Cardioprotective effect of erythropoietin in rats with acute myocardial infarction through JNK pathway

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Abstract. – OBJECTIVE: To explore the molecular mechanism of the cardioprotective effect of erythropoietin (EPO) in the rats with acute myocardial infarction (AMI) via the c-Jun N-terminal kinase (JNK) pathway.

MATERIALS AND METHODS: The rat AMI models were constructed and randomly divided into the EPO group, AMI group, and Sham group. At 2 weeks after successful modeling, the cardiac function-related indicators of rats were determined, and after the rats were sacrificed, the left ventricular weight (LVW) index was measured. The enzyme-linked immunosorbent assay (ELISA) and the terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling (TUNEL) assay were employed to determine the levels of the related serum inflammatory factors and myocardial apoptosis, respectively. The apoptosis and the expression levels of the JNK pathway-related messenger ribonucleic acids (mRNA) and proteins in the myocardial tissues of all groups of rats were determined via Real Time-Polymerase Chain Reaction (RT-PCR) and immunohistochemistry.

RESULTS: After intervention with EPO, the EPO group had a substantially lower left ventricular end-diastolic pressure, but a remarkably higher left ventricular systolic pressure than the AMI group (p<0.05), and the LVW and LVW/body weight (BW) notably declined in the EPO group compared with those in AMI group (p<0.05). According to the ELISA results, the inflammatory factors were substantially raised in the AMI group compared with those in the other two groups (p<0.05) and significantly lowered after treatment with EPO (p<0.05). The TUNEL assay results revealed that EPO treatment could reverse the pathological changes in AMI to decrease the apoptosis to a large extent. It was detected via RT-PCR and immunohistochemistry that, compared with those in the Sham group, the expression level of the anti-apoptosis gene B-cell lymphoma 2 (Bcl-2) was substantially decreased, but the expression levels of pro-apoptosis gene Bax, JNK, and c-Jun were evidently elevated in the AMI group (p<0.05). Moreover, the expression level of Bcl-2 was remarkably raised (p<0.05) and the expression levels of Bax, JNK, and c-Jun remarkably declined (p<0.05) in the EPO group after intervention with EPO.

CONCLUSIONS: EPO can inhibit the inflammatory responses and decrease the myocardial apoptosis to protect the heart of the AMI rats, and its mechanism of action is related to the inhibition of the expression of the JNK pathway.

Key Words: Erythropoietin, Acute myocardial infarction, JNK pathway, Rats, Cardiac protection.

Introduction

Acute myocardial infarction (AMI) mainly occurs when blood flow is decreased and blocked due to coronary artery stenosis, thus causing patchy ruptures to coronary arteries, and once progressing to the severe stage, it will trigger various secondary reactive disorders and even necrosis¹. AMI is characterized by sudden and rapid onset, severe consequences, and high mortality rate, seriously endangering people’s health²,³. With the constant innovation of basic research and advances in clinical practices, the treatment regimens for myocardial infarction patients have been gradually improved over the past several decades. Erythropoietin (EPO), a related glycoprotein, can regulate multiple processes including cell proliferation⁴, and it circulates in bone marrow during the prenatal period and the growth and development period to accelerate the proliferation and development of cells⁵,⁶. Some investigations⁷,⁸ have proven
that EPO, a natural cytokine, can serve as a potential potent neuroprotective agent to intervene with several points of damage cascade. The studies of multiple animal models of the central nervous system ischemia and reperfusion have demonstrated that EPO has a relatively strong cerebral protective effect. It can repair the motion function of the cerebral cortex and ventricles in stroke-prone spontaneously hypertensive rats. Additionally, EPO is able to greatly reduce the number of terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling (TUNEL)-positive cells in ischemic penumbra where the abundance of the messenger ribonucleic acids (mRNAs) encoding EPO receptor (EPOR) is raised probably to enhance the neuroprotective function of EPO and prevent infarcts from expanding. The above findings have dramatically elevated the value of EPO as the potential treatment strategy for such diseases as AMI and have focused on the molecular mechanism of its anti-apoptosis effect.

As a representative of a group of mitogen-activated protein kinases (MAPKs), c-Jun N-terminal kinase (JNK) mediates stress-induced events including cell apoptosis. JNK promotes cell death by directly regulating its mechanism, and it is activated when the cells are exposed to various forms of stresses. The inhibition of the JNK pathway has been reported to be able to effectively treat the neuronal apoptosis in ischemic-cerebral diseases. However, the regulatory mechanism of JNK in cell death remains to be fully understood. EPO and EPOR, related to JNK, possess the cell specific-downstream signaling pathways such as the MAPK pathway, and the activation of these pathways participates in the regulation of the gene expression with the binding between EPO and EPOR to produce a neuroprotective effect. The MAPK pathway is implicated in such neuronal injuries as hypoxia-ischemia, but the influence of EPO on MAPK has not yet been elucidated.

Therefore, the present study aims to verify whether EPO can protect myocardium by repressing cell apoptosis, explore the potential cardioprotective effect of continuous administration of EPO immediately after AMI, and further investigate the mechanism of apoptosis probably due to the activation of the JNK pathway by evaluating the cardiac function indicators, serum inflammatory levels, and cell apoptosis, as well as the related mRNA and protein expression levels of apoptosis genes and the JNK pathway.

**Materials and Methods**

**Establishment of Animal Model**

A total of 30 male Sprague-Dawley (SD) rats were purchased and underwent permanent ligation of the left anterior descending coronary artery under sterile conditions to establish the AMI model after 1 week of adaptive feeding. This study was approved by the Animal Ethics Committee of Heze Municipal Hospital Animal Center. Then, they were randomly assigned into AMI group and EPO group. Another 10 SD rats not ligated were set as Sham group. At 24 h after surgery, the rats in EPO group were intraperitoneally or subcutaneously injected with 2,000 U/kg EPO, and those in the other two groups were given the equal amount of normal saline.

**Measurement of Serum Inflammatory Factors by Enzyme-Linked Immunosorbent Assay (ELISA)**

The venous blood was drawn from the tails of the rats, placed in the tubes without anticoagulants, and centrifuged to isolate the serum. In the experiment, the levels of the serum inflammatory factors were determined using the ELISA (Roche, Basel, Switzerland) kit based on the operation steps in the instructions and the particular conditions. Finally, the absorbance of each group was measured using a microplate reader.

**Determination of Left Ventricular Weight (LVW) Index and Cardiac Function**

After being continued to be routinely fed for 4 weeks, all groups of rats were intubated through the right common carotid artery to measure the cardiac function-related hemodynamic indexes and then executed to weigh the body weight (BW) under sterile conditions. Subsequently, the heart of the rats was quickly anatomized and removed to measure LVW. Finally, the LVW index was calculated.

**TUNEL Apoptosis Assay**

Each group of myocardial cells was cultured separately. Then, the paraffin-embedded sections prepared were subjected to cell apoptosis assay using the TUNEL cell apoptosis assay kit (Roche, Basel, Switzerland), and the sealed section samples were labeled by the fluorescent developer. Finally, the TUNEL-positive cells were observed under a fluorescence microscope.
Detection of Gene Expression Via Reverse Transcription-Polymerase Chain Reaction (RT-PCR)

The total RNAs were extracted from the myocardial tissues of rats in each group using TRIzol (Invitrogen, Carlsbad, CA, USA) reagent and reversely transcribed into complementary deoxyribonucleic acids (cDNAs) chains once the RNA purify and concentration were eligible (the use of isopropanol should be noticed), followed by PCR amplification and determination of the target gene expression levels via RT-PCR. The primer sequences of the target genes and the internal reference glyceraldehyde 3-phosphate dehydrogenase (GAPDH) were designed based on the sequences in GenBank, which are listed in Table I. Finally, the mRNA expressions of the related genes were calculated using the 2^{-\Delta\Delta Ct} method.

Immunohistochemistry

The dissected heart was soaked in formalin for 7 d and then rinsed using flowing water overnight, dehydrated in gradient alcohol, immersed, and embedded in paraffin and sectioned, followed by de-paraffinization and hydration using ethanol. Subsequently, the sections were subjected to antigen retrieval and biotin blocking. After incubation with the primary antibodies at 4°C overnight, they were incubated with the secondary antibodies for 1 h, developed using diaminobenzidine (DAB), counter-stained with hematoxylin, dehydrated, transparentized, and sealed. Finally, the images acquired under a microscope were scanned using IPP software, and the protein expression level of each group was calculated.

Statistical Analysis

All data were statistically analyzed using Statistical Product and Service Solutions (SPSS) 22.0 (IBM Corp. Released 2010. IBM SPSS Statistics for Windows, Armonk, NY, USA) and presented as (x ± s). The univariate analysis was performed for the inter-group comparison of data, and p<0.05 denoted statistical significance.

Results

Hemodynamic Indexes of Rats

According to the detection results of the rat cardiac function indicators in each group (Table II), the left ventricular end-diastolic pressure (LVEDP) in the AMI group was remarkably higher than that in the Sham group (p<0.05), and the left ventricular systolic pressure (LVSP) was evidently lower than that in the Sham group (p<0.05). Moreover, after intervention with EPO, EPO had a significantly lower LVEDP, but an evidently higher LVSP than AMI group (p<0.05).

Table I. PCR primers.

<table>
<thead>
<tr>
<th>MRNA</th>
<th>Sequence</th>
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<tbody>
<tr>
<td>JNK</td>
<td>F: 5’-TTGGAACACCATGTCTGAA-3’</td>
</tr>
<tr>
<td></td>
<td>R: 5’-ATGTACGGGTGTGGAGAGGC-3’</td>
</tr>
<tr>
<td>C-Jun</td>
<td>F: 5’-ACAGAGCATGACCCCTGAAAC-3’</td>
</tr>
<tr>
<td></td>
<td>R: 5’-CCGTTGCTGGACTGGATTTAT-3’</td>
</tr>
<tr>
<td>Bel-2 associated X protein (Bax)</td>
<td>F: 5’-GAACAGATCATGAAGAGCACGAGG-3’</td>
</tr>
<tr>
<td></td>
<td>R: 5’-CAGTTTCATCTCCAATTTCGCC-3’</td>
</tr>
<tr>
<td>Bel-2</td>
<td>F: 5’-GGTGCCTTGAGATCTCTGG-3’</td>
</tr>
<tr>
<td></td>
<td>R: 5’-CCATCGATCTTTGACAGATCTC-3’</td>
</tr>
<tr>
<td>GAPDH</td>
<td>F: 5’-TGACTTCAACACGGCGACACCCA-3’</td>
</tr>
<tr>
<td></td>
<td>R: 5’-CACCCTGTGTGCTGATGCAAGAA-3’</td>
</tr>
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</table>

Note: After intervention with EPO, the LVEDP in EPO group is distinctly lower than that in the AMI group, and the LVSP is remarkably higher than that in the AMI group. *p<0.05 vs. Sham group, and #p<0.05 vs. AMI group.

Table II. Hemodynamic indexes of rats (x ± s).

<table>
<thead>
<tr>
<th>Group</th>
<th>LVEDP (mmHg)</th>
<th>LVSP (mmHg)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sham</td>
<td>2.03 ± 1.01</td>
<td>165.13 ± 5.71</td>
</tr>
<tr>
<td>AMI</td>
<td>2.71 ± 0.46*</td>
<td>103.28 ± 6.49*</td>
</tr>
<tr>
<td>EPO</td>
<td>1.23 ± 0.65*</td>
<td>145.73 ± 7.75*</td>
</tr>
</tbody>
</table>

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LVW Index of Rats
The relative LVW was significantly increased in the AMI group ($p<0.05$), suggesting that the left ventricle was thickened. After intervention with EPO, the EPO group exhibited substantially lower LVW and LVW/BW than the AMI group ($p<0.05$), implying that EPO can reduce the relative LVW (Table III).

Changes in Inflammatory Factors
Table IV describes the changes in the levels of the serum inflammatory factors interleukin-6 (IL-6), tumor necrosis factor-α (TNF-α), and C-reactive protein (CRP) in all groups of rats, and it was found that the levels of the three inflammatory factors were remarkably higher in the AMI group than those in the other two groups ($p<0.05$), and they were evidently lowered in the EPO group ($p<0.05$).

Myocardial Apoptosis Determined
According to the TUNEL staining results (Figure 1), there were almost no apoptotic myocardial cells in the Sham group, while the AMI group had large numbers of apoptotic myocardial cells, and after intervention with EPO, the level of

Table III. LVW index determined ($\bar{x} \pm s$).

<table>
<thead>
<tr>
<th>Group</th>
<th>LVW (mg)</th>
<th>LVW/BW (mg/g)</th>
</tr>
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<tbody>
<tr>
<td>Sham</td>
<td>598.36 ± 98.71</td>
<td>2.07 ± 0.13</td>
</tr>
<tr>
<td>AMI</td>
<td>726.13 ± 102.13*</td>
<td>3.11 ± 0.21*</td>
</tr>
<tr>
<td>EPO</td>
<td>613.25 ± 99.53*</td>
<td>1.87 ± 0.17*</td>
</tr>
</tbody>
</table>

Note: Compared with those in the AMI group, the LVW and LVW/BW are notably decreased in EPO group. *$p<0.05$ vs. Sham group, and *$p<0.05$ vs. AMI group.

Table IV. Inflammatory factors ($\bar{x} \pm s$).

<table>
<thead>
<tr>
<th>Group</th>
<th>CRP (μg/L)</th>
<th>IL-6 (μg/L)</th>
<th>TNF-α (ng/L)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sham</td>
<td>28.1 ± 4.1</td>
<td>20.5 ± 3.1</td>
<td>29.1 ± 5.1</td>
</tr>
<tr>
<td>AMI</td>
<td>60.5 ± 5.3*</td>
<td>56.7 ± 4.5*</td>
<td>68.2 ± 7.8*</td>
</tr>
<tr>
<td>EPO</td>
<td>40.2 ± 3.5*</td>
<td>38.4 ± 2.9**</td>
<td>47.2 ± 3.5**</td>
</tr>
</tbody>
</table>

Note: EPO group has lower levels of inflammatory factors than AMI group ($p<0.05$). *$p<0.05$ vs. Sham group, and *$p<0.05$ vs. AMI group.

Figure 1. Level of apoptosis evaluated via TUNEL staining (magnification: 40×). There is massive apoptosis of myocardial cells in the AMI group, and the number of apoptotic cells is evidently lowered in the EPO group.
myocardial apoptosis was remarkably lowered in the EPO group, suggesting that EPO can protect myocardial cells from injuries.

**Gene Expression Detected Via RT-PCR**

As shown in Figure 2, the RT-PCR results revealed that compared with the Sham group, the AMI group exhibited a substantially decreased expression level of Bcl-2 ($p<0.05$), markedly raised expression level of Bax ($p<0.05$), and significantly elevated mRNA expression levels of JNK and c-Jun ($p<0.05$). Moreover, the results demonstrated that after intervention with EPO, the EPO group had a dramatically raised expression level of Bcl-2 ($p<0.05$), a substantially lowered expression level of Bax ($p<0.05$), and evidently decreased mRNA expression levels of JNK and c-Jun ($p<0.05$). The above findings indicate that EPO can suppress the expression of JNK pathway genes.

**Expressions of Related Proteins Detected Via Immunohistochemistry**

The related protein expression levels of apoptosis genes and the JNK pathway in myocardial tissues of all groups of rats were determined using immunohistochemistry. According to the results (Figure 3), the AMI group exhibited a substantially decreased protein expression level of Bcl-2 ($p<0.05$) and significantly elevated protein expression levels of JNK and c-Jun ($p<0.05$), and after intervention with EPO, the protein expression of Bcl-2 is markedly elevated, and the protein expressions of JNK and c-Jun are significantly decreased in the EPO group ($p<0.05$). *$p<0.05$ vs. Sham group, and $p<0.05$ vs. AMI group.
expression level of Bcl-2 was remarkably raised \((p<0.05)\), the protein expression levels of JNK and c-Jun was remarkably lowered in the EPO group \((p<0.05)\).

**Discussion**

AMI is manifested as myocardial ischemic necrosis, myocardial inability to relax normally, and contract and blocking or forced stopping of blood flow, causing severe ischemia to myocardium\(^{19,20}\). AMI seriously influences normal myocardial functions, and once it progresses into the disease at a severe stage, various secondary reaction disorders will be triggered, posing economic and social burdens on the individuals and their families. It has been confirmed that EPO can effectively stimulate cell proliferation and differentiation, but the mechanism of EPO regulating cell apoptosis remains less fully understood. The present investigation explored the cardiac function indicators, the serum inflammatory levels, and cell apoptosis, as well as the related mRNA and protein expression levels of apoptosis genes and the JNK pathway after EPO treatment, to verify whether EPO can suppress cell apoptosis to protect the myocardium. It was found that myocardial infarction rats had effectively improved the cardiac function after intervention with EPO. Additionally, the EPO treatment evidently lowered the inflammatory factors and reversed the AMI-induced myocardial injury, thus reducing apoptotic cells. According to the RT-PCR and immunohistochemistry results, after intervention with EPO, the expression level of the anti-apoptosis gene Bcl-2 was considerably raised, and the expression levels of JNK and c-Jun were evidently lowered.

According to several reports\(^{21,22}\), EPO plays favorable roles in ischemia or mechanical injury in rats and the cerebral ischemia in clinical patients, as well as autoimmune-mediated central nervous system inflammation. EPO can resist apoptosis, oxidation, and inflammation in the process of damage to different tissues and the central nervous system\(^{23}\), and it has been proven to be able to activate the anti-apoptotic signal cascades in the central nervous system and PNS involving JAK2\(^{24}\). In addition, EPO represses p-JNK and c-Jun to prevent motion neuronal cell death\(^{25}\). Hence, it is speculated in this analysis that the JNK/c-Jun signaling pathway may be critical in the myocardial infarction-related apoptosis. The existing data have shown that the phosphorylation of JNK and the activation of its substrate c-Jun, as well as the decrease in Bcl-2 shortly after myocardial infarction, hypothesized that the JNK/c-Jun signaling pathway probably participates in the induction of cell apoptosis. The expression level of anti-apoptosis gene Bcl-2 is largely associated with cell apoptosis\(^{26}\). EPO alters the Bcl-2/Bax ratio in the gerbils with cerebral ischemia, thereby promoting the expression of the anti-apoptotic Bcl-2\(^{27}\), which is consistent with the results of this work.

It has recently been demonstrated\(^{28}\) that, as an important player in various cellular processes, JNK is first identified as a serine/threonine kinase that phosphorylates c-Jun, a member in AP1 family under ultraviolet light, thus enhancing the abilities of c-Jun to activate and transcribe, and that the increase in c-Jun expression is regarded as a positive regulator of apoptosis. It is now clarified that JNK plays very different roles in the regulation of cell proliferation and survival, although JNK protein was initially considered as the protein activated by stress and apoptosis-inducing factors. JNK can be activated by growth factors that accelerate the proliferation and survival of hematopoietic cells, including EPO and other regulators\(^{29,30}\). However, the role of JNK activation in the growth factor-related signaling has not yet been clarified. JNK1 can destroy the T-cell differentiation and mediate immune deficiency in a targeted manner. Moreover, the knockout of JNK1 and JNK2 can cause embryonic death from the 11\(^{th}\) to the 12\(^{th}\) d after pregnancy. The activated JNK, therefore, acts as the inducer of cell apoptosis.

**Conclusions**

We demonstrated that EPO can inhibit inflammatory responses and decrease myocardial apoptosis to protect the heart of AMI rats by the mechanism of action related to the inhibition of the expression of the JNK pathway. The present work provides important experimental support for the treatment of AMI with EPO and serves as a theoretical reference for subsequent research of the mechanism by which the JNK pathway regulates cell apoptosis.

**Conflict of Interest**

The Authors declare that they have no conflict of interests.
Erythropoietin in rats with acute myocardial infarction

References


