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Review Article

Effects of N-acetylcysteine combined with ischemic postconditioning on acute lung injury induced by diabetic myocardial ischemia-reperfusion in mice

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Abstract: To inquire into the effect of N-Acetylcysteine (NAC) combined with ischemic postconditioning (IPostC) on diabetic myocardial ischemia/reperfusion (MI/R) induced acute lung injury (ALI). Thirty healthy male mice were randomly divided into a sham operation (SO) group (n=10), an ischemia/reperfusion (I/R) group (n=10) and an ischemia postconditioning group (IPostC, n=10). The mice in the SO group did not receive any treatment after thoracotomy, those in the I/R group underwent 15 min of reperfusion after 60-minutes of ischemia, while those in the IPostC group received three cycles of 5-minute I/R immediately after 60 min of ischemia, followed by 15 min of reperfusion. The wet/dry lung weight ratio (W/D), total lung injury score, alveolar permeability index, myocardial infarction (MI) range, inflammatory factors, oxidative stress factors, vascular endothelial growth factor (VEGF) and HIF-1α levels were measured in each group. The W/D, total lung injury score, alveolar permeability index, MI range, monocyte chemoattractant protein-1 (MCP-1) and malondialdehyde (MDA) levels in the lung tissue were the highest in the I/R group, followed by the IPostc group, and the lowest in the SO group (P<0.05). As to superoxide dismutase (SOD), glutathione (GSH), C-reactive protein (CRP) and tumor necrosis factor-α (TNF-α), they presented at the highest levels in the SO group (P<0.05), seconded by the IPostC group, and the lowest in the I/R group (P<0.05). HIF-1α and VEGF in the IPostC group were noticeably elevated among all three groups, followed by the I/R and SO groups successively (both P<0.05). NAC combined with IPostC may improve the MI/R induced ALI in diabetic mice through the HIF pathway and antioxidation.

Keywords: Diabetic myocardial ischemia/reperfusion, acute lung injury, N-acetylcysteine combined with IPostC, HIF pathway

Introduction

MI/R injury refers to the phenomenon of alleviating the original ischemic injury in the tissue, where the structural damage or dysfunction is further aggravated when the blood flow is restored after prolonged ischemia [1]. Acute lung injury (ALI) is a term used to describe an acute diffuse lung injury and subsequent acute respiratory failure caused by various pathogenic factors outside of the heart [2, 3]. Studies have found that the incidence of myocardial ischemia in diabetic patients is 1.45-2.99 times higher than that in non-diabetic patients, and blood hyperviscosity can lead to hemodynamic disturbances in pulmonary microcirculation and damage to the pulmonary vascular wall, resulting in reduced lung function [4, 5]. Therefore, the study of pulmonary injury caused by MI/R in diabetic patients is of great clinical significance.

NAC is a highly effective free radical scavenger and antioxidant containing sulfhydryl groups. Recent studies have found that NAC has good effects on I/R injury [6, 7]. Ischemic postconditioning (IPostC) refers to a repeated 2-4 time application of transient local coronary ischemia before reperfusion after the myocardial ischemic injury attack, thus providing a certain protective effect on the normal myocardium [8]. The effects induced by IPostC share the same effect for not only on the myocardium, but also on the lungs [9, 10]. IPostC reduces the concen-

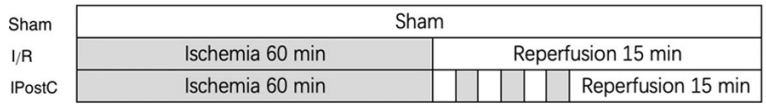

Figure 1. Grouping diagram. The gray area represents ischemic intervention, and the white area represents blood perfusion. Mice in the SO group received no treatment after thoracotomy, while those in the I/R group underwent 60 min of ischemia and 15 min of reperfusion. After 60 minutes of ischemic treatment in the IPostC group, three 5-minute I/R cycles were performed immediately, followed by 15-minute reperfusion.

tration of malondialdehyde (MDA), a lipid metabolite in lung tissues, and increases the activity of superoxide dismutase (SOD), an antioxidant free radical enzyme, which can reduce the oxidative damage of the lungs by anti-lipid peroxidation and inhibiting the production of oxygen free radicals [11]. Numerous studies have demonstrated that IPostC upregulates interleukin-6 (IL-6), TNF-α of pro-inflammatory cytokines and interleukin-10 (IL-10) an anti-inflammatory cytokine; thereby regulating the relative balance between pro- and anti-inflammatory responses, so as to reduce reperfusion injury and improve lung function [12].

Therefore, this study established a MI/R injury model to detect the degree of lung injury, oxidative injury and the expression of HIF signaling pathway-related proteins and inflammatory factors in three groups of mice. As well as to probe into the effects of NAC combined with IPostC on ALI induced by MI/R in diabetes, so as to provide references for clinical treatment in the future.

Methods

Materials and methods

Thirty male db/db and C57BL/6 mice, aged 15 weeks, were selected and bred in a SPF level laboratory in Xinjiang Medical University. The feeding environment was clean and well ventilated, with a room temperature of 20-25°C and a humidity of 55%. The mice were fed with SPF experimental mouse chow provided by Jiangsu Xietong Pharmaceutical Bioengiietongneering Co., Ltd, Nanjing, China. Animal model preparation: According to the principle of similar body weight, mice were equally grouped into a SO group, an I/R group and an IPostC group at random. This experiment was conducted with the approval of the hospital ethics committee, and the experimental process was in accordance with the Guide for the Care and Use of Experimental Animals [13].

MI/R injury model establishment

After thoracotomy, mice in the SO group were threaded only on the myocardium along the inferior edge of the left atrial appendage, but the left anterior descending coronary artery was not ligated. While those in the I/R group were ligated with the left anterior descending artery of the coronary artery along the lower margin of the left atrial appendage after thoracotomy, and then treated with I/R, and the time of ischemia was 60 min. After the ligation line was released, the reperfusion was continued for 15 minutes. In the IPostC group, the left anterior descending branch of the coronary artery was ligated along the inferior edge of the left atrial appendage after thoracotomy for 60 minutes, and three I/R cycles were performed immediately after the ligation line was loosened. The operation method was to loosen the ligature and perform reperfusion for 5 min, tighten the ligature and re-perfuse for 5 min, thus repeating three cycles in turn, and then loosen the ligature for a 15-minute reperfusion. The grouping diagram is shown in **Figure 1**.

Indicator detection

(1) Pathological examination of lung tissues: After successful modeling of mice in each group, the left lower lung lobes were removed and fixed, embedded in wax blocks, sectioned, stained with H&E and examined at 400x magnification. The lung injury degree was comprehensively assessed by the Smith scoring system [14], with the specific criteria of using 2 different sections of each specimen and mean values from 5 field/sections. Five indicators, including alveolar and interstitial inflammatory cell infiltration, pulmonary edema (PE), alveolar and interstitial hemorrhage, pulmonary atelectasis and formation of transparent membranes, were applied to score lung injury degree, with the corresponding manifestations and score as follows: normal pulmonary vessels, alveoli, interstitium and bronchus was scored as 0 points; the lesion area was less than 25% of the entire field of view (FOV) was scored as 1 point; the lesion range covered 25%-50% of the

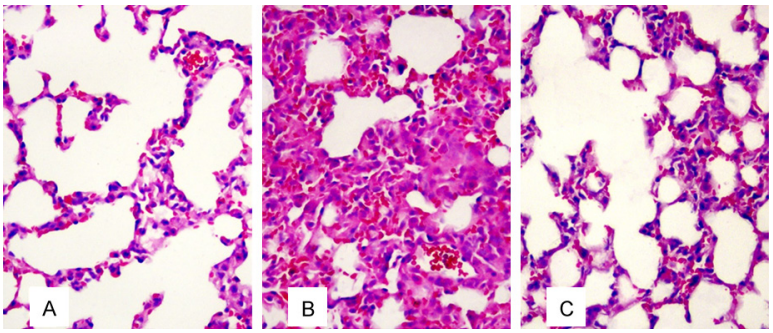

Figure 2. Lung histopathological observations in the sham operation group, I/R group and IPostC group (H&E x400). A. Alveolar epithelial cells were intact in the sham-operated group without inflammatory cell infiltration; B. In the I/R group, the cells are disorderly arranged, the alveolar septum was broken, and the interstitial exudation was severe; C. In the lung tissue of the IPostC group, the cell layer structure was basically normal, and most of the cell membranes were intact and smooth.

entire FOV was scored as 2 points; the lesion range covered 50%-75% of the entire FOV was scored as 3 points; and the lesion area covered greater than 75% of the entire FOV was scored as 4 points. The sum of the above items was taken as the total lung injury score. (2) The degree of PE was determined by W/D. After weighing the wet weight of the right lung tissue, it was put into a 60°C dryer for 48 hours to be dehydrated, and then the dry weight was weighed. The W/D was counted as an index of edema. (3) The alveolar permeability index was measured; that is, the ratio of bronchoalveolar lavage fluid (BALF) to plasma protein content was calculated. The BALF preparation method was used to clamp the right main bronchus with blood forceps, and repeatedly lavaged the left lung 4-5 times with 1 ml of sterile PBS buffer. Plasma preparation was performed by taking blood from the heart and centrifuging at 2500 rpm and 4°C for 5 min to obtain the supernatant. Plasma protein content was determined by the BCA method. (4) Detection of inflammatory factors: IL-6, TNF-α as pro-inflammatory, IL-10 as anti-inflammatory, in the plasma and BALF were detected by ELISA. (5) Detection of oxidative damage: MDA and SOD content in plasma and BALF were examined by ELISA. (6) Detection of proteins related to HIF signaling pathway: the upper lobe of the left lung was cryopreserved at -80°C, weighed, homogenized on ice with the lysate, and the protein was extracted from the supernatant by 12000 rpm centrifugation 15 min at 4°C. HIF-1α and VEGF were measured with Western Blot.

Statistical methods

The data were statistically processed with SPSS 22.0 (IBM, SPSS, Chicago, IL, USA), and the measurement data are described in the form of (x ± sd). One-way ANOVA was employed for inter-group comparisons, and least significant difference (LSD) was adopted for pairwise inter-group comparisons among multiple groups. $P<0.05$ indicated that there was a statistically significant difference.

Results

Histopathological observation of lung

H&E staining results of lung tissues of the three groups of mice showed clear lung structure, complete alveolar epithelial cells, no inflammatory cell infiltration, and basophilic nuclei in the sham operation group. In the I/R group, the alveolar structure was seriously damaged and the cells were arranged in a disordered way. The epithelial cells were infiltrated by inflammatory cells, and the alveolar septa was fractured. In addition, there was massive hyperemia and consolidation in the alveoli and alveolar walls, and severe interstitial exudation, and a large number of neutrophils and inflammatory cells were seen. The cell layer structure of the IPostC group was basically normal, and most of the cell membranes were intact and smooth (**Figure 2**).

Determination of PE degree

The IPostC and I/R groups exhibited noticeably enhanced W/D compared to the SO group ($P<0.05$), and the W/D in the IPostC group was markedly reduced compared with the I/R group ($P<0.05$) (**Figure 3**).

Comparison of total lung injury scores of mice in the three groups

The total score of lung injury was evidently elevated in the IPostC and I/R groups over the SO group ($P<0.05$). While that in the IPostC group was notably lower than the I/R group ($P<0.05$) (**Table 1**).

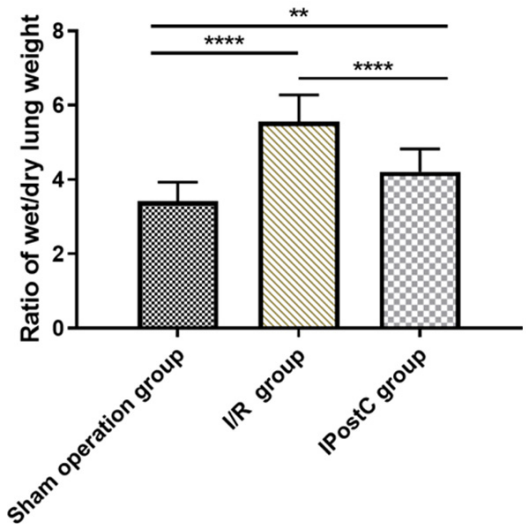

Figure 3. Comparison of PE degree of mice in the three groups. The lung tissue W/D in the I/R group and the IPostC group was significantly higher than that in the SO group (P<0.05), while the lung tissue W/D in the IPostC group was notably lower than that in the I/R group (P<0.05).

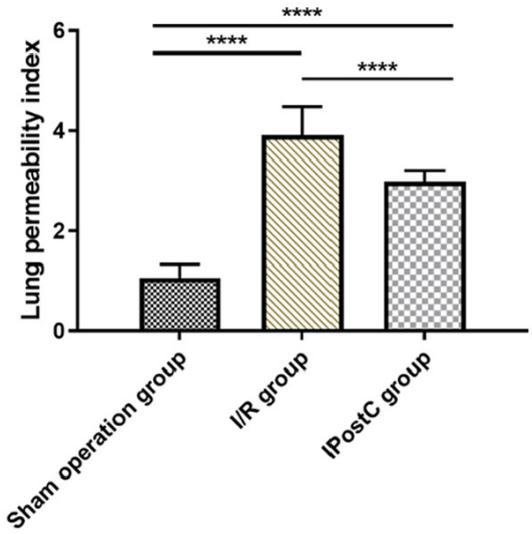

Figure 4. Comparison of alveolar permeability indexes of mice in the three groups. The alveolar permeability indexes of the lung tissues in the I/R group and the IPostC group were significantly higher than those in the SO group (P<0.05), while the alveolar permeability indexes in the IPostC group were noticeably lower than those in the I/R group (P<0.05).

Table 1. Comparison of total lung injury scores of mice in the three groups

Groups	
SO group (n=10)	1.87±0.24
I/R group (n=10)	5.23±0.46*
IPostC group (n=10)	3.87±1.12*,#
F	56.250
P	<0.01

Note: *indicates compared with the SO, P<0.05; #indicates compared with the I/R group, P<0.05.

Table 2. Comparison of MI ranges of mice in the three groups

Groups	MI range (%)
SO group (n=10)	44.37±4.06
I/R group (n=10)	57.35±5.21*
IPostC group (n=10)	48.24±5.21*,#
F	18.520
P	<0.01

Note: *indicates compared with the SO group, P<0.05; #indicates compared with the I/R group, P<0.05.

Determination of alveolar permeability indexes in the three groups

The pulmonary alveolar permeability index in the IPostC and I/R groups was boosted compared with the SO group (P<0.05). Comparasion between IPostC and I/R groups, revealed that the pulmonary alveolar permeability index in the former was dramatically lower than the latter (P<0.05) (**Figure 4**).

Comparison of MI ranges of mice in the three groups

The IPostC and I/R groups presented noticeably higher MI ranges than the SO group (P<0.05). Comparison between the IPostC and I/R groups, revealed that the MI range in the former group was evidently lower than that in the latter (P<0.05) (**Table 2**).

Comparison of inflammatory factor levels

The IPostC and I/R groups presented obviously down-regulated CRP and TNF-α over the SO group (P<0.05). Those in the IPostC group were dramatically elevated compared to the I/R group (P<0.05). In terms of MCP-1, it presented markedly enhanced levels in the IPostC and I/R groups over the SO group (P<0.05), and its level in the IPostC group was dramatically lower compared with the I/R group (P<0.05) (**Table 3**).

Determination of oxidative damage degree

Concerning oxidative damage degree, it was found that the SOD and GSH content in the IPostC and I/R groups were remarkably reduced compared to those in the SO group (P<0.05).

Table 3. Comparison of expression levels of inflammatory factors

Groups	CRP (mg/L)	TNF-α (ng/L)	MCP-1 (ng/L)
SO group (n=10)	42.15±5.33	65.87±6.23	7.18±0.91
I/R group (n=10)	23.35±0.88*	31.78±3.78*	21.56±2,87*
IPostC group (n=10)	32.78±3.45*,#	44.43±5.12*,#	15.68±1.89*,#
F	9.928	112.3	121.4
P	0.002	<0.01	<0.01

Note: *indicates compared with the SO group, P<0.05; #indicates compared with the I/R group, P<0.05.

Table 4. Comparison of oxidative damage degree among the three groups of mice

Groups	SOD (U/L)	GSH (U/L)	MDA (nmol/L)
SO group (n=10)	42.35±5.26	66.78±6.71	7.32±0.97
I/R group (n=10)	24.57±3.28*	30.42±3.41*	20.87±2,63*
IPostC group (n=10)	36.51±3.27*,#	45.27±5.16*,#	14.21±1.76*,#
F	50.16	120.4	125.7
P	<0.01	<0.01	<0.01

Note: *indicates compared with the SO group, P<0.05; #indicates compared with the I/R group, P<0.05.

Table 5. Comparison of the expression levels of HIF-1α and VEGF in the three groups of mice

Groups	VEGF	HIF-1α
SO group (n=10)	0.223±0.073	0.203±0.042
I/R group (n=10)	0.357±0.068*	0.314±0.061*
IPostC group (n=10)	0.513±0.087*,#	0.473±0.073*,#
F	36.07	51.09
P	<0.01	<0.01

Note: *indicates compared with the SO group, P<0.05; #indicates compared with the I/R group, P<0.05.

Comparison between the IPostC and I/R groups, revealed that the SOD and GSH content in the former were remarkably higher than those in the latter (P<0.05). As for MDA, its content in the IPostC was the most elevated among the three group, followed by the I/R and SO groups in succession (both P<0.05) (**Table 4**).

Comparison of HIF-1α and VEGF levels

The IPostC and I/R groups showed remarkably enhanced HIF-1α and VEGF over the SO group (P<0.05). Compared with the I/R group, the HIF-1α and VEGF in the IPostC group were greatly elevated (P<0.05) (**Table 5**; **Figure 5**).

Discussion

Being one of the primary causes of death in diabetic patients, ischemic heart disease is prone to cause MI/R injury during the process [15]. While in turn, MI/R injury releases a flood of oxygen free radicals and inflammatory factors acting on the lungs, which is a major cause of ALI [16, 17].

NAC is the precursor of intracellular reduced glutathione and has been used as a mucus solubilizer in the treatment of various respiratory diseases [18]. The latest studies have shown that NAC has other pharmacological effects, such as scavenging free radicals, reducing the production of inflammatory cytokines, chemokines and adhesion molecules, and is protective of MI/R injury [19, 20]. Also, it is well established that IPostC is an effective method to reduce MI/R injury [21]. Therefore here, the effect of NAC combined with IPostC on the improvement of MI/R induced ALI in diabetic rats was investigated. Lung injury degree, oxidative injury degree, expression of HIF signaling pathway related proteins and inflammatory factors in three groups of mice were compared. The basic pathological changes of pulmonary I/R injury are manifested in increased pulmonary capillary permeability, enhanced intravascular exudation, protein leakage, which is characterized by PE, leading to obvious disturbance of pulmonary gas exchange. Therefore, lung W/D, total score of lung injury and alveolar permeability indexes are important indicators to reflect PE, especially in identifying intra-alveolar edema and lung injury [22, 23]. In this study, the total scores of W/D, lung injury and alveolar permeability indexes were evidently higher in the I/R group, seconded by the IPostC group, and the lowest in the SO group, suggesting that NAC combined with IPostC could reduce MI/R induced ALI in diabetes, and was protective of I/R lung. The MI range in the IPostC and I/R groups was dramatically higher than that in the SO group (P<0.05), while that in the IPostC group was obviously lower compared with the I/R group. Similar to

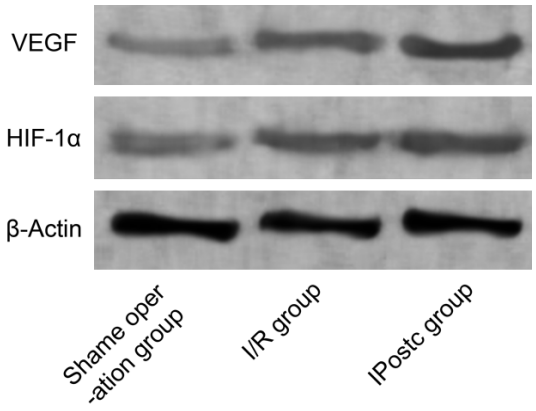

Figure 5. Comparison of HIF-1α, VEGF expression levels of mice in the three groups. The expression levels of HIF-1α and VEGF in the I/R group and the IPostC group were significantly higher than those in the SO group ($P<0.05$), and the HIF-1α, VEGF levels in the IPostC group were significantly higher than those in the I/R group ($P<0.05$).

our results, Abe et al. [24] used a rat MI/R model to observe the effect of NAC on MI range in different drug delivery methods, and found that NAC group greatly reduced the infarction area, indicating that NAC combined with ischemic preconditioning can reduce the MI range in mice.

ALI induced by I/R usually involves multiple inflammatory mediators and their effector cells, and presents a cascade of amplified inflammatory secondary injury and diffuse pulmonary parenchymal injuries [25]. The migration and aggregation of inflammatory factors lead to an "Inflammatory cascade", i.e., cascade reaction, which then leads to the massive release of a variety of inflammatory factors and oxygen free radicals, resulting in the proliferation of inflammatory mediators and the damage of pulmonary capillary endothelial cells and alveolar epithelial cells [26, 27]. While oxidative stress exerts marked effects on the onset and progression of diabetes and its complications. When antioxidant therapy is applied, the damage of oxidative stress to tissues will be reversed, thereby delaying the process and damage of diabetes [28]. Therefore, we detected inflammatory factor levels and oxidative damage degree in the three groups. It was noticed that the SO group showed the highest CRP, TNF-α, SOD and GSH levels among the three groups, followed by the IPostC group and I/R group successively, with significant differences. Regarding oxidative damage indexes represented by MCP-1 and MDA, their contents in the I/R were the highest, followed by the IPostC group and SO groups in succession, and the differences were statistically significant. NAC plays a role in combination with the aggregation of IPostC, and the protective mechanism of NAD IPostC. We hypothesize, thismay be to reduce the generation of oxygen free radicals, inhibit the release of inflammatory mediators and lower the aggregation of neutrophils.

The HIF signaling pathway is effective in MI/R triggered ALI [29]. At the end of the study, HIF-1α and VEGF levels in lung tissues were examined with Western Blot. It showed that their levels were dramatically boosted in the IPostC and I/R groups than the SO group, and their levels in the IPostC group were evidently higher compared with the I/R group, suggesting that the protective effect of NAC combined with IPostC on diabetic MI/R induced ALI in rats was related to the HIF pathway. The possible mechanism is that increased expression of VEGF, the protein product of the target gene of HIF-1α, promotes glycolysis in damaged alveolar epithelial cells, increases blood supply in ischemic tissue, inhibits the generation of apoptotic cells, and thus alleviates lung injury caused by reperfusion. The very mechanism, however, remains to be further explored.

Although this study confirmed the effects of NAC combined with IPostC on diabetic MI/R induced ALI, there are still several shortcomings. First of all, this study only used mice as the research subjects, whether other animals have similar results needs further investigation. Secondly, this study found that HIF signaling pathway is effective in MI/R induced ALI, its specific regulatory relationship needs to be investigated in future studies.

Conclusion

NAC combined with IPostC may improve ALI in diabetic mice induced by MI/R through the HIF pathway and antioxidant activity.

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Disclosure of conflict of interest

None.

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