Nicotinamide ribose ameliorates myocardial ischemia/reperfusion injury by regulating autophagy and regulating oxidative stress

CHEN YUAN[1-3], HENG YANG[1,2], WANQI LAN[1,2], JUESHENG YANG[1,2] and YANHUA TANG[1,2]

[1]The Second Affiliated Hospital, Jiangxi Medical College, Nanchang University, Nanchang, Jiangxi 330006; [2]Department of Cardiovascular Surgery, The Second Affiliated Hospital of Nanchang University, Nanchang, Jiangxi 330000; [3]East China Institute of Digital Medical Engineering, Shangrao, Jiangxi 334100, P.R. China

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Abstract. Nicotinamide riboside (NR) has been reported to play a protective role in myocardial ischemia-reperfusion (I/R) injury when used in association with other drugs; however, the individual effect of NR is unknown. In the present study Evan's blue/triphenyl tetrazolium chloride staining, hematoxylin and eosin staining, echocardiography, western blotting, reverse transcription-quantitative PCR, and the detection of myocardial injury-associated markers and oxidative stress metabolites were used to explore the ability of NR to alleviate cardiac I/R injury and the relevant mechanisms of action. In a mouse model of I/R injury, dietary supplementation with NR reduced the area of myocardial ischemic infarction, alleviated pathological myocardial changes, decreased inflammatory cell infiltration and attenuated the levels of mitochondrial reactive oxygen species (ROS) and creatine kinase myocardial band (CK-MB). In addition, echocardiography suggested that NR alleviated the functional damage of the myocardium caused by I/R injury. In H9c2 cells, NR pretreatment reduced the levels of lactate dehydrogenase, CK-MB, malondialdehyde, superoxide dismutase and ROS, and reduced cell mortality after the induction of hypoxia/reoxygenation (H/R) injury. In addition, the results indicated NR activated sirt 1 via the upregulation of nicotinamide adenine dinucleotide (NAD^+) and protected the cells against autophagy. The sirt 1 inhibitor EX527 significantly attenuated the ability of NR to inhibit autophagy, but had no significant effect on the ROS content of the H9c2 cells. In summary, the present study suggests that NR protects against autophagy by increasing the NAD^+ content in the body via the sirt 1 pathway, although the sirt 1 pathway does not affect oxidative stress.

Correspondence to: Dr Juesheng Yang or Professor Yanhua Tang, Department of Cardiovascular Surgery, The Second Affiliated Hospital of Nanchang University, 1 Minde Road, Nanchang, Jiangxi 330000, P.R. China
E-mail: mniyjs162000@163.com
E-mail: tyh6565@163.com

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Introduction

Acute myocardial infarction (AMI) is increasing in incidence of worldwide, and has gradually become an important cause of mortality (1). With the continuous development of interventional techniques, the treatment of coronary heart disease and AMI is becoming more advanced. However, ischemia/reperfusion (I/R) injury may occur as treatment progresses (2), which is an urgent clinical issue (3). Myocardial I/R injury can offset the benefits of reperfusion therapy and even worsen the prognosis of AMI (4,5). I/R injury is defined as reduced cardiac systolic function, reperfusion arrhythmia, dysfunctional myocardial metabolic capacity, oxidative damage, inflammation, cardiac dysfunction and other injuries following the recovery of the blood supply to ischemic myocardial tissues (6). Myocardial ischemia causes numerous cellular changes, including changes in cell membrane potential and intracellular ion distribution, such as increased Ca^{2+} and Na^+ influx, cell swelling and rupture, and cell acidosis (7,8). It is widely considered that the upregulated release of reactive oxygen species (ROS) is a major cause of early I/R injury (9). Under normal physiological conditions, ROS are continuously produced in the myocardium, but can be removed via a cellular clearance mechanism. However, in the pathological state of hypoxia/reoxygenation, the disordered myocardial metabolism produces a large quantity of ROS that cannot be cleared away, which may lead to pathological changes such as DNA injury and protease degeneration (10). Other mechanisms of myocardial I/R injury are known, including intracellular Na^+ and Ca^{2+} accumulation, pH reduction, mitochondrial dysfunction, increased free radical formation and nitric oxide metabolism disorder (11-13). The occurrence of autophagy also plays an important role (14).

Autophagy is a key metabolic pathway by which eukaryotic cells maintain homeostasis when stimulated by various physicochemical factors, such as inflammation, starvation, anoxia and reoxygenation injury. It is also a multi-step intracellular catabolic process involved in the pathophysiological processes of various diseases. During autophagy, damaged organelles and macromolecules are enclosed in specialized vesicles called

autophagosomes. The resulting autophagosomes are eventually transferred to lysosomes for fusion and degradation of their contents (15). Autophagy has been shown to be involved in the process of myocardial I/R injury (16). Mitochondrial autophagy involves the mitochondria in hypoxic cardiomyocytes being surrounded by autophagosomes and undergoing phagocytosis (17). Therefore, autophagy and oxidative stress are important factors to be considered in the treatment of myocardial I/R injury.

Nicotinamide riboside (NR) is a precursor of nicotinamide adenine dinucleotide (NAD^+) that has potential health benefits due to the production of NAD^+ in the body. NAD^+ plays a key role in cellular oxidation-reduction reactions, including catabolic and anabolic reactions such as glycolysis and the tricarboxylic acid cycle (18-20). NAD^+-depleting enzymes are mediators of aging that are mainly induced by stressors such as DNA damage, oxidative stress and inflammation. These include sirtuins, which are NAD^+-dependent deacetylases/deacylases that deplete NAD^+ when removing acetyl or other acyl groups from proteins (21). Sirtuin 1 (sirt 1) is a member of the sirtuin family, which has been shown to participate in myocardial I/R injury via the regulation of autophagy (22).

NR can participate in the regulation of autophagy and oxidative stress (21). However, the effect of NR on myocardial I/R injury is not clear. Therefore, the aim of the present study was to investigate the role of NR in hypoxia/reoxygenation (H/R)-induced myocardial cell injury to evaluate the therapeutic potential of NR as a treatment for I/R injury.

Materials and methods

Animals. A total of 24 wild-type C57BL/6 male mice (8-10 weeks old; body weight, 24.5±3.7 g) were provided by the Shanghai Model Organisms Center, Inc. All protocols were approved by the Animal Care Ethics Committee of Zhonghong Boyuan Biotechnology Co., Ltd. (K-2023-0513-1; Nanchang, China) and implemented according to the Guide for the Care and Use of Experimental Animals of the National Institutes of Health. The validity of the approval was verified by the Ethics Committee of Nanchang University (Nanchang, China). The mice were placed in a constant temperature of 20-26˚C and humidity of 40-70% environment with a 12-h light/dark cycle and 5 g daily food and 6-7 milliliters of water. The NR treatment was administered as a once daily dose of oral NR (Shanghai Macklin Biochemical Co., Ltd.) via gavage, which was guaranteed to provide a 450 mg/kg dose. The treatment was administered for 14 days. According to a previous study (23), the administration of NR to mice at a dose of 450 mg/kg and duration of ≥14 days significantly increases the body content of NAD^+. The mice were randomly divided into four groups (n=6/group): Control group, no treatment; sham group, opening of the chest without ligation of the left anterior descending (LAD) coronary artery; I/R group, 30-min LAD ligation followed by 24-h reperfusion; and I/R + NR group, pre-treatment with NR followed by I/R as described for the I/R group.

Myocardial I/R injury. A mouse myocardial I/R injury model was established using the aforementioned mice. During modeling, anesthesia was induced using 2.5% isoflurane and maintained with 1% isoflurane inhalation. After fixing the mice in a stable position, the chest cavity was opened, the LAD arterial branch was ligated with a 6-0 silk thread, and then the chest cavity was closed following release of the ligature. Following 30 min of ligation, the ligature was released to allow reperfusion for 24 h, after which the I/R model was constructed (24). The mice were then euthanized using CO_2 at a flow rate of 60% volume displacement/min followed by cervical dislocation. Serum was collected from the mice for determination of CK-MB, and their hearts were excised and some were frozen for later use.

Echocardiography detection. To evaluate the effect of NR supplementation on I/R injury in the mice, echocardiography was performed to detect the structure and function of the heart and basic data were obtained after 24-h perfusion, namely the left ventricular ejection fraction (EF) and left ventricular shortening fraction (SF).

Evan's blue/triphenyl tetrazolium chloride (TTC) and hematoxylin and eosin (H&E) staining. Infarction was measured using the TTC staining method. Following euthanasia, the hearts were immediately extracted from the mice and the coronary arteries were perfused with PBS. The blood was washed away with normal saline and the left anterior coronary artery was re-occluded. Evan's blue dye (~1 ml, 2%) was injected into the heart via the aorta until the heart turned blue. The heart was then cut into 5 or 6 transverse sections and incubated in 1% TTC solution at 37˚C for 20 min. The non-ischemic area was stained blue, the white tissue was the infarcted area, and the white/red tissue was the area at risk (AAR). ImageJ software was used to calculate the AAR.

With regard to H&E staining, the heart was first embedded in paraffin, fixed with 12% formalin at room temperature for 12 h and sliced into 5-μm sections. The sections were then dewaxed and stained at room temperature for 15 min with hematoxylin solution. After this, the sections were rinsed with running water for 1 h, dehydrated with ethanol and stained with eosin solution for 5 min at room temperature. The stained tissues were dehydrated again and soaked in xylene. Finally, the slices were covered with a glass coverslip and observed at x200 magnification using an optical microscope and bright field illumination at room temperature.

Cell culture and H/R treatments. H9c2 cells (cat. no. AW-CELLS-R0006) were obtained from Anwei Biotechnology Co., Ltd. (Shanghai, China) and cultured in Dulbecco's modified Eagle's medium (DMEM; Gibco; Thermo Fisher Scientific, Inc.) supplemented with 10% fetal bovine serum (FBS; Gibco; Thermo Fisher Scientific, Inc.) in 37˚C. Prior to the simulation of I/R injury, the cytogenic medium was discarded, the cells were rinsed with PBS, DMEM without FBS was added, and the cells were cultured for 24 h. Firstly, the effect of treatment with different concentrations of NR alone on the survival of the H9C2 cells was investigated. The cells were pretreated with different concentrations of NR and their survival status was determined using a Cell Counting Kit-8 (CCK-8) assay. Secondly, the minimum effective concentration of NR for protecting against H/R injury was determined. The cells were pretreated with different concentrations of NR

followed by H/R treatment, and cell survival status was detected using the CCK-8 assay. In these assays, various dilutions of NR (0, 0.01, 0.1, 1, 5 and 10 mM) were established using DMEM. The cells were seeded in six-well plates, and the different dilutions of NR were added. The NR pre-treatment of the cells was performed for 24 h in 37˚C (25). The cells were then placed in a three-gas anoxic incubator (1% O_2 + 94% N_2 + 5% CO_2) for 3 h. After this hypoxic treatment, the cell culture medium was replaced with DMEM containing 10% FBS and the plate was placed in a normal 37˚C incubator with 95% air and 5% CO_2 and cultured for another 3 h. Then the cell culture supernatant was collected and transferred to an Eppendorf tube, which was placed in a -80˚C freezer for later use. Control group, normal cell culture without any treatment; The H/R group was cultured at 37˚C for 3 h in an anaerobic incubator, while the H/R+NR group was pretreated with NR for 24 h before undergoing hypoxia reoxygenation. The H/R+NR+EX527 and NR+EX527 groups were pretreated for 24 h before undergoing hypoxia reoxygenation. According to the instructions provided for EX527 (HY-15452, MedChemExpress, USA) by the manufacturer, a concentration of 10 μM was used.

NAD^+ measurements. Previous studies (23,26) have shown that the oral administration of NR increases the content of NAD^+ in mice. To determine whether NR also has this effect *in vitro*, the NAD^+ content of the cells was measured after NR pretreatment and H/R culture. The NAD^+ content was determined using an enzyme-linked immunosorbent assay (ELISA) kit (AAT-B15258; Amplite®; AAT Bioquest, Inc.), according to the manufacturer's instructions.

Lactate dehydrogenase (LDH) and creatine kinase myocardial band (CK-MB) determination by ELISA. The cells were tested using an LDH Assay Kit (AK141; BIOSS) and the cells and mouse cardiac tissue were tested using an CK-MB ELISA Kit (H191-1-1; Nanjing Jiancheng Bioengineering Institute) according to the manufacturers' instructions. Finally, the absorbance was measured at a wavelength of 450 nm using a microplate reader (Mindray Medical International, Ltd.) (27).

ROS measurements. An ROS detection kit (E-004-1-1; Nanjing Jiancheng Bioengineering Institute) was used to analyze the H9c2 cells and mouse cardiac tissue according to the manufacturer's instructions. Blank and sample holes were set up in the enzyme assay plate. Following the addition of enzyme labeling reagent and color rendering, the absorbance of each hole was measured using a microplate reader at a wavelength of 450 nm.

Malondialdehyde (MDA) and superoxide dismutase (SOD) measurement by ELISA. The MDA and SOD contents of the H9c2 cells were analyzed according to the instructions provided by the manufacturers of the MDA Assay Kit (cat. no. AK289; BIOSS) and SOD Assay Kit (AK061; BIOSS). The absorbance for MDA at 532 and 600 nm and for SOD in 550 nm were measured using a microplate reader.

Cell viability measurement by CCK-8 assay. Cell viability was measured in 96-well plates according to the instructions of the CCK-8 assay kit (Beyotime Institute of Biotechnology). In brief, 10 μl CCK-8 reagent was added to each well of the plate and the cells were cultured at 37˚C for 3 h in the absence of light. Finally, the absorbance of each well at 450 nm was measured using a microplate reader.

Trypan blue assay. Following digestion of the H9c2 cells with trypsin solution (Beijing Solarbio Science & Technology Co., Ltd.), Trypan blue reagent (Beijing Solarbio Science & Technology Co., Ltd.) was added to the cell mixture. After 3 min at room temperature, 20 μl cell suspension was put on a cell counter plate and inserted into a cell counter to analyze the survival status of the cells.

Reverse transcription-quantitative PCR (RT-qPCR) analysis. After treatment, RNA was extracted from the cells using TRIzol® reagent (Invitrogen; Thermo Fisher Scientific, Inc.), and the RNA was reverse transcribed into cDNA using an iScript™ cDNA Synthesis Kit (Bio-Rad Laboratories, Inc.) in 70˚C for 5 min. Then an SYBR Green PCR kit (Bio-Rad Laboratories, Inc.) was used for qPCR with the following thermocycling conditions: 95˚C for 5 min, 95˚C for 15 sec and 60˚C for 45 sec, for 40 cycles. The relative expression of the target gene was calculated by the $2^{-\Delta\Delta Cq}$ method with GADPH normalization (24). The primers used were GAPDH forward: 5'-GGGTGTGAACCACGAGAAAT-3' and reverse: 5'-ACTGTGGTCATGAGCCCTTC-3'; sirt 1 forward: 5'-CCAGACCTCCCAGACCCTCAAG-3' and reverse: 5'-GTGACACAGAGACGGCTGGAAC-3'.

Western blot analysis. The cells were digested with trypsin, collected and then lysed at low temperature or on ice with a mixture of radioimmunoprecipitation assay buffer and phenylmethylsulfonyl fluoride (both from Beyotime Institute of Biotechnology). After lysis for 30 min, the supernatant was obtained by centrifugation at 4˚C and 12,000 g for 15 min. The protein lysate (30 μg protein/lane) was subjected to 10-15% SDS-PAGE, and the gel was transferred to a polyvinylidene fluoride membrane after electrophoresis. The membranes were blocked in QuickBlock™ Blocking Buffer for Western Blot (Beyotime Institute of Biotechnology) for 10 min at room temperature, and then incubated overnight at 4˚C with primary antibodies targeting P62 (18420-1-Ag; 1:5,000; Proteintech Group, Inc.), Beclin 1 (11306-1-Ap; 1:1,000; Proteintech Group, Inc.), GADPH (60004-1-Ig; 1:50,000; Proteintech Group, Inc.), microtubule-associated protein 1A/1B-light chain 3 (LC3; cat. no. 206019; 1:1,000; Zen-Bio, inc.) and sirt 1 (ab189494; 1:1,000; Abcam). Following primary antibody incubation, clean the membrane three times with TBST for 10 min each time. Subsequently, the membrane was incubated with the secondary rabbit antibody (ab205718; 1:10,000; Abcam) at room temperature for 1 h. After incubation, the membrane was washed three times with Tris-buffered saline containing 0.01% Tween-20 for 10 min each time. Finally, a GelView 6000M System (BoLuTeng, China) was used to capture images of the membrane. ImageJ software v1.8.0 (National Institutes of Health) was used to densitometrically analyze the blots (27).

Statistical analysis. Each group of experiments was repeated three times, and all data are presented as the mean ± standard deviation. The data were analyzed using GraphPad Prism version 9.0 (Dotmatics). One-way ANOVA followed by Tukey's

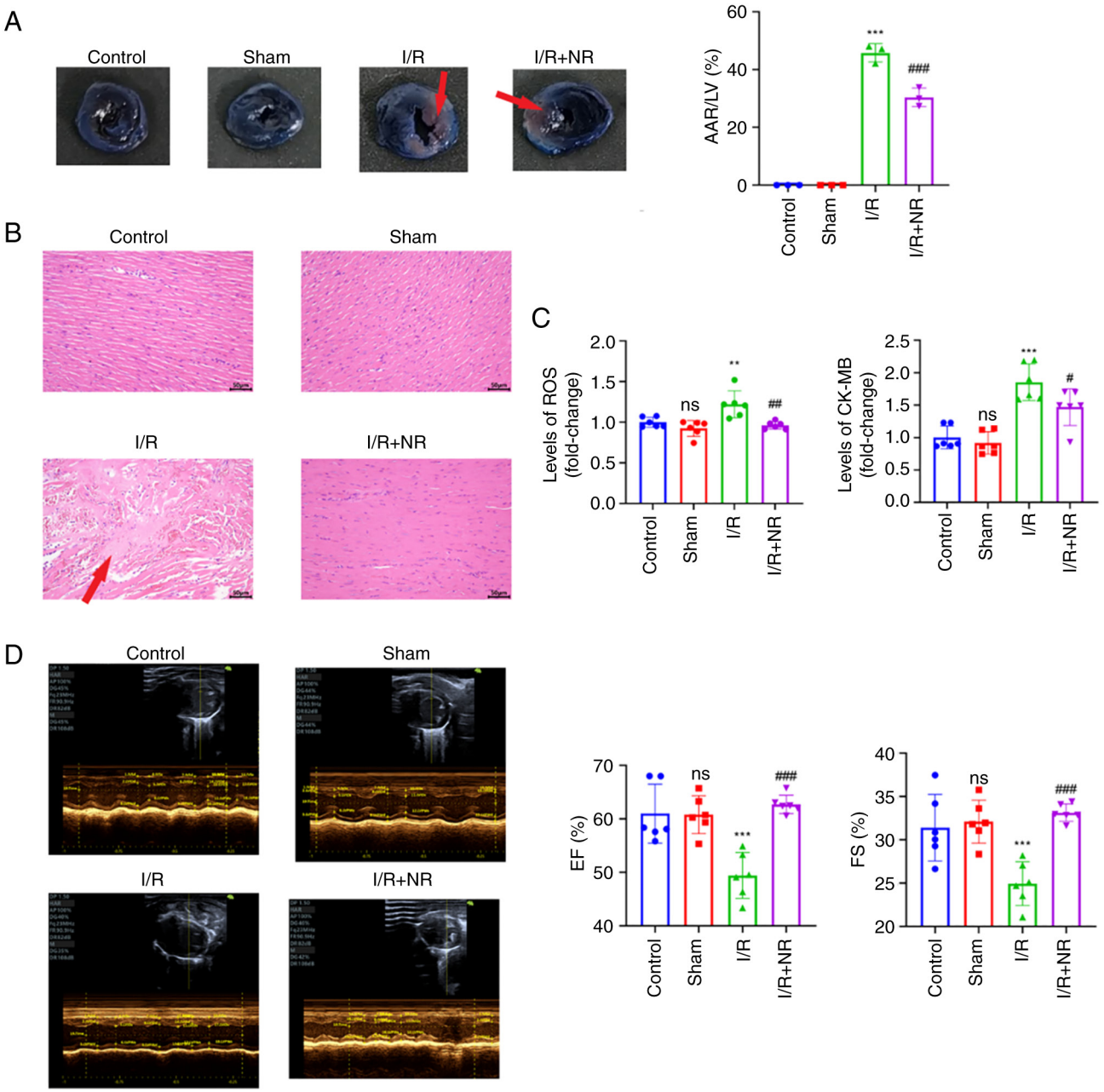

Figure 1. NR treatment ameliorates I/R injury in mice. (A) Representative images of Evan's blue and triphenyl tetrazolium chloride staining, and the ratio of the AAR to the LV area in each group. The red arrows indicate the infarcted area. (B) Representative images of hematoxylin and eosin staining (scale bar, 50 μm). The red arrow indicates the lesion area. (C) NR treatment reduces the levels of ROS and CK-MB in mice after I/R injury. (D) Representative echocardiographic images of each group and the changes in EF and FS in each group. **P<0.01 and ***P<0.001 vs. Sham group. $^{\#}$P<0.05, $^{\#\#}$P<0.01 and $^{\#\#\#}$P<0.001 vs. the I/R group. NR, nicotinamide riboside; I/R, ischemia/reperfusion; AAR, area at risk; LV, left ventricular; ROS, reactive oxygen species; CK-MB, creatine kinase myocardial band; EF, left ventricular ejection fraction; FS, fractional shortening; ns, not significant.

post hoc test was used to compare data among groups. P<0.05 was considered to indicate a statistically significant difference.

Results

NR alleviates the effects of myocardial I/R injury in mice. To explore the potential of NR supplementation in the treatment of I/R, an I/R model with NR pre-treatment was established in mice. Following euthanasia of the mice, Evan's blue/TTC staining and pathological staining were performed to observe the area of myocardial infarction and the histopathological changes in the mice. The Evan's blue/TTC staining results in Fig. 1A show that treatment with NR significantly reduced the AAR following myocardial I/R injury in mice. In addition, H&E staining revealed that the NR treatment ameliorated the pathological changes induced by I/R injury in the mouse heart (Fig. 1B). Under an optical microscope, it was observed that the structure of the heart tissue in the control and sham groups was normal, with orderly and uninterrupted arrangement of the myocardial fibers, no inflammatory cell infiltration and no evident pathological changes. However, the hearts of the I/R group had a large number of broken myocardial fibers that had a loose and disorderly appearance, with an increased distance between myocardial fibers compared with those in

the control and sham groups, accompanied by bleeding and inflammatory cell infiltration. The heart tissue structure of the I/R + NR group was almost normal, with more orderly myocardial fibers, mild edema, and only a small amount of bleeding and inflammatory cell infiltration. Measurement of the ROS and CK-MB levels in the mice revealed that treatment with NR prior to I/R significantly reduced the levels of ROS and CK-MB compared with those in the I/R group (Fig. 1C). In addition, echocardiography demonstrated that the pretreatment of the mice with NR significantly increased the EF and FS compared with those in the I/R group (Fig. 1D). These results indicate that NR has a protective effect against myocardial I/R injury.

Cytotoxicity of NR to H9c2 cells and its protective effect against H/R injury. To explore the optimal concentration of NR for H9c2 cells, the cells were first treated with different concentrations of NR, and cell viability was detected by CCK-8 assay. As shown in Fig. 2A, all concentrations of NR had no significant effect on cell viability. Secondly, the same concentrations were used to explore the optimal concentration of NR for protection against H/R injury. As Fig. 2B shows, concentrations from 1 to 10 mM had a significant protective effect, which increased as the concentration of NR increased. A concentration of 10 mM was selected for use in subsequent assays

NR prevents the H/R-induced injury of H9c2 cardiomyocytes. H/R is recognized to simulate the pathological state of myocardial cell I/R injury (27). A blank control group was established and cells were exposed to H/R in a medium with or without NR for 24 h. The results of Trypan blue staining indicated that cell viability was significantly decreased under H/R conditions compared with those in the control group, while the viability of cells treated with NR and exposed to H/R was significantly higher than that of the cells exposed to H/R without NR treatment, as shown in Fig. 3A. In addition, the level of LDH in the H/R group was significantly higher than that in the control group, while the level of LCH in the H/R + NR group was significantly reduced compared with that in the H/R group (Fig. 3B). An increase in CK-MB can be regarded as a marker of myocardial damage (16). Subsequently, an ELISA was used to detect the CK-MB level of the cells. The CK-MB level was significantly increased under H/R conditions compared with that in the control group, while the H/R-induced increase in the level of CK-MB was reversed by NR treatment (Fig. 3C). These findings indicate that NR treatment protects against the H/R injury of H9c2 cardiomyocytes.

NR alleviates oxidative stress in H9c2 cardiomyocytes. Myocardial oxidative stress is regarded as an early marker of myocardial I/R injury (24). Therefore, ROS, MDA and SOD levels were detected using ELISAs to evaluate the oxidative stress level of the cells. It was found that the levels of ROS and MDA in the H/R group were significantly increased compared with those in the control group, while the levels of all three variables were significantly decreased in the H/R + NR group compared with those in the H/R group, the level of SOD is contrary (Fig. 4). These results demonstrate the ability of NR to alleviate the oxidative stress caused by I/R injury in H9c2 myocardial cells.

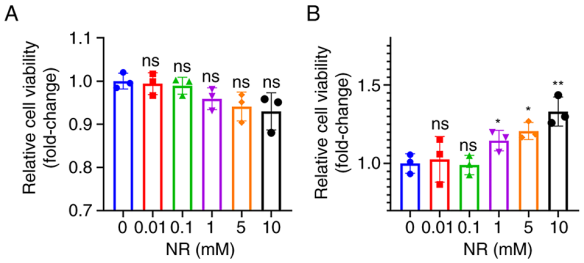

Figure 2. Cytotoxicity and protective effect of NR against hypoxia/reoxygenation-induced toxicity in H9c2 cardiomyocytes. (A) Cytotoxicity of NR in H9c2 cardiomyocytes. (B) Effect of different concentrations of NR on the cytotoxicity induced by hypoxia/reoxygenation activity in H9c2 cells. Cell viability was determined via Cell Counting Kit-8 assay. *P<0.05 and **P<0.01 vs. the vehicle control group. NR, nicotinamide riboside; ns, not significant.

NR increases sirt 1 expression and regulates autophagy. Sirt 1 is a deacetylase that relies on NAD^+ activation (21). Therefore, the NAD^+ content of the H9c2 myocardial cells was first detected and then the expression of sirt 1 was detected using RT-qPCR and western blotting to determine whether NR increases sirt 1 expression. The results of an NAD^+ ELISA (Fig. 5A) showed that pretreatment with NR increased the content of NAD^+ in the H/R exposed cells, and RT-qPCR and western blotting results showed a significant concurrent increase in sirt 1 expression (Fig. 5B). Secondly, through the preliminary detection of autophagy-associated protein expression, it was found that supplementation of the culture medium with NR regulated autophagy. The protein levels of P62, Beclin 1 and LC3II/LC3I in the H/R group were increased compared with those in the control group, and decreased in the H/R + NR group compared with those in the H/R group (Fig. 5C). These results showing that NR treatment reduces the protein levels of P62, beclin 1 and LC3II indicate that NR has the ability to regulate autophagy.

NR upregulates sirt 1 to improve the protective effect of autophagy on H/R injury in H9c2 cardiomyocytes. Sirt 1 is an NAD^+-dependent deacetylase, and NAD^+ is its exclusive substrate (28,29). As aforementioned, Sirt 1 has been shown to regulate autophagy. However, it is unclear whether NR regulates autophagy through sirt 1. Therefore, a sirt 1 inhibitor (EX527) was used to investigate the underlying mechanism. Western blotting showed that NR significantly increased expression of sirt 1 in the H9c2 cells under H/R conditions, and the use of EX527 with H/R and NR significantly reduced the content of sirt 1 in the cells (Fig. 6A). In addition, as shown in Fig. 6B, NR significantly reduced the protein levels of beclin 1, P62 and LC3II/LC3I in H9c2 cardiomyocytes challenged with H/R, while the addition of EX527 significantly attenuated the NR-induced reduction in the expression levels of all proteins under these conditions. However, the use of EX527 did not significantly change the levels of ROS in the NR treated cells under H/R conditions (Fig. 6C). Therefore, it may be concluded that NR protects H9c2 cardiomyocytes from H/R injury by the upregulation of sirt 1, but the upregulation of sirt 1 does not ameliorate oxidative stress.

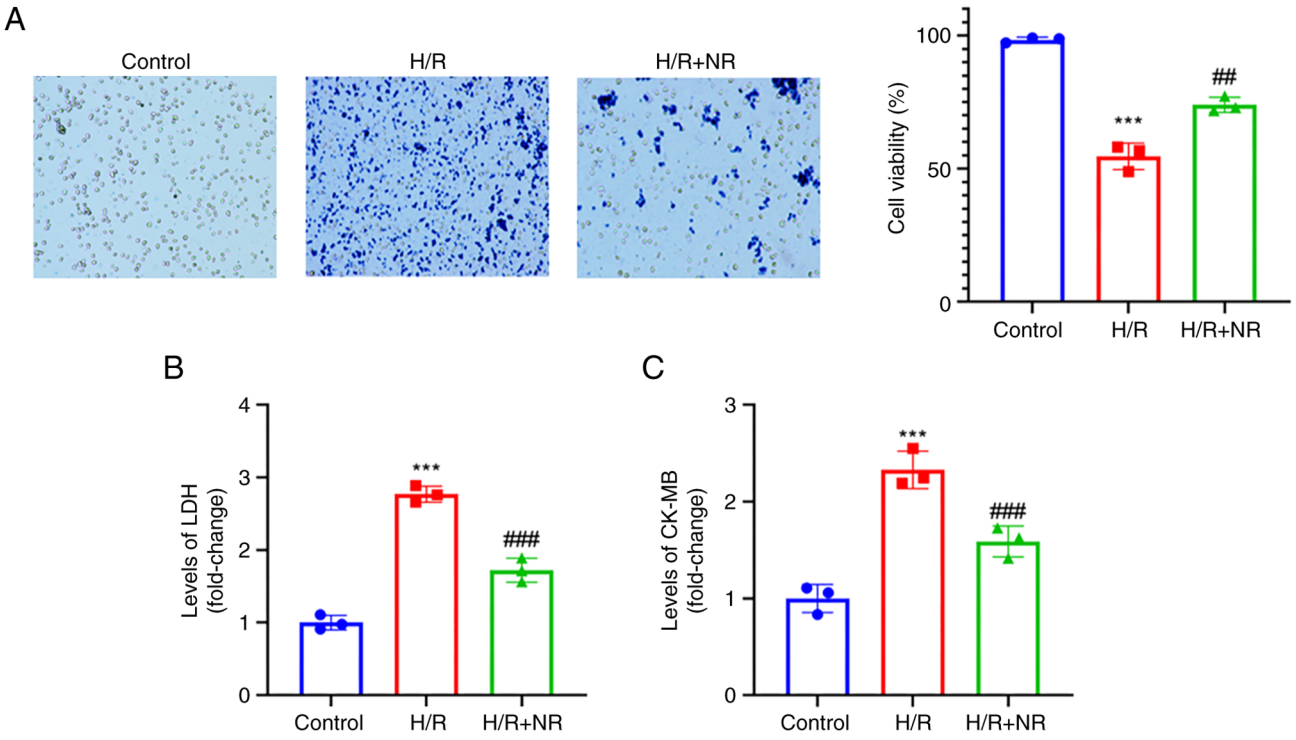

Figure 3. NR prevents H/R-induced damage in H9c2 cardiomyocytes. (A) NR pretreatment reduces the mortality rate of H9c2 cells after H/R injury as determined using Trypan blue staining. Representative images are shown. NR alleviates the exudation of (B) LDH and (C) CK-MB. ***P<0.001 vs. control; $^{\#\#}$P<0.01 and $^{\#\#\#}$P<0.001 vs. H/R group. NR, nicotinamide riboside; H/R, hypoxia/reperfusion; LDH, lactate dehydrogenase; CK-MB, creatine kinase myocardial band.

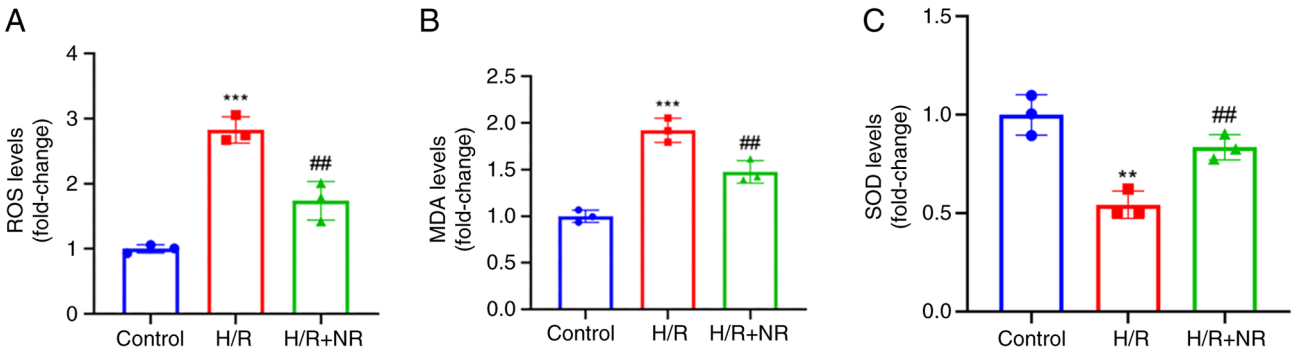

Figure 4. NR mitigates oxidative stress in H/R-challenged H9c2 cardiomyocytes. NR pretreatment reduces the levels of (A) ROS, (B) MDA and (C) SOD. **P<0.01 and ***P<0.001 vs. control; $^{\#\#}$P<0.01 vs. the H/R group. NR, nicotinamide riboside; H/R, hypoxia/reperfusion; ROS, reactive oxygen species; MDA, malondialdehyde; SOD, superoxide dismutase.

Discussion

Myocardial I/R injury is commonly associated with cardiac surgery, coronary artery bypass grafting, coronary artery thrombolysis and coronary artery occlusive stent recanalization (30,31). As the incidence rate of myocardial infarction has increased and coronary interventions have matured, myocardial I/R has gradually become a frequent pathophysiological phenomenon in clinical practice. Therefore, it has become urgently necessary to develop methods for the reduction of I/R injury.

Currently, only one study has investigated the role of NR in myocardial I/R injury. The analysis of combinations of promising cardioprotective compounds with various routinely used drugs, it was concluded that NR may have a protective effect on myocardial I/R injury (32); however, there has not yet been any basic research on the application of NR in I/R injury.

NR is an NAD^+ intermediate and a precursor of NADH (33). NR has been shown to play an important role in insulin sensitivity and liver health (34,35). In addition, the supplementation of NR has been shown to prolong the lifespan of mice (23). The effect of NR on heart function has also been studied, and it was found that the supplementation of NR preserved the cardiac function of mice with dilated heart disease (26). Sirt 1 is an NAD^+-dependent enzyme that has been shown to serve a key role in autophagy (36). Autophagy is a protective reaction that occurs when cells are damaged. It removes denatured proteins and rupture aged organelles to maintain homeostasis within cells (37). However, it has been reported that during myocardial I/R, myocardial cells undergo excessive autophagy,

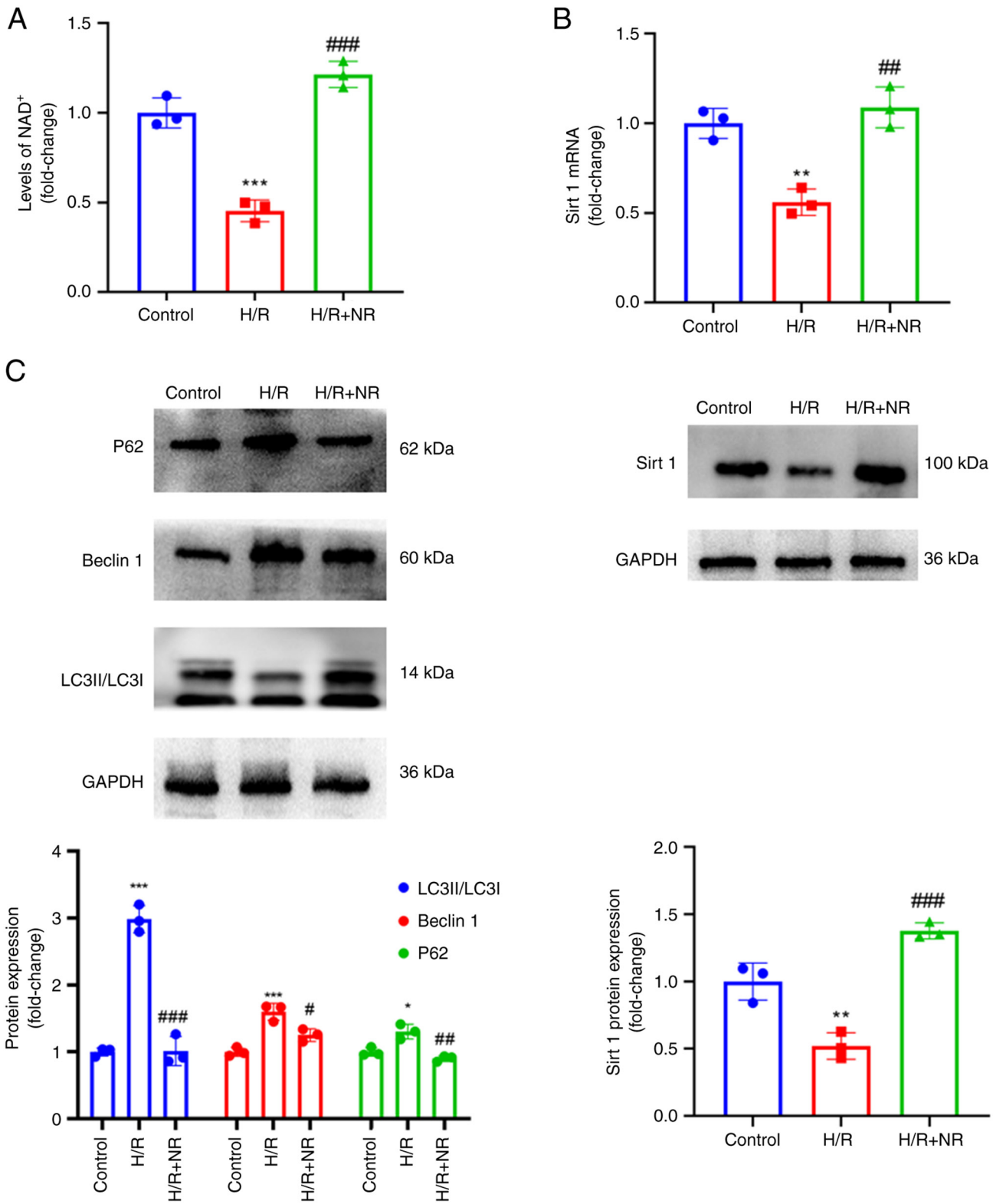

Figure 5. Effect of NR on the levels of NAD+, sirt 1 and autophagy markers in H/R-challenged H9c2 cardiomyocytes. (A) NR pretreatment increases NAD+ levels in H/R-challenged cells. (B) Quantitative evaluation of sirt 1 expression at the mRNA level. (C) Representative western blots and quantification of the expression levels of sirt 1 and the autophagy-associated proteins P62 and beclin 1, and the LC3II/LC3I ratio. The protein levels of the autophagy-associated proteins were reduced by NR pretreatment. *P<0.05, **P<0.01 and ***P<0.001 vs. control; $^{\#}$P<0.05; $^{\#\#}$P<0.01 and $^{\#\#\#}$P<0.001 vs. H/R. NR, nicotinamide riboside; NAD+, nicotinamide adenine dinucleotide; sirt 1, sirtuin 1; H/R, hypoxia/reperfusion; LC3, microtubule-associated protein 1A/1B-light chain 3.

resulting in the abnormal accumulation of autophagic material and anomalous degradation of proteins and organelles (38). In the present study, it was found that when H9c2 cardiomyocytes underwent H/R, the protein levels of P62, LC3II and Beclin 1 in the cells increased, indicating that autophagy occurred in the cardiomyocytes during H/R injury and the autophagic flux increased. When NR was supplemented prior to H/R, the protein levels of P62, LC3II and beclin 1 decreased. Moreover,

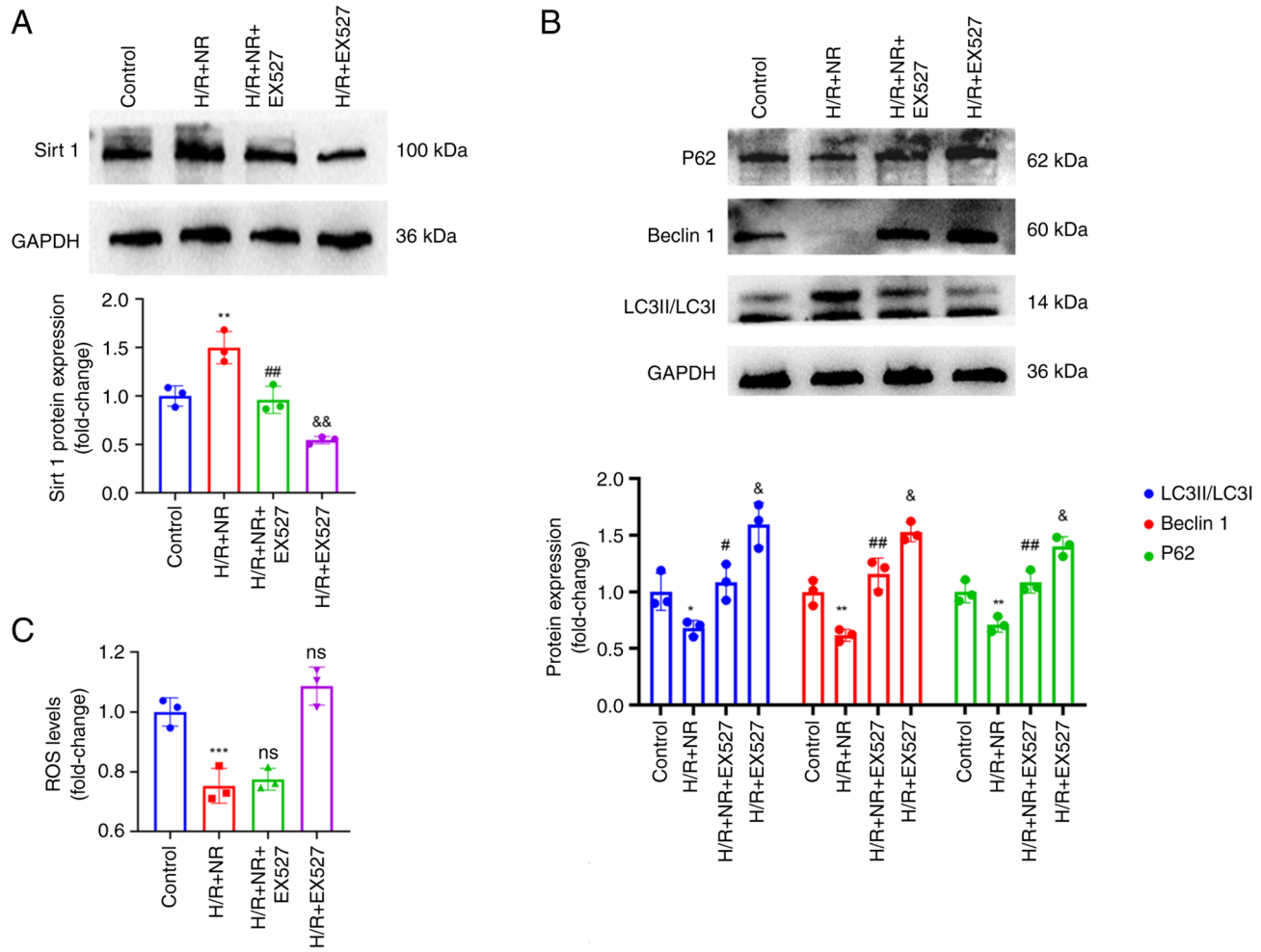

Figure 6. Sirt 1 inhibitor EX527 attenuates the protective effects of NR in H/R-challenged H9c2 cardiomyocytes. (A) Representative western blots and quantitative evaluation of the expression of sirt 1. (B) Representative western blots and quantitative evaluation of the protein expression of P62, beclin 1, LC3II/LC3I and GADPH. (C) Changes in ROS levels after the application of EX527. EX527 counteracts the effect of NR pretreatment in the reduction of autophagy-associated protein expression but has no significant effect on oxidative stress. *P<0.05, **P<0.01 and ***P<0.001 vs. H/R; $^{\#}$P<0.05 and $^{\#\#}$P<0.01 vs. H/R + NR; $^{\&}$P<0.05 and $^{\&\&}$P<0.01 vs. H/R + NR + EX527. Sirt 1, sirtuin 1; NR, nicotinamide riboside; dinucleotide; H/R, hypoxia/reperfusion; LC3 microtubule-associated protein 1A/1B-light chain 3; ns, not significant.

the present study has demonstrated that NR has a protective effect against H/R injury in H9c2 cardiomyocytes. This suggests that when cardiomyocytes undergo H/R injury, excessive autophagy causes cell damage, and NR plays a protective role via the inhibition of excessive autophagy. In addition, previous studies have shown that while autophagy is protective in myocardial I/R injury, excessive autophagy can exacerbate I/R injury, leading to an increase in cardiomyocyte apoptosis and greater cardiac dysfunction (39,40).

The mitochondrial ROS produced by the interaction between the dysfunctional respiratory chain and oxygen during I/R are nonspecific products (41). During tissue ischemia, the excessive production of mitochondrial ROS may be associated with inflammation and hypoxia, which lead to cell injury (42). One of the main mechanisms by which oxidative stress regulates cell damage is considered to be the opening of mitochondrial permeability transition pores (43). In the present study, it was found that under H/R conditions, cell viability was impaired and apoptosis occurred with elevated levels of LDH and CK-MB, accompanied by oxidative stress. A significant increase in ROS levels was observed following H/R, which is consistent with previous research results (44). NR, as a precursor of NAD^+, can directly participate in oxidative reduction, thereby reducing the level of oxidative stress in cardiomyocytes. In the present study, it was observed that after treatment with NR, the reduction in cell viability caused by H/R and the average reduction in inflammatory exudation were alleviated. Therefore, it may be concluded that NR serves a protective role in H/R injury via the alleviation of oxidative stress in myocardial cells.

Myocardial I/R injury comprises myocardial ischemia injury and reperfusion injury, and the latter may sometimes offset the benefits of treatment, or even aggravate the original myocardial injury. The mechanism of myocardial I/R injury is unclear, but oxidative stress is often considered an important factor (45). In the present study, NR was demonstrated to alleviate the damage induced by oxidative stress in H9c2 cardiomyocytes, with mitigation of damage via the regulation of autophagy through the regulation of sirt 1. EX527 treatment offset the protective effect of NR against H/R injury, indicating that the protective role of NR is mediated via the regulation of sirt 1. However, the lack

of sirt 1 inhibition had no significant effect on oxidative stress. It is prudent to consider carefully whether sirt 1 has no regulatory impact on oxidative stress, or whether the inhibitory effects of sirt 1 offset those on NAD^+ in NR treated cells without significant effect; further studies are required to investigate this.

One limitation of this study is that knockout mice were not used, which would have enhanced the comprehensiveness of the study. Secondly, sirt 1 levels were not assessed in cells treated with sirt 1 inhibitor-alone. In summary, despite these limitations, the present study demonstrates the potential of NR in providing protection against myocardial I/R injury.

In summary, the present research suggests that treatment with NR improved the cardiac function of mice with myocardial I/R injury, and reduced the generation of myocardial injury- and oxidative stress-associated biomarkers. NR may protect against autophagy by increasing the NAD^+ content in the body via the Sirt 1 pathway; however, the sirt 1 pathway does not appear to affect oxidative stress. These findings provide a novel potential approach for the clinical treatment of myocardial I/R 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

CY was responsible for the conception, design and quality control of the study, and reviewed and edited the manuscript. CY, HY and WL performed literature search, data extraction and statistical analyses, were major contributors to writing the manuscript . JY and YT participated in revising the manuscript and statistical analyses. CY and HY confirm the authenticity of all the raw data. All authors read and approved the final version of the manuscript.

Ethics approval and consent to participate

All protocols were approved by the Animal Care Ethics Committee of Jiangxi Zhonghong Boyuan Biotechnology Co., Ltd. (approval no. K-2023-0513-1). The validity of this approval was verified by the Ethics Committee of Nanchang University (Nanchang, China).

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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