames per second to obtain cardiac coronary angiograms.

Echocardiography (Echo) examination - At 48 h post operation, the Vivid 7 dimension ultrasound system from GE, USA with 3.5 MHz probe (for pigs) and 30-40 MHz probe (for rats) was used to measure left ventricular end-systolic volume (LVESV), left ventricular end-diastolic volume (LVEDV) and left ventricular ejection fraction (LVEF). Three cardiac cycles of apical four-chamber and apical two-chamber sections were dynamically acquired. Echo results were interpreted by two radiologists who were blinded to the animal groups.

Magnetic resonance imaging of hearts - To confirm the heart structural and functional changes, magnetic resonance imaging (MRI) was performed in all pigs at 48 h post surgery using a 1.5 T whole body imager (Magnetom Sonata scanner, Siemens Medical Solutions, Erlangen, Germany). Fast gradient echo sequences for chest scan were employed. The specific absorption rates (SAR) were limited to 2.2 W/kg, and the average SAR was 1.4 W/kg.

Plasma Sample Preparation - To determine the metabolic features in MI pigs with or without DM, the coronary sinus blood samples of Bama mini-pigs (anticoagulated with sodium citrate) were centrifuged at 13,000 rpm for 20 min to obtain a plasma samples. Then, vortex and mix the plasma samples with methanol in a ratio of 1:4 for 2 min, centrifuge at 13,000 rpm, and 4°C for 15 min. Collect 1200 μL of the supernatant, transfer it to a new 1.5 mL polypropylene tube, put it in a vacuum drying oven, and evaporate to dryness. Add 150 μL of 50% methanol to dissolve the residue in each test tube. The solutions were filtered with a 0.22 - μm Millipore filter before

injection.

Measurement of intracellular AA - A commercially available AA measurement kit (Jiancheng Institute of Biological Engineering, Nanjing City, Jiangsu Province, China, A037) was used to determine the intracellular AA level. The experiment was performed according to the manufacturer's instructions, and the results were expressed as a percentage relative to the control value.

siRNA Knockdown - H9C2 cells were seeded in a 6-well dish (1.2 million/well). Twenty-four hours later, cells were transfected with either small interfering RNA (siRNA) against rat Pink1 (Ambion, ID#180640 as previously validated (Haque M. E., et al., 2008)) or with a scrambled control siRNA using Lipofectamine RNAiMAX (Invitrogen) according to the manufacturer's instruction. The scrambled control siRNA has no significant homology with any known gene sequence (Ambion, ID#4611). In brief, resuspend 3 microliters of siRNA stock solution (40 mmol/L) and an equal volume of RNAiMax transfectant in 1 mL of Opti-MEM medium, and mix well. Cardiomyocytes were co-incubated with the RNAiMax for 6 hours, followed by the addition of 1 mL of culture medium containing 20% serum. 24 hours later, cells were harvested and analyzed by Western blot to confirm knockdown of Parkin. Parallel cell cultures were subjected to HG (72 hours) and OGD (3 hours) in the presence or absence of AA (25 μM) for cell viability assay and the measurement of mitophagy/mitochondrial turnover associated proteins expression level.

Preparation of cytosolic and mitochondrial fractions - Cytosolic and mitochondrial fractions were prepared using a commercially available cytosolic/mitochondrial fraction kit (C3601, BiYunTian, Institute of Biotech, Shanghai, China) referring to previous study (Cao H, et al., 2012). Briefly, 3×10^7 cells were collected and washed three times with ice-cold PBS at 600×g. Then the cell pellet was resuspended in 2 mL of extraction buffer, incubated at 4°C for 20 min, and homogenized in a Dounce homogenizer. The cell homogenate was centrifuged at 750 g for 10 minutes at 4°C. Take the supernatant and further centrifuge (12,000 g) at 4°C for 15 min. The resulting precipitate is the mitochondrial fraction. The supernatant is centrifuged at 100,000 g for another 1 hour at 4°C to obtain the cytoplasmic fraction.

Measurement of intracellular ROS and mitochondrial ROS (mtROS) - Intracellular ROS were measured using 2',7'-dichlorofluorescein diacetate (DCFH-DA) (D6883, Sigma-Aldrich, Saint Louis, MO, USA) and dihydroethidium (D11347, Molecular Probes, Eugene, OR, USA) following the manufacturer's protocol (Barlow J,et al.,2015; Natalicchio A,et al., 2015). The fluorescence intensity of each group was determined by flow cytometer (BD Accuri C6) and the results were expressed as a percentage relative to the value of the control group.

mtROS in H9C2 were determined using MitoSOX red (Molecular Probes, Eugene, OR, USA), a fluorogenic dye with high selectivity of superoxide in the mitochondria of live cells. At the end of the treatment, MitoSOX red (3.0 μM) was added to the cell

culture medium and continued to be incubated in a cell incubator at 37°C in the darkness for 15 min. Subsequently, cells were collected by trypsinization and washed in warm HBSS. The fluorescence intensity of each group was measured by flow cytometry using Accuri C6 (BD Biosciences, San Jose, CA, USA) and quantified as fold change compared with baseline.

Measurement of mitochondrial membrane potential (ΔΨm) - In order to quantitatively analyze the changes in the membrane potential of mitochondria with or without AA treatment, the fluorescent indicator 5,5',6,6'-tetrachloro- 1,1',3,3'-tetraethyl benzimidazolylcarbcyanine iodide (JC-1) (No.70014, Biotium, Hayward, CA, USA) was used to measure the mitochondrial membrane potential in different groups with a fluorescence plate reader.

Briefly, cells were seeded into a black 96-well plate (Nunc, Denmark) at a density of 2×10^3 cells in 200 μL culture medium/well, and JC-1 (2.5 μg/mL) was added. After incubating for 30 min in the dark, cells were washed twice with PBS to remove unbound JC-1. Mitochondrial membrane potential is expressed as the fluorescence emission rate (580/520 nm) relative to the fluorescence intensity of the control cell membrane. In addition, the Leica confocal microscope was used to observe and photograph the cells.

Measurement of cell viability - Cell viability was measured using an MTT assay (M5655, Sigma-Aldrich, St. Louis, Missouri, USA). Dehydrogenase in mitochondria can convert MTT into formazan crystals. The main experimental operations were as follows: H9C2 cells were seeded in a 96 - well plate at a density of 5×10^3 cells / well, then MTT stock solution (5 mg/mL in PBS) was added to make the final concentration of MTT in the medium 0.5 mg/mL. After incubating for 4 hours at 37°C, the supernatant was removed and the intracellular formazan crystals were dissolved in 150 μL of dimethyl sulfoxide. Then，the 150 μL of the dimethyl sulfoxide solution of formazan crystals was transfer into a 96-well plate, and measured the absorbance at 570 nm using an automatic microplate reader (Tecan Infinite 200 microplate reader, Männedorf, Switzerland). Cell viabilities were calculated by comparing results to control cells.

TUNEL assay - The TUNEL (TdT-mediated dUTP nick-end labeling) assay was performed using a commercially available in situ apoptosis detection kit (No.11684795910, Roche Molecular Biochemicals, Mannheim, Germany) following the manufacturer's suggestion. As mentioned before (Tsai CY, et al., 2013), first, fix the cells with 4% paraformaldehyde solution for 30 minutes at room temperature. After washing 3 times with PBS, cells were permeabilized with cell permeation solution (0.1% Triton X-100 in 0.1% sodium citrate) at 4°C for 2 min. After washing with PBS, TUNEL reagent containing terminal deoxynucleotidyl transferase and fluorescent isothiocyanate-dUTP was added and incubated together. Finally, stain the nucleus with 1 μg/mL DAPI (4',6-dimidyl-2-phenylindole) for 30 min (indicated in

blue). Under the microscope, use excitation wavelengths in the range of 450-500 nm and detection wavelengths in the range of 515-565 nm (green) to observe the number of TUNEL-positive heart cells containing apoptotic bodies and the total number of cardiomyocytes stained with DAPI. Three digital images with similar total cell counts were selected from each cover glass for counting and averaging. The results were expressed as the number of TUNEL positive cells divided by the total number of cells visible in the same field, which is the apoptosis index (AI). Take the average of three independent experiments and perform statistical analysis.

Determination of intracellular ATP content - The ATP content in H9C2 cells was measured using a commercially available ATP assay kit according to the instructions for use (S0026 Biyuntian) (Zhu SS, et al., 2011). At the same time, each group of cell lysates was subjected to protein quantification (BCA method). The intracellular ATP content is expressed as nmol/mg prot.

Histological analysis - At study endpoints, rats were euthanized by an intraperitoneal injection of sodium pentobarbital (120 mg/kg body weight), the pigs were euthanized by intravenous injection of KDP (ketamine, 35 mg/kg + diazepam, 1.5 mg/kg + 10% potassium chloride, 15-20 mL). The heart was quickly removed, its cross-section was cut into 10 mm thick tissue pieces, and stained with 2,3,5-triphenyltetrazolium chloride (TTC) to determine the size of the infarct. The infarct size was identified as the ratio of area of infarct size (non-TTC-stained area) to area of left ventricular in the same section.

Moreover, around 1 cubic centimeter of myocardial tissue in the infarciton border area was collected and fixed for 24 hours in formalin. Then fixed heart tissue were embedded in paraffin. Sliced sections (10 μm) were stained with hematoxylin and eosin (H&E Sigma, Shanghai, China) to observe the difference in the degree of damage of myocardial cells in different groups under a microscope.

To ensure the quality of myocardial tissue staining results, this study was conducted by two pathologists with a blind method.

Transmission electron microscopy - For electron microscopy, heart tissue samples (0.5 cm×0.5 cm) were fixed in a cacodylate buffer containing 1% osmium tetroxide at 4°C for 1 hour. Then tannic acid was used as an en bloc stain to enhance the contrast of the elastic sheet. (Heegaard AM et al., 2007). Subsequently, the heart was rinsed in water, then dehydrated into propylene oxide in a series of graded ethanol-containing solutions, embedded in epoxy resin, stained with Azure II-methylene blue, treated with uranyl acetate and lead citrate, and examined under a CM Philips transmission electron microscope (KE Electronics, Tofts, UK).

Malondialdehyde (MDA) measurement – MDA of cardiomyocytes in the peri-infarct region of rats with or without AA treatment were measured with commercial kits according to the manufacturer's recommendations as previously

described (G. Fiskum,et al,2004). MDA levels were expressed as nmol/mg protein.

Total Antioxidant Capacity (T-AOC) Assay - There are a variety of antioxidants in the myocardial tissue, including antioxidant macromolecules, antioxidant small molecules and enzymes, which can eliminate various ROS produced in the myocardial tissue to prevent the generation of oxidative stress induced by ROS. The total level of various antioxidant macromolecules, antioxidant small molecules and enzymes within a system reflects the total antioxidant capacity within the system. The T-AOC of the serum in the experiment was measured using a commercially available T-AOC Assay Kit (Beyotime,) according to the instructions for user.

Immunoblot analysis - H9C2 cell samples and animal cardial tissue samples were homogenized in Western blot analysis buffer containing 10 mM Tris- HCl (pH 7.4), 150 mM NaCl, 1% (v/v) triton X-100, 1% sodium deoxycholate, 0.1% SDS, 5 mM EDTA, 1 mM PMSF, 0.28 kU/L aprotinin, 50 mg/L leupeptin, 1 mM benzamidine, and 7 mg/L pepstain A (all chemicals were purchased from Sigma-Aldrich, USA). The homogenate was then centrifuged two times at 12000 rpm for 15 min each time at 4°C, and the supernatant was retained and preserved at −20 °C for later use. Protein concentrations were then determined using BCA kit (Pierce Chemical, USA). Take each sample proteins (20 μg) and load them on a 10% SDS-PAGE gel, use constant current electrophoresis, and then transfer the protein on the gel to the PVDF membrane through a semi-dry electrotransfer unit (Bio-Rad). The membranes were blocked with Tris-buffered saline containing 0.1% Tween-20 (TBST) and 5% nonfat dry milk for 1 hour, and then respectively incubated with anti-LC3 (abcam), p62/ SQSTM1 (abcam), Drp1 (Cell Signal), FIS1 (American Research Products), PINK1 (abcam), Parkin (abcam), Caspase 3 or cytochrome C antibodies overnight at 4°C. The membranes were washed 3 times with TBST, and incubated with the horseradish-conjugated second antibody in TBST for 2 hours. After striping, GAPDH (Sigma) or VDAC1 (Sigma) antibody was used to hybridize with the membrane. An ECL system (ChemiScope, Shanghai) was used to test their reactivity. The signal intensity of primary antibody binding was quantitatively analyzed by ChemiScope analysis, and was normalized to the signal intensity of GAPDH or VDAC1.