

Protective effect of N-acetylcysteine activated carbon release microcapsule on myocardial ischemia-reperfusion injury in rats

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Abstract. With the development of science and technology, and development of artery bypass, methods such as cardiopulmonary cerebral resuscitation have been practiced in recent years. Despite this, some methods fail to promote or recover the function of tissues and organs, and in some cases, may aggravate dysfunction and structural damage to tissues. The latter is typical of ischemia-reperfusion (IR) injury. Lipid peroxidation mediated by free radicals is an important process of myocardial IR injury. Myocardial IR has been demonstrated to induce the formation of large numbers of free radicals in rats, which promotes the peroxidation of lipids within unsaturated fatty acids in the myocardial cell membrane. Markers of lipid peroxidation include malondialdehyde, superoxide dismutase and lactic dehydrogenase. Recent studies have demonstrated that N-acetylcysteine (NAC) is able to dilate blood vessels, prevent oxidative damage, improve immunity, inhibit apoptosis and the inflammatory response and promote glutathione synthesis in cells. NAC also improves the systolic function of myocardial cells and cardiac function, prevents myocardial apoptosis, protects ventricular remodeling and vascular remodeling, reduces opiomelanocortin levels in the serum and increases the content of nitric oxide in the serum, thus improving vascular endothelial function. Therefore, NAC has potent pharmacological activity; however, the relatively fast metabolism of NAC, along with its large clinical dose and low bioavailability, limit its applications. The present study combined NAC with medicinal activated carbons, and prepared N-acetylcysteine activated carbon sustained-release

microcapsules (ACNACs) to overcome the limitations of NAC. It was demonstrated that ACNACs exerted greater effective protective effects than NAC alone on myocardial IR injury in rats.

Introduction

Cardiovascular disease, particularly ischemic heart disease, has become a worldwide health problem affecting all economic groups of society (1). In recent years, many organs and tissues may have undergone reperfusion following ischemia with the establishment and promotion of treatment methods, including coronary artery bypass grafting, thrombolytic therapy, cardiac surgery extracorporeal circulation, cardiopulmonary cerebral resuscitation and organ transplantation (2-4). In most cases, ischemia-reperfusion (I/R) may help organs and tissues to repair themselves. However, sometimes I/R may also cause damage to them and became a serious threat to recovery. This phenomenon is called ischemia-reperfusion injury (5).

The World Health Organization predicts that by 2030, ischemic heart disease will become the second largest disease threatening human health (6). Myocardial ischemia caused by coronary artery infarction is the most important cause of ischemic heart disease (7). With social-economic development in China, changes to the living environment and lifestyle and the rise of the aging population, high incidence and mortality of chronic diseases, especially cardiovascular and cerebrovascular diseases, have been a heavy burden on society (8). Additionally, epidemiological studies showed that the age of onset tended to be younger in recent years (9).

Oxygen derived free radicals serve an important role in tissue injury during ischemia and reperfusion of the heart (10,11). There is substantial evidence that reactive oxygen including superoxide anion, hydrogen peroxide and hydroxyl radicals are responsible for myocardial injury during ischemia-reperfusion (12-14). MDA is a product of lipid peroxidation that indirectly reflects the generation of free radicals and injury degree of myocardial tissues (15); SOD is scavenging agent of superoxide radicals, which serves an important protective role in anti myocardial cell injury (16). At the same time, myocardial cell membrane lipid peroxidation increases cell

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membrane permeability and a large amount of LDH in cells is leaked into the intercellular space and body fluid (17).

N-acetylcysteine (NAC) is an acetyl compound of L-cysteine with an active mercapto group (18). In the past it has been used clinically as a mucolytic in respiratory diseases (19,20). More recently, studies have demonstrated that NAC dilates blood vessels, prevents oxidative damage, improves immunity, inhibits apoptosis and the inflammatory response and promotes the synthesis of glutathione in cells (21-25). With regard to the liver, NAC has been demonstrated to exert strong anti-fibrotic effects and preventative effects against fatty liver disease (26-28). With regard to the heart, NAC may improve the systolic function of myocardial cells and cardiac function, resist myocardial apoptosis, protect ventricular remodeling and vascular remodeling, reduce opiomelanocortin levels in the serum and improve the content of nitric oxide (NO) in the serum, and thus improve vascular endothelial function (29-33). Therefore, NAC has strong pharmacological effects (34,35), though its unfavorable effects include its relatively fast metabolism in the body, large clinical dose and low bioavailability, and side effects such as flush, nausea and vomiting (35-37). By contrast, medicinal activated carbons are sufficiently absorbed and biocompatible (35), and slowly release drugs during the metabolism of absorbed drugs, which overcomes the limitations of acetylcysteine and improves drug action time and bioavailability (38). The present study prepared N-acetylcysteine activated carbon sustained-release microcapsules (ACNACs) (39) by effectively combining NAC with medicinal activated carbons through an orthogonal experiment. The curative effect of ACNAC in rat liver has previously been documented (26,27,40-41). To determine the effect of ACNAC in the heart, the current study investigated the protective effect of ACNAC on myocardial ischemia-reperfusion (IR) injury in rats.

Materials and methods

Drugs and equipment. NAC was from Wuhan Grand Hoyo Co., Ltd. (Wuhan, China; batch number: 20110607); medicinal activated carbon was from Zhejiang Hangzhou Hangmu Timber Industry Co., Ltd. (Hangzhou, China; batch number: 120907) metoprolol tartrate injection was from Shandong East San Lu Pharmaceutical Co., Ltd. (Jining, China); malondialdehyde (MDA) assay kit (cat no. A003-1), superoxide dismutase (SOD) assay kit (cat no. A001-3), lactic dehydrogenase (LDH) assay kit (cat no. A020-2), NO assay kit (cat no. A012-1), NO synthase (NOS) assay kit (cat no. A014-1-1) and Coomassie brilliant blue protein assay kit were purchased from Nanjing Jiancheng Bioengineering Institute (Nanjing, China); a Sartorius BS124S precision balance and PowerLab biological signal acquisition and analysis system were from AD Instruments (ML880, Sydney, Australia); a HX-300 small animal respiratory ventilator was from Chengdu Taimeng Technology Co., Ltd. (Chengdu, China); an Electrothermal Constant-temperature Dry Box was from Tianjin City Taisite Instrument Co., Ltd. (Tianjin, China); and a rotary paraffin microtome was from Jinhua Yidi Medical Appliance Co. Ltd. (YD-1508, Jinhua, China).

Preparation of animal model. A total of 64 male Sprague-Dawley rats (weight, 226.835 ± 21.646 g; 45 days old) were used. Animals

were obtained from the Experimental Animal Center of the Zhejiang Academy of Medical Sciences (Hangzhou, China), and had access to a standard commercial diet and water *ad libitum* with the exception of preoperative fasting for 12 h. Rats were kept in rooms maintained at $22 \pm 1^\circ\text{C}$ under a 12-h light/dark cycle throughout the experiments. All experiments were carried out in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals (42), and approved by the Animal Care Committee of Xixi Hospital of Hangzhou Affiliated to Zhejiang University of Traditional Chinese Medicine (Hangzhou, China). The rats were randomly divided into the following eight groups ($n=8$ per group): Normal, sham, IR, metoprolol (Meto) control, NAC control, ACNAC1 (low-dose), ACNAC2 (moderate-dose) and ACNAC3 (high-dose).

The animals in groups ACNAC1, ACNAC2 and ACNAC3 were administered with 20, 40, 80 mg/kg ACNACs, respectively, via gavage. The animals in the NAC and Meto control groups were administered with an equivalent concentration of 80 mg/kg NAC and 20 mg/kg metoprolol solvent, respectively, via gavage. Normal saline of equivalent volume was administered to animals in the IR and sham groups. IR models were prepared 30 min after drug treatments, as described previously (43,44). All groups received ligature sutures, and the ligature in all groups excluding the normal and sham groups were tensioned after 5 min. The ligatures were loosened after 45 min, and limb lead electrocardiogram (ECG) and carotid blood pressure were observed for 2 h, after which a plastic tubing ball end a mosquito clamp was used to oppress and ligature the anterior descending left coronary artery. During the experiment, cyanosis in the left ventricular posterior wall and an increased ST section in a synchronous lead ECG were defined as signs of successful ligation, and a gradual change of cyanosis to red in the left ventricular posterior wall and 50% decrease in the ECG ST section were defined as signs of successful reperfusion. Rats were euthanized via exposure to gradually increasing concentrations of isoflurane and carbon dioxide gas (30% gradual-fill chamber vol/min) (45,46). Blood and heart tissue samples were then immediately stored at -80°C for later use. The number of animals used and their suffering was minimized. Following the procedure, animals were treated and specimens were prepared in strict accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals.

Determination of SOD, MDA and LDH indices. At 2 h after reperfusion, the heart was isolated and immediately washed with normal saline, and ~ 100 mg myocardial tissue from the ischemic area was cut and ground to obtain a 10% tissue homogenate. The mixture was homogenized 15 times at 4°C prior to centrifugation at $1,0625 \times g$ for 15 min at 4°C . The supernatant was retained. SOD activity and MDA content were determined with SOD and MDA assay kits, respectively, and measured with a UV visible light photometer, according to the manufacturer's instructions. At 2 h after reperfusion, abdominal aorta blood was also collected and centrifuged at $1,0625 \times g$ for 15 min at 4°C to obtain serum. LDH level was measured with a UV visible light photometer, according to the instructions of the LDH assay kit.

Determination of NO content and NOS activity. The reserved serum samples (stored at -80°C) were thawed at room

temperature and NO content was) was measured according to the kit instructions, based on measurements of optical density (OD) obtained using a 721 Spectrophotometer (Shanghai Optical Instrument Factory, Co., Ltd., Shanghai, China) at a wavelength of 550 nm. The following formula was used: NO content in serum ($\mu\text{mol/l}$)=(OD value of sample tube-OD value of blank tube)/(OD value of standard tube-OD value of blank tube)x standard tube concentration (20 $\mu\text{mol/l}$)x sample dilution.

The reserved heart samples were thawed at room temperature and myocardial tissue homogenate was prepared. The mixture was homogenized 15 times at 4°C prior to centrifugation at 10,625 x g for 15 min at 4°C. The supernatant was retained and the OD values of total NOS (TNOS) in the sample tube, inducible NOS (iNOS) in the sample tube and constitutive NOS (cNOS) in the sample tube were determined with a 721 Spectrophotometer at a wavelength of 530 nm. Protein content (in mg prot/l) in the sample tubes was also measured with the Coomassie brilliant blue kit. Measurements were obtained with a 721 Spectrophotometer at wavelength 595 nm. The following formula was used:

$$\text{TNOS activity(U/mg prot)} = \frac{\text{OD}_{\text{TNOS-Sample}} - \text{OD}_{\text{TNOS-Blank}}}{\text{Nanomolar extinction coefficient of colored compound}} \times \frac{\text{Total volume of reaction solution}}{\text{Sampling volume}} \times \frac{1}{\text{Length of light path} \times \text{Reaction time}} \div \text{Protein concentration (mgprot/l)}$$

$$\text{iNOS activity(U/mg prot)} = \frac{\text{OD}_{\text{iNOS-Sample}} - \text{OD}_{\text{iNOS-Blank}}}{\text{Nanomolar extinction coefficient of colored compound}} \times \frac{\text{Total volume of reaction solution}}{\text{Sampling volume}} \times \frac{1}{\text{Length of light path} \times \text{Reaction time}} \div \text{Protein concentration (l)}$$

$$\text{cNOS (U/mg prot)activity} = \text{TNOS (U/mg prot)activity} - \text{iNOS (U/mg prot)activity}$$

Recording of reperfusion arrhythmia duration. The judgment of arrhythmia on Lambeth, Conventions standards, and arrhythmia reperfusion scores were assigned according to the scoring system by Walker *et al* (47). The system is as follows: 0, No arrhythmia; 1, Accidental ventricular premature contraction, VPC; 2, Frequent VPC; 3, Accidental ventricular tachycardia, VT; 4, Frequent VT or Accidental Ventricular fibrillation (VF); and 5, Frequent VF or death.

Hematoxylin and eosin staining of myocardial tissues to detect pathological changes. Left ventricular anterior wall tissue from each group was treated in 4% paraformaldehyde for 24 h at room temperature, dehydrated with alcohol prepared according to a set gradient (75, 85, 90, 95 and 100%) and then embedded in paraffin blocks. Sections 4 μm thick were deparaffinated in dimethylbenzene, embedded in xylene I for 20 min, xylene II for 20 min, absolute ethyl alcohol I for 10 min, absolute ethyl alcohol II for 10 min, 95% alcohol for 5 min, 90% alcohol for 5 min, 80% alcohol for 5 min and 70% alcohol for 5 min successively prior to washing with water. Cell nuclei were stained with hematoxylin, and the cell cytoplasm was stained with eosin. Sections were then dehydrated and sealed with neutral resin. A light microscope (magnification, x200; Nikon Corporation; Tokyo, Japan) was

used to observe the structures of myocardial tissues from each group.

Statistical analysis. Measurement data were presented as the mean \pm standard deviation, and statistical analysis was performed with SPSS 16.0 software (SPSS, Inc., Chicago, IL, USA). One-way analysis of variance was used for comparison of multiple groups, and the LSD method was used as a post hoc test. $P < 0.05$ was considered to indicate a statistically significant difference.

Results

Determination of SOD, MDA and LDH indices. The SOD activation and MDA and LDH levels are presented in Fig. 1. Compared with the normal group, the IR group exhibited significantly reduced SOD (76.41 \pm 3.19 vs. 55.66 \pm 7.11 U/mg prot; $P < 0.05$) activation and significantly increased MDA (1.36 \pm 0.02 vs. 2.54 \pm 0.34 nmol/mg prot; $P < 0.05$) content and serum LDH (484 \pm 16 vs. 1,915 \pm 262 U/l; $P < 0.05$) levels; the difference in SOD (76.41 \pm 3.19 vs. 75.97 \pm 4.06 U/mg prot; $P > 0.05$) activation, MDA (1.36 \pm 0.02 vs. 1.27 \pm 0.03 nmol/mg prot; $P > 0.05$) and serum LDH levels (484 \pm 16 vs. 491 \pm 16 U/l; $P > 0.05$) in the Sham group was not significant; the NAC group exhibited significantly reduced SOD (76.41 \pm 3.19 vs. 67.07 \pm 7.60 U/mg prot; $P < 0.05$) activation and significantly increased MDA (1.36 \pm 0.02 vs. 1.73 \pm 0.23 nmol/mg prot; $P < 0.05$) content and serum LDH (484 \pm 16 vs. 917 \pm 61 U/l; $P < 0.05$) levels; the Meto group exhibited significantly reduced SOD (76.41 \pm 3.19 vs. 67.82 \pm 7.21 U/mg prot; $P < 0.05$) activation and significantly increased MDA (1.36 \pm 0.02 vs. 1.76 \pm 0.20 nmol/mg prot; $P < 0.05$) content and serum LDH (484 \pm 16 vs. 917 \pm 62 U/l; $P < 0.05$) levels; the ACNAC1 group exhibited significantly reduced SOD (76.41 \pm 3.19 vs. 60.43 \pm 5.89 U/mg prot; $P < 0.05$) activation and significantly increased MDA (1.36 \pm 0.02 vs. 1.84 \pm 0.17 nmol/mg prot; $P < 0.05$) content and serum LDH (484 \pm 16 vs. 1,128 \pm 177 U/l; $P < 0.05$) levels; the ACNAC2 group exhibited significantly reduced SOD (76.41 \pm 3.19 vs. 66.82 \pm 7.14 U/mg prot; $P < 0.05$) activation and significantly increased MDA (1.36 \pm 0.02 vs. 1.741 \pm 0.232 nmol/mg prot; $P < 0.05$) content and serum LDH (484 \pm 16 vs. 918 \pm 46 U/l; $P < 0.05$) levels; the difference in SOD (76.41 \pm 3.19 vs. 69.33 \pm 6.09 U/mg prot; $P > 0.05$) and MDA (1.36 \pm 0.02 vs. 1.57 \pm 0.13 nmol/mg prot; $P > 0.05$) content in the ACNAC3 group was not significant, while the serum LDH (484 \pm 16 vs. 818 \pm 49 U/l; $P < 0.05$) was significantly increased.

In the Sham group, SOD (75.97 \pm 4.06 vs. 55.67 \pm 4.29 U/mg prot; $P < 0.05$) activation was significantly increased and MDA (1.27 \pm 0.03 vs. 2.54 \pm 0.41 nmol/mg prot; $P < 0.05$) content and serum LDH (491 \pm 16 vs. 1,915 \pm 262 U/l; $P < 0.05$) levels were significantly reduced when compared with the IR group. In the NAC group, SOD (67.07 \pm 7.60 vs. 55.67 \pm 4.29 U/mg prot; $P < 0.05$) activation was significantly increased and MDA (1.73 \pm 0.23 vs. 2.54 \pm 0.41 nmol/mg prot; $P < 0.05$) content and serum LDH (917 \pm 61 vs. 1,915 \pm 262 U/l; $P < 0.05$) levels were significantly reduced when compared with the IR group. In the Meto group, SOD (67.82 \pm 7.21 vs. 55.67 \pm 4.29 U/mg prot; $P < 0.05$) activation was significantly increased and MDA (1.76 \pm 0.20 vs. 2.54 \pm 0.41 nmol/mg prot; $P < 0.05$) content and serum LDH (917 \pm 62 vs. 1,915 \pm 262 U/l; $P < 0.05$) levels were

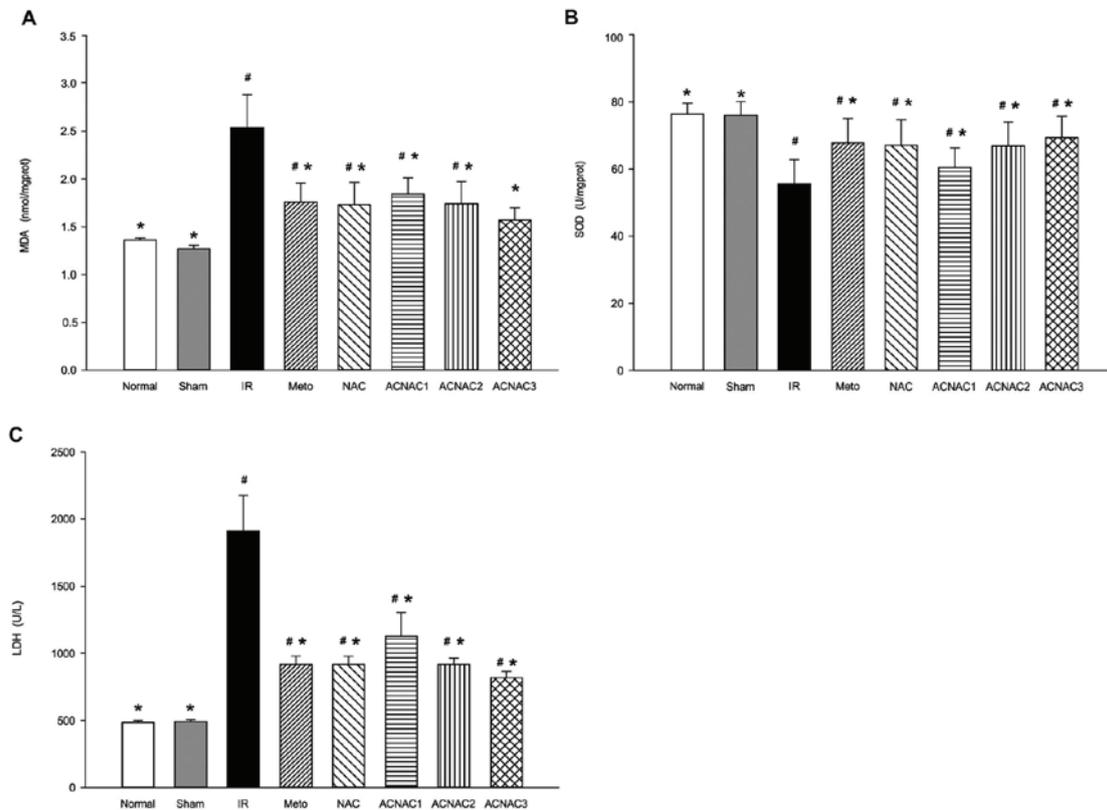


Figure 1. Effect of ACNAC on the activation of SOD, MDA and LDH in the myocardium of IR rats. (A) MDA content, (B) SOD activity and (C) LDH levels were determined following exposure of the rat groups to IR. Data are presented as the mean \pm standard deviation. * P <0.05 vs. IR group, # P <0.05 vs. normal group. SOD, superoxide dismutase; MDA, malondialdehyde; LDH, lactate dehydrogenase; IR, ischemia-reperfusion; Meto, metoprolol; NAC, N-acetylcysteine; ACNAC, N-acetylcysteine activated carbon release microcapsule; ACNAC1/2/3, ACNAC low/moderate/high-dose groups.

significantly reduced when compared with the IR group. In the ACNAC1 group, SOD (60.43 ± 5.89 vs. 55.67 ± 4.29 U/mg prot; $P < 0.05$) activation was significantly increased and MDA (1.84 ± 0.17 vs. 2.54 ± 0.41 nmol/mg prot; $P < 0.05$) content and serum LDH ($1,128 \pm 177$ vs. $1,915 \pm 262$ U/l; $P < 0.05$) levels were significantly reduced when compared with the IR group. In the ACNAC2 group, SOD (66.82 ± 7.14 vs. 55.67 ± 4.29 U/mg prot; $P < 0.05$) activation was significantly increased and MDA (1.74 ± 0.23 vs. 2.54 ± 0.41 nmol/mg prot; $P < 0.05$) content and serum LDH (918 ± 46 vs. $1,915 \pm 262$ U/l; $P < 0.05$) levels were significantly reduced when compared with the IR group. In the ACNAC3 group, SOD (70.49 ± 6.22 vs. 55.67 ± 4.29 U/mg prot; $P < 0.05$) activation was significantly increased and MDA (1.57 ± 0.15 vs. 2.54 ± 0.41 nmol/mg prot; $P < 0.05$) content and serum LDH (818 ± 49 vs. $1,915 \pm 262$ U/l; $P < 0.05$) levels were significantly reduced when compared with the IR group. The difference between the SOD (67.82 ± 7.21 vs. 67.07 ± 7.60 U/mg prot; $P > 0.05$) activation, MDA (1.76 ± 0.20 vs. 1.73 ± 0.23 nmol/mg prot; $P > 0.05$) content and serum LDH (917 ± 62 vs. 917 ± 61 U/l; $P > 0.05$) in the Meto and NAC groups was not significant. The difference between the SOD (67.82 ± 7.21 vs. 66.82 ± 7.14 U/mg prot; $P > 0.05$) activation, MDA (1.76 ± 0.20 vs. 1.74 ± 0.23 nmol/mg prot; $P > 0.05$) content and serum LDH (917 ± 62 vs. 918 ± 46 U/l; $P > 0.05$) in the Meto and ACNAC2 groups was not significant.

Determination of NO content and NOS activity. The NO content and NOS activity are presented in Fig. 2. Compared with the

normal group, the NO content of the IR group was significantly reduced (64.667 ± 3.31 vs. 17.41 ± 3.06 $\mu\text{mol/l}$; $P < 0.05$); the NO content of the Sham group was not significantly reduced (64.67 ± 3.31 vs. 64.60 ± 4.70 $\mu\text{mol/l}$; $P > 0.05$); the NO content of the NAC group was significantly reduced (64.67 ± 3.31 vs. 42.63 ± 3.78 $\mu\text{mol/l}$; $P < 0.05$); the NO content of the Meto group was significantly reduced (64.67 ± 3.31 vs. 43.44 ± 3.22 $\mu\text{mol/l}$; $P < 0.05$); the NO content of the ACNAC1 group was significantly reduced (64.67 ± 3.31 vs. 32.94 ± 5.01 $\mu\text{mol/l}$; $P < 0.05$); the NO content of the ACNAC2 group was significantly reduced (64.67 ± 3.31 vs. 43.13 ± 3.66 $\mu\text{mol/l}$; $P < 0.05$); the NO content of the ACNAC3 group was significantly reduced (64.67 ± 3.31 vs. 47.08 ± 2.83 $\mu\text{mol/l}$; $P < 0.05$).

Compared with the IR group, the NO content of the Sham group was significantly increased (17.41 ± 3.06 vs. 64.60 ± 4.70 $\mu\text{mol/l}$; $P < 0.05$); the NO content of the NAC group was significantly increased (17.41 ± 3.06 vs. 42.63 ± 3.78 $\mu\text{mol/l}$; $P < 0.05$); the NO content of the Meto group was significantly increased (17.41 ± 3.06 vs. 43.44 ± 3.22 $\mu\text{mol/l}$; $P < 0.05$); the NO content of the ACNAC1 group was significantly increased (17.41 ± 3.06 vs. 32.94 ± 5.01 $\mu\text{mol/l}$; $P < 0.05$); the NO content of the ACNAC2 group was significantly increased (17.41 ± 3.06 vs. 43.13 ± 3.66 $\mu\text{mol/l}$; $P < 0.05$); and the NO content of the ACNAC3 group was significantly increased (17.41 ± 3.06 vs. 47.08 ± 2.83 $\mu\text{mol/l}$; $P < 0.05$).

Regarding NOS activity, the activity of TNOS (0.205 ± 0.008 vs. 0.706 ± 0.006 U/mg prot; $P < 0.05$) and cNOS (0.193 ± 0.014 vs. 0.677 ± 0.006 U/mg prot; $P < 0.05$) was significantly reduced

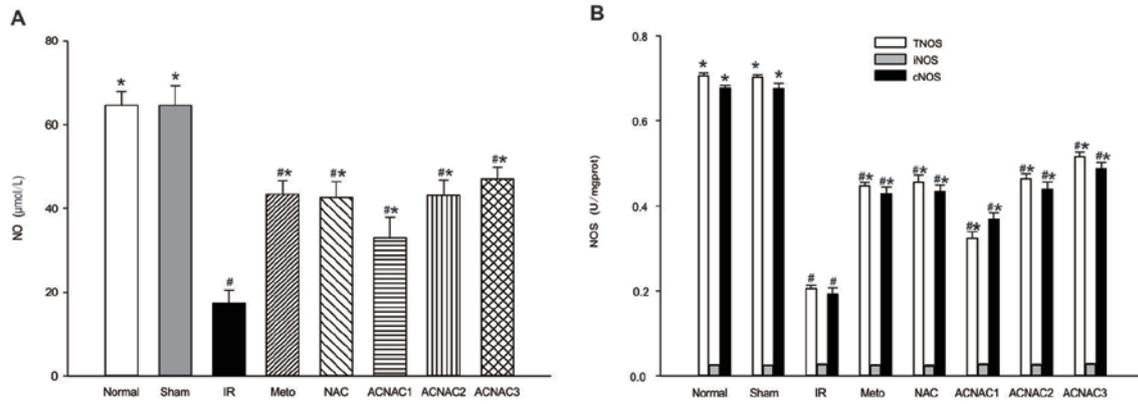


Figure 2. Effect of ACNAC on NO content and NOS activity in the myocardium of IR rats. (A) NO content; (B) TNOS, iNOS and cNOS activity. Data are presented as the mean \pm standard deviation. * $P < 0.05$ vs. IR group, # $P < 0.05$ vs. normal group. NO, nitric oxide; NOS, nitric oxide synthase; T/i/cNOS, total/inducible/constitutive NOS; IR, ischemia-reperfusion; Meto, metoprolol; NAC, N-acetylcysteine; ACNAC, N-acetylcysteine activated carbon release microcapsule; ACNAC1/2/3, ACNAC low/moderate/high-dose groups.

in the IR group when compared with the normal group, while iNOS activity did not differ significantly between the two groups (0.026 ± 0.002 vs. 0.025 ± 0.001 U/mg prot; $P > 0.05$). Regarding NOS activity, the activity of TNOS (0.703 ± 0.005 vs. 0.706 ± 0.006 U/mg prot; $P > 0.05$), cNOS (0.676 ± 0.012 vs. 0.677 ± 0.006 U/mg prot; $P > 0.05$) and iNOS (0.024 ± 0.001 vs. 0.025 ± 0.001 U/mg prot; $P > 0.05$) activity did not differ significantly between the Sham group and the normal group. Regarding NOS activity, the activity of TNOS (0.455 ± 0.017 vs. 0.706 ± 0.006 U/mg prot; $P < 0.05$) and cNOS (0.434 ± 0.015 vs. 0.677 ± 0.006 U/mg prot; $P < 0.05$) was significantly reduced in the NAC group when compared with the normal group, while iNOS (0.023 ± 0.003 vs. 0.025 ± 0.001 U/mg prot; $P > 0.05$) activity did not differ significantly between the two groups. Regarding NOS activity, the activity of TNOS (0.447 ± 0.008 vs. 0.706 ± 0.006 U/mg prot; $P < 0.05$) and cNOS (0.429 ± 0.015 vs. 0.677 ± 0.006 U/mg prot; $P < 0.05$) was significantly reduced in the Meto group when compared with the normal group, while iNOS (0.024 ± 0.002 vs. 0.025 ± 0.001 U/mg prot; $P > 0.05$) activity did not differ significantly between the two groups. Regarding NOS activity, the activity of TNOS (0.324 ± 0.015 vs. 0.706 ± 0.006 U/mg prot; $P < 0.05$) and cNOS (0.369 ± 0.015 vs. 0.677 ± 0.006 U/mg prot; $P < 0.05$) was significantly reduced in the ACNAC1 group when compared with the normal group, while iNOS (0.026 ± 0.002 vs. 0.025 ± 0.001 U/mg prot; $P > 0.05$) activity did not differ significantly between the two groups. Regarding NOS activity, the activity of TNOS (0.464 ± 0.011 vs. 0.706 ± 0.006 U/mg prot; $P < 0.05$) and cNOS (0.439 ± 0.017 vs. 0.677 ± 0.006 U/mg prot; $P < 0.05$) was significantly reduced in the ACNAC2 group when compared with the normal group, while iNOS (0.025 ± 0.002 vs. 0.025 ± 0.001 U/mg prot; $P > 0.05$) activity did not differ significantly between the two groups. Regarding NOS activity, the activity of TNOS (0.515 ± 0.011 vs. 0.706 ± 0.006 U/mg prot; $P < 0.05$) and cNOS (0.488 ± 0.014 vs. 0.677 ± 0.006 U/mg prot; $P < 0.05$) was significantly reduced in the ACNAC3 group when compared with the normal group, while iNOS (0.027 ± 0.002 vs. 0.025 ± 0.001 U/mg prot; $P > 0.05$) activity did not differ significantly between the two groups.

Compared with the IR group, the activity of TNOS (0.205 ± 0.008 vs. 0.703 ± 0.005 U/mg prot, $P < 0.05$) and cNOS (0.193 ± 0.014 vs. 0.676 ± 0.012 U/mg prot; $P < 0.05$) in the Sham

group was significantly increased, while iNOS (0.026 ± 0.002 vs. 0.024 ± 0.001 U/mg prot; $P > 0.05$) activity did not differ significantly between the two groups. Compared with the IR group, the activity of TNOS (0.205 ± 0.008 vs. 0.455 ± 0.017 U/mg prot; $P < 0.05$) and cNOS (0.193 ± 0.014 vs. 0.434 ± 0.015 U/mg prot; $P < 0.05$) in the NAC group was significantly increased, while iNOS (0.026 ± 0.002 vs. 0.023 ± 0.003 U/mg prot; $P < 0.05$) was significantly decreased. Compared with the IR group, the activity of TNOS (0.205 ± 0.008 vs. 0.447 ± 0.008 U/mg prot; $P < 0.05$) and cNOS (0.193 ± 0.014 vs. 0.429 ± 0.015 U/mg prot; $P < 0.05$) in the Meto group was significantly increased, while iNOS (0.026 ± 0.002 vs. 0.024 ± 0.002 U/mg prot; $P > 0.05$) activity did not differ significantly between the two groups. Compared with the IR group, the activity of TNOS (0.205 ± 0.008 vs. 0.324 ± 0.015 U/mg prot; $P < 0.05$) and cNOS (0.193 ± 0.014 vs. 0.369 ± 0.015 U/mg prot; $P < 0.05$) in the ACNAC1 group was significantly increased, while iNOS (0.026 ± 0.002 vs. 0.026 ± 0.002 U/mg prot; $P > 0.05$) activity did not differ significantly between the two groups. Compared with the IR group, the activity of TNOS (0.205 ± 0.008 vs. 0.464 ± 0.011 U/mg prot; $P < 0.05$) and cNOS (0.193 ± 0.014 vs. 0.439 ± 0.017 U/mg prot; $P < 0.05$) in the ACNAC2 group was significantly increased, while iNOS (0.026 ± 0.002 vs. 0.025 ± 0.002 U/mg prot; $P > 0.05$) activity did not differ significantly between the two groups.

Compared with the IR group, the activity of TNOS (0.205 ± 0.008 vs. 0.515 ± 0.011 U/mg prot; $P < 0.05$) and cNOS (0.193 ± 0.014 vs. 0.488 ± 0.014 U/mg prot; $P < 0.05$) in the ACNAC3 group was significantly increased, activity did not differ significantly between the two groups.

Effect of ACNACs on the duration and score of reperfusion arrhythmia. The reperfusion arrhythmia scores are presented in Fig. 3. The reperfusion arrhythmia score of the IR group was significantly increased when compared with the normal group (4.16 ± 0.57 vs. 0 min; $P < 0.05$). The difference in reperfusion arrhythmia score of the Sham group when compared with the normal group was not significant (0.34 ± 0.05 vs. 0 min; $P > 0.05$). The reperfusion arrhythmia score of the NAC group was significantly increased when compared with the normal group (2.25 ± 0.60 vs. 0 min; $P < 0.05$). The reperfusion

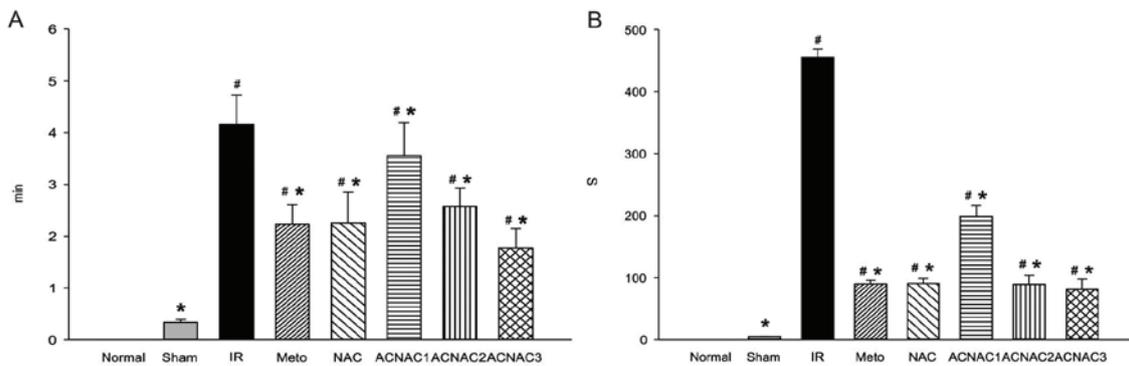


Figure 3. Effect of ACNAC on the duration and score of reperfusion arrhythmia. (A) Duration of reperfusion arrhythmia; (B) score of reperfusion arrhythmia. Data are presented as the mean \pm standard deviation. * $P < 0.05$ vs. IR group, # $P < 0.05$ vs. normal group. IR, ischemia-reperfusion; Meto, metoprolol; NAC, N-acetylcysteine; ACNAC, N-acetylcysteine activated carbon release microcapsule; ACNAC1/2/3, ACNAC low/moderate/high-dose groups; S, score.

arrhythmia score of the Meto group was significantly increased when compared with the normal group (2.22 ± 0.40 vs. 0 min; $P < 0.05$). The reperfusion arrhythmia score of the ACNAC1 group was significantly increased when compared with the normal group (3.57 ± 0.63 vs. 0 min; $P < 0.05$). The reperfusion arrhythmia score of the ACNAC2 group was significantly increased when compared with the normal group (2.50 ± 0.43 vs. 0 min; $P < 0.05$). The reperfusion arrhythmia score of the ACNAC3 group was significantly increased when compared with the normal group (1.78 ± 0.36 vs. 0 min; $P < 0.05$).

The reperfusion arrhythmia score of the Sham group was significantly decreased when compared with the IR group (0.34 ± 0.05 vs. 4.16 ± 0.57 min; $P < 0.05$). The reperfusion arrhythmia score of the NAC group was significantly decreased when compared with the IR group (2.25 ± 0.60 vs. 4.16 ± 0.57 min; $P < 0.05$). The reperfusion arrhythmia score of the Meto group was significantly decreased when compared with the IR group (2.25 ± 0.60 vs. 4.16 ± 0.57 min; $P < 0.05$). The reperfusion arrhythmia score of the ACNAC1 group was significantly decreased when compared with the IR group (3.57 ± 0.63 vs. 4.16 ± 0.57 min; $P < 0.05$). The reperfusion arrhythmia score of the ACNAC2 group was significantly decreased when compared with the IR group (2.50 ± 0.43 vs. 4.16 ± 0.57 min; $P < 0.05$). The reperfusion arrhythmia score of the ACNAC3 group was significantly decreased when compared with the IR group (1.78 ± 0.36 vs. 4.16 ± 0.57 min; $P < 0.05$).

In addition, compared with the normal group, the duration of reperfusion arrhythmia in the IR group was significantly increased (0 vs. 446.56 ± 21.81 sec; $P < 0.05$). Compared with the normal group, the difference in duration of reperfusion arrhythmia in the Sham group was not significant (0 vs. 4.94 ± 0.49 sec; $P < 0.05$). Compared with the normal group, the duration of reperfusion arrhythmia in the NAC group was significantly increased (0 vs. 88.64 ± 9.85 sec; $P < 0.05$). Compared with the normal group, the duration of reperfusion arrhythmia in the Meto group was significantly increased (0 vs. 89.71 ± 5.87 sec; $P < 0.05$). Compared with the normal group, the duration of reperfusion arrhythmia in the ACNAC1 group was significantly increased (0 vs. 198.85 ± 17.92 sec; $P < 0.05$). Compared with the normal group, the duration of reperfusion arrhythmia in the ACNAC2 group was significantly increased (0 vs. 89.15 ± 14.89 sec; $P < 0.05$).

Compared with the normal group, the duration of reperfusion arrhythmia in the ACNAC3 group was significantly increased (0 vs. 84.71 ± 12.92 sec; $P < 0.05$).

In the Sham group, the duration of reperfusion arrhythmia was significantly reduced when compared with the IR group (4.94 ± 0.49 vs. 446.56 ± 21.81 ; $P < 0.05$). In the NAC group, the duration of reperfusion arrhythmia was significantly reduced when compared with the IR group (88.64 ± 9.85 vs. 446.56 ± 21.81 ; $P < 0.05$). In the Meto group, the duration of reperfusion arrhythmia was significantly reduced when compared with the IR group (89.71 ± 5.87 vs. 446.56 ± 21.81 ; $P < 0.05$). In the ACNAC1 group, the duration of reperfusion arrhythmia was significantly reduced when compared with the IR group (198.85 ± 17.92 vs. 446.56 ± 21.81 ; $P < 0.05$). In the ACNAC2 group, the duration of reperfusion arrhythmia was significantly reduced when compared with the IR group (89.15 ± 14.89 vs. 446.56 ± 21.81 ; $P < 0.05$). In the ACNAC3 group, the duration of reperfusion arrhythmia was significantly reduced when compared with the IR group (84.71 ± 12.92 vs. 446.56 ± 21.81 ; $P < 0.05$).

Pathological changes in the myocardial tissues. HE staining was used to identify pathological changes in the myocardium following ACNAC treatment and IR injury (Fig. 4). Cardiac muscle fibers in the normal and sham-operated groups (Fig. 4A and B) were complete and exhibited a regular arrangement, clear structure and uniform coloring. The morphology of the nucleus appeared normal, the cell membrane was complete, and no degeneration or necrosis of cells was observed. In the IR group (Fig. 4C), the cardiac muscle fibers exhibited uneven coloring and a disordered arrangement, and some cells were broken and necrotic. Rupture, dispersal and disappearance of the nucleus were also observed, and there was edema of the intercellular space and a high level of inflammatory cell infiltration. Compared with the IR group, the cardiac muscle fibers in the Meto and ACNAC3 groups (Fig. 4D and H) were relatively complete. Fiber arrangement was relatively ordered with uniform coloring, however, a few inflammatory cells had infiltrated and the intercellular space exhibited mild edema. Compared with the IR group, there was an improvement in non-uniform staining and disorganization of myocardial fibers and some of the damaged cells were reduced in the NAC, ACNAC1 and ACNAC2 group (Fig. 4E-G).

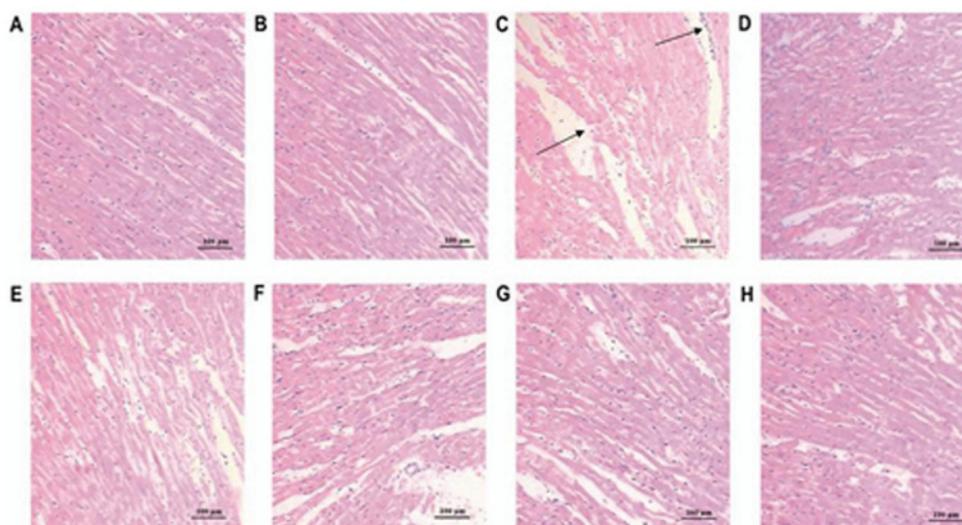


Figure 4. Hematoxylin and eosin staining of the myocardium from different rat groups (magnification, x200). (A) Normal group; (B) Sham group; (C) IR group; (D) Meto group; (E) NAC group; (F) ACNAC1 group; (G) ACNAC2 group; (H) ACNAC3 group. IR, ischemia-reperfusion; Meto, metoprolol; NAC, N-acetylcysteine; ACNAC, N-acetylcysteine activated carbon release microcapsule; ACNAC1/2/3, ACNAC low/moderate/high-dose groups.

Discussion

The majority of coronary heart diseases involving myocardial damage are caused by a reduction in the volume of coronary blood flow and imbalances in myocardial oxygen demand due to coronary artery lesion (48,49). Coronary heart disease is among the main diseases that threaten human health worldwide (50). At present, thrombolytic therapy, coronary artery intervention therapy and coronary artery bypass grafting are the more effective therapeutic strategies for acute myocardial infarction, and generally these technologies are capable of saving dying ischemic myocardial cells, decreasing infarct size and improving heart function (51). Treatment of myocardial reperfusion injury using the above methods is a current focus of research in the medical community (52,53). Lipid peroxidation mediated by free radicals is an important step during myocardial IR injury. It has been observed in rats that myocardial IR leads to the formation of a large number of free radicals (54-58). This promotes lipid peroxidation in unsaturated fatty acids of the myocardial cell membrane, and produces lipid peroxides that react with intracellular structures, including nucleic acid and proteins, which ultimately changes cell structure and function and leads to myocardial cell damage (59,60). The content of MDA is also increased, which interferes with the cell membrane and leads to greater damage to cell membrane structure and function (61). This typically manifests as a change in cell membrane fluidity and permeability, leakage of myocardial enzymes, reduction of cell membrane ATP activity, disorders of ion transportation and abnormal ion distribution (62). In turn, aberrant ion distribution due to intracellular calcium overload may stimulate after depolarizations and trigger activity, inducing arrhythmia and cardiac dysfunction (63). In addition, impairment of energy metabolism caused by ischemic myocardial necrosis and mitochondrial dysfunction may lead to a decrease in cardiac function (64). Therefore, the content of MDA may indirectly reflect the generation of oxygen free radicals and degree of tissue damage (65,66). LDH, as a specific enzyme

of the myocardial cytoplasm, is an additional marker of myocardial damage, as the leakage of LDH typically occurs only when the cell membrane is damaged. Thus the degree of LDH leakage may indirectly reflect the degree of myocardial damage (67,68). Furthermore, SOD is an intracellular antioxidant, which removes superoxide anions and protects the body against oxidative damage by free radicals, and thus the level of SOD may indirectly reflect the body's ability to scavenge oxygen free radicals and prevent lipid peroxidation (69,70).

Results of the present study indicated that compared with the IR group, SOD activation was significantly increased in rats of the ACNAC3 group, while MDA content and serum LDH were significantly decreased. SOD activation, MDA content and serum LDH levels did not differ significantly among the Meto, NAC and three ACNAC groups. These results suggest that ACNAC may inhibit peroxidation by free radicals and stimulate oxidase activation in myocardial tissue. This is a preliminary indication that lipid peroxidation mediated by free radicals is an important underlying mechanism of ACNAC regarding its alleviative role in myocardial ischemia injury.

NO, which is synthesized by a family of NOS enzymes, including neuronal, inducible, and endothelial NOS (n/i/eNOS), serves a key role in cardiovascular physiology and pathology (71). eNOS has been reported to inhibit the progression of myocardial infarction (72), ameliorate myocardial I/R injury (73) and left ventricular hypertrophy (74,75), and prevent the onset of heart failure (76). However, it remains controversial whether NO exerts a protective or cytotoxic effect in myocardial IR injury (77,78). Though as an important signal molecule in the body, a specific concentration range of NO is required to protect and maintain cardiac muscle cells and cardiac function (79-81).

L-arginine is the substrate of NOS, and the reaction generates citrulline and NO. In signaling pathways involving NO, NOS is a key rate-limiting enzyme. Due to its differential expression pattern in different tissues, NOS is as cNOS and iNOS, of which cNOS is also divided into nNOS and eNOS.

eNOS primarily serves roles in the regulation of arterial blood pressure and blood flow (82). eNOS is mainly expressed in myocardial cells, vascular endothelial cells, the endocardium and platelets, and it maintains physiological function through continuous synthesis of basic NO (82-84). iNOS is mainly expressed in endothelial and vascular smooth muscle cells (85,86). Previous studies have indicated that ischemia and reperfusion may induce endothelial cell damage and dysfunction and activate endogenous NOS inhibitors, thus reducing the total activity of NOS (82) and eNOS (87-90). When the activity of iNOS is increased, it may produce high levels of NO; however, the activation process of iNOS is relatively slow and requires 4-6 h to be expressed abundantly, reaching a peak some 48 (91-93).

It has been previously observed that in addition to the direct effect of NO on myocardial systolic function (94-96), NO influences myocardial cell oxygen metabolism (97), regeneration (98), hypertrophy (99) and apoptosis (100). It may also enhance the mechanical efficiency of the myocardium (101) and reduce myocardial oxygen consumption (102). In the process of IR, NO maybe a protective agent in the heart, and may prevent IR-related tissue damage (103).

NAC is a molecule containing a sulfhydryl group, and it may interact with the electrophilic group of reactive oxygen species, which generates a sulfhydryl intermediate. In this way, NAC serves a direct role as an antioxidant, alleviates oxidative stress-related injury to tissues and enhances the biological function of NO by preventing reactions between NO and free radicals (104,105).

Spectrophotometry data in the present study demonstrated that compared with the IR group, the NO content, TNOS and cNOS activity of the ACNAC3 group were significantly increased, while iNOS activity did not differ significantly. Due to the relatively long activation process of iNOS, the unaltered expression of iNOS may have been due to the short reperfusion period of 2 h used in the current study. These data suggest that ACNAC may alleviate myocardial ischemia injury by increasing the NO content of myocardial tissue, which may be initially increased by the promotion of TNOS activity in the myocardial tissue. ACNAC also reduced arrhythmia score and shortened reperfusion arrhythmia duration, which may be beneficial to the recovery of heart function and indicates the protective effects of ACNAC against arrhythmia.

The present study preliminarily concluded that the underlying mechanism of ACNAC regarding its alleviative role in myocardial reperfusion injury may be related to its effects on lipid peroxidation mediated free radicals, and the NO pathway. However, whether ACNAC is related to other mechanisms requires further comprehensive studies.

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