Int J Clin Exp Med 2020;13(2):478-485
www.ijcem.com /ISSN:1940-5901/IJCEM0099156

Original Article

Heart injury alleviated by erythropoietin and the relationship between cardiomyocyte apoptosis and p-Akt protein in rats with myocadial infarction

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Received July 2, 2019; Accepted January 7, 2020; Epub February 15, 2020; Published February 28, 2020

Abstract: Objective: To investigate the protective effect of erythropoietin (EPO) on myocardial infarction in rats with cardiac injury and its mechanism. Methods: 60 healthy female Sprague-Dawley rats aged 8 weeks were randomly divided into mock surgical (MS) group, myocardial infarction (MI) group and EPO group, with 20 in each group. 4 weeks after modeling, among the three groups of rats, the left ventricular ejection fraction (LVEF), fractional shuotening (FS), LDH and CK-MB expression of related biochemical indicators, infarct size, cardiomyocytes apoptosis, and the expression of p-Akt protein in myocardial tissues were detected. Among the myocardial infarction rats, the correlation between myocardial apoptosis index and p-Akt protein expression in myocardial tissues was analyzed. Results: The LVEF value, FS value, and expression of LDH and CK-MB in the MS group were better than those in MI and EPO group (P<0.05). The indicators in the EPO group were better than those in the MI group (P<0.05); the myocardial infarct size and cardiomyocyte apoptosis in the MS group were smaller than those in the MI group and the EPO group (P<0.05). However, the myocardial infarct size and cardiomyocyte apoptosis in the EPO group were lower than those in the MI group (P<0.05). The p-Akt protein in the MS group was higher than that in the MI group and EPO group. The p-Akt protein in the EPO group was increased than that in the MI group (P<0.05). Among the myocardial infarction rats, there was a negative correlation between myocardial apoptosis index and p-Akt protein expression in myocardial tissues (r=-0.623, P<0.05). Conclusion: For rats with myocardial infarction, EPO can effectively improve their cardiac function, and reduce myocardial infarct size and myocardial apoptosis. The mechanism of protective effect on cardiomyocytes may be relied on the regulation of Akt anti-apoptotic signaling pathway.

Keywords: Erythropoietin, myocardial infarction, rat, heart injury

Introduction

As one of the diseases that pose a serious threat to human life and health, cardiovascular disease not only has a higher morbidity, but also has a youth oriented tendency [1, 2]. Myocardial infarction is a serious cardiovascular disease. It is mainly caused by the occlusion of local blood vessels in the heart, which causes irreversible liquefaction necrosis of cardiomyocytes. Irreversible heart function damage and ventricular remodeling are induced. Eventually, heart failure may also occur [3, 4]. At present, the treatment of myocardial infarction is mostly focused on improving the myocardial blood supply in the early myocardial infarction area. The necrotic myocardium is unable to be effectively repaired yet [5, 6]. As one of the main mechanisms that induce cardiomyocyte death, cardiomyocyte apoptosis plays an important role in the myocardial ischemia injury. Therefore, for the exploration of cardiomyocyte protection, more studies have been set out investigating cardiomyocyte anti-apoptosis mechanism [7]. In the past, Akt-dependent anti-apoptotic signaling mechanism is a hot research topic in the mechanism of protective effect on cardiomyocytes. The anti-apoptotic effect of Akt on cardiomyocyte is mainly achieved through the activity of caspase against apoptotic signaling pathway [8].

Erythropoietin (EPO) is a glycoprotein hormone produced by the kidney and embryonic liver. It

can promote red blood cell production and inhibit apoptosis of erythroid progenitors when EPO binds to erythropoietin receptors [9]. In recent years, studies have found that EPO has a relatively direct protective effect on the heart, and that EPO can also repair heart injury [10]. For example, clinical studies have found that that EPO can markedly improve the cardiac function in patients with heart failure [11]. Ongoing studies have proved that EPO may increase the anoxic tolerance of ischemic cardiomyocytes [12]. However, there is still no detailed study on the protective mechanism of EPO in myocardial infarction. Therefore, in order to explore the mechanism of EPO in myocardial infarction, the following investigation was conducted.

Materials and methods

Animals and materials

Animals: 60 healthy female Sprague-Dawley rats were 8 weeks old, weighing 171.61±7.52 g. All rats were purchased from Shanghai Slack Laboratory Animals Co., Ltd., with production license of SCXK (Shanghai) 2012-0002. At a constant temperature of 22°C, the rat was kept in a plastic box with bedding material on the bottom. The relative humidity was between 50% and 65%. The rat was free to eat and drink. The operation on rats in the modeling process was in accordance with the standards of the experimental animal ethics committee of our hospital. Experimental equipment: medical small animal ventilator was purchased from Shanghai Yuyan Scientific Instrument Co., Ltd.; the surgical instruments were purchased from Shenzhen RWD Life Science Co., Ltd.; 1% pentobarbital sodium was purchased from Shanghai Sinopharm Chemical Reagent Company; TUNEL apoptotic kit was purchased from Beijing Solarbio Technology Co., Ltd., and the item number was T2190.

Establishment of rat myocardial infarction model

The rats were anesthetized by injecting 1% pentobarbital sodium anesthetic into the enterocoelia at a dose of 0.1 ml/10 g. 20 rats from MS group were only conducted with thoracotomy without coronary artery ligation, and other 40 rats were for the model group. The model group rats were successfully anesthetized, and then they were fixed on the mouse plate in a natural supine state. The trachea was incised, the cannula was performed, and the ventilator was connected. After cannula was successful, the small animal ECG detector was connected to the limbs of the rats. The electrocardiogram changes during the operation were recorded. Next, the skin was cut along the 3rd to 4th intercostal space from the left side of the mouse. The wound was fixed to expose the heart. The left coronary artery was ligated at about 2.5 mm below the left atrial appendage. After ligation, when the myocardium of left ventricular anterior wall became white, the heartbeat became faint, the ST-T segment elevation occurred on the electrocardiogram, and the wide malformation lasted for more than 30 min, the myocardial infarction model was established successfully [13]. 400,000 units of penicillin were injected into the postoperative enterocoelia to prevent infection. The rats were awakened at room temperature. 40 successful modeling rats were randomly divided into MI group and EPO group, with 20 in each group. After modeling, EPO was injected into the enterocoelia of rats of EPO group at a dose of 1000 U/kg. An equal amount of physiological saline was injected into the enterocoelia of rats of MI group. At the 4th week of successful modeling, the cardiac function of the rats was detected by echocardiography. The rats were first connected with the echocardiographic detector. Next, the left ventricular ejection fraction (LVEF) and fractional shuotening (FS) of the rats were recorded and evaluated. The average was taken from all indicators over 3 consecutive cardiac cycles.

Detection of biochemical indicators

5 mL of blood was taken from the abdominal aorta of the rats 4 weeks after modeling. It was placed at 4°C for 30 min, and then centrifuged at 3000 r/min for 10 min. The serum was taken, and the content of serum lactate dehydrogenase (LDH) and creatine kinase isoenzyme (CK-MB) in each group of rats was detected by chemical colorimetry.

Detection of myocardial infarct size

After the above tests were completed, the rats were sacrificed by cervical dislocation, followed by removing the heart. The bloodiness was rinsed off with PBS solution. The gauze was

Table 1. Cardiac function at the 4th week after modeling in each group of rats (%)

Index	Mock surgical group n=20	Myocardial infarction n=20	EPO group n=20	F	P
LVEF	81.32±3.14	41.04±1.54	68.25±4.36	811.0	<0.001
FS	42.76±4.05	21.93±1.17	32.64±3.57	213.3	<0.001

used to dry the water. Subsequently, it was frozen in a refrigerator at -20°C for 15 min. After serial sections, it was placed into 1% TTC solution, and then incubated at 37°C for 15 min in the dark. After that, the percentage of infarct size was calculated. Infarct size percentage = infarct size/total area of heart section × 100%.

TUNEL staining

The myocardial tissue was first embedded in paraffin, sectioned, dewaxed and dehydrated. 20 ug/ml of proteinase K was added and the tissue protein was digested at room temperature. After that, it was washed for 4 times with distilled water. The TUNEL reaction solution was added to incubate for 50 min. The anti-fluorescein-dUTP was added to the sections for incubation. Finally, DAB was developed and the count was observed under a light microscope. The nucleus of apoptotic-negative cells showed blue, and the nucleus of apoptosis-positive cells was brown-yellow. Under an optical microscope with 400 × magnification, 6 different visual fields in the infarcted area were randomly selected. Apoptosis was observed and calculated. Apoptosis index = the number of apoptotic positive cell nucleus/total number of cell nucleus × 100%.

Western blot

The tissue suspension was first prepared, and lysate was added for tissue lysis. It was mixed on ice, and then centrifuged at 1300 r/min for 10 min. Protein was separated by 10% SDS-PAGE and transferred to a PVDF membrane. 5% skim milk was added, and then it was blocked overnight at 4°C. After blocking overnight, anti-p-Akt (1:1000, Shanghai Enzyme Biotechnology Co., Ltd.) and β-actin (1:1000, Shanghai Enzyme Biotechnology Co., Ltd.) mice monoclonal antibodies were added. It was incubated at 4°C overnight. HRP-labeled goat anti rabbit IgG antibody (secondary antibody, 1:1000, Shanghai Enzyme Biotechnology Co., Ltd.) was added to incubate for 1 h at room temperature. After that, it was rinsed with PBS. Finally, ECL developing liquid was developed.

Statistical methods

In this study, the collected data were statistically analyzed by SPSS18.0 software (Bizinsight (Beijing) Information Technology Co., Ltd.). The measurement data were expressed by mean ± standard deviation, and t test was used for analysis between the two groups. The variance analysis was used for comparison among groups. LSD/t test was used for the pairwise comparison. Repeated analysis of variance was used for the comparison at different time points. P<0.05 was considered statistically different.

Results

Cardiac function at the 4th week after modeling in each group of rats

At the 4th week after modeling, the LVEF value of the MS group was 81.32±3.14%, and the FS value was 42.76±4.05%; the LVEF value of the MI group was 41.04±0.54%, and the FS value was 21.93±1.17%; the LVEF value of the EPO group was 68.25±4.36%, and the FS value was 32.64±3.57%. The LVEF and FS values of the MS group were higher than those of the MI group and the EPO group (P<0.05). However, the LVEF and FS values of the EPO group were significantly higher than those of the MI group (P<0.05) (**Table 1** and **Figure 1**).

LDH and CK-MB expression in three groups of rats

At the 4th week after modeling, the LDH value of the MS group was 1618.79±125.34 U/L, and the CK-MB value was 12.71±3.27 U/L; the LDH value of the MS group was 3017.85± 163.75 U/L, and the CK-MB value was 215.82± 14.66 U/L; the LDH value of EPO group was 2016.46±131.38 U/L, and the CK-MB value was 104.32±8.96 U/L. The LDH value and CK-MB values of the MS group were lower than those of the MI group and the EPO group (P<0.05). However, the LDH value and CK-MB values of the EPO group were significantly higher than those of the MI group (P<0.05) (**Table 2**).

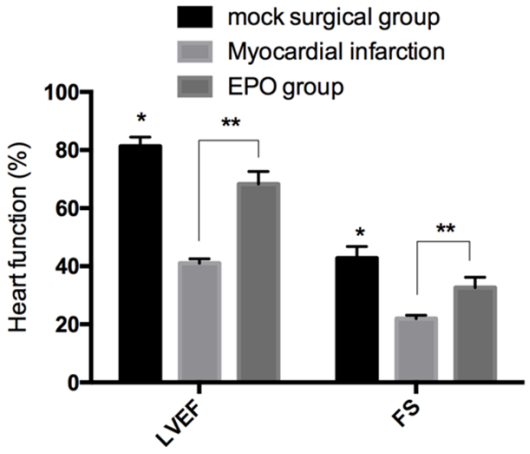

Figure 1. The cardiac function at the 4th week after modeling in each group of rats. The LVEF and FS values of the MS group were higher than those of the MI group and the EPO group (P<0.05). However, the LVEF and FS values of EPO group were higher than those of the MI group, and the difference was statistically significant (P<0.05). Note: *was compared with **, P<0.05; **meant P<0.05.

Comparison of myocardial infarct size in rats

The myocardial infarct size of the rat was compared. It was found that the myocardial infarct size of the MI group, the MS group and the EPO group was 26.43±1.51%, 0% and 16.75±1.03%, respectively. The myocardial infarct size of MS group was smaller than that of the MI group and the EPO group. The myocardial infarct size of the EPO group was significantly smaller than that of the MI group (P<0.05) (**Figure 2**).

Cardiomyocyte apoptosis index of three groups of rats

In the MS group, only a few cardiomyocytes apoptosis occurred, and the apoptotic index was 0.47±0.05%. The myocardial apoptosis index of the MI group was 30.13±3.27%. The cardiomyocyte apoptosis index of the EPO group was 21.06±2.24%. The cardiomyocyte apoptosis index of the MS group was lower than that of the MI group and the EPO group. The cardiomyocyte apoptosis index of the EPO group was significantly lower than that of the MI group (P<0.05) (**Figure 3**).

Comparison of p-Akt protein expression of myocardial tissue in each group of rats

The expression of p-Akt protein in myocardial tissue of MS group was 1.03±0.07%. The expression of p-Akt protein in myocardial tissue of MI group was 0.43±0.03%. The expression of p-Akt protein in myocardial tissue of EPO group was 0.79±0.05%. The p-Akt protein in the MS group was higher than that in the MI group and the EPO group. The p-Akt protein in the EPO group was significantly higher than that in the MI group (P<0.05) (**Figures 4** and **5**).

Correlation analysis between myocardial apoptosis index and p-Akt protein expression in myocardial tissue of rats with myocardial infarction

There was a negative correlation between cardiomyocyte apoptosis index and p-Akt protein expression in myocardial tissue of rats with myocardial infarction (r=-0.623, P<0.05) (**Figure 6**).

Discussions

Myocardial infarction is a cardiovascular disease with high mortality and disability rate. It has a serious impact on patients' quality of life [14]. For patients with myocardial infarction, the severity and prognosis of the patients are closely related to the size of myocardial infarct and the number of myocardial apoptosis. Therefore, the current clinical treatment for myocardial infarction is mostly focused on reducing myocardial infarct size and anti-cardiomyocyte apoptosis [15, 16]. EPO is originally a hematopoietic cell growth factor for treating anemia. However, in recent years, studies show that EPO can inhibit apoptosis and promote angiogenisis. It has also been found that EPO has certain protective effects on the cardiomyocytes in myocardial infarction [17, 18]. However, there are few studies about the protective mechanism of EPO on the cardiomyocyte in myocardial infarction.

In our study, the protective mechanism of EPO on cardiomyocytes was analyzed by establishing a rat model of myocardial infarction. First, at the 4th week after EPO intervention, the cardiac function of the EPO group was better than that of the MI group. What's more, the serum LDH and CK-MB expressions were also lower than those of the MI group. Although there are still differences in the cardiac function index and the expression of LDH and CK-MB between EPO group and MS group, it was improved a lot compared with the MI group. Previous studies [19] using the rabbit model with myocardial

Table 2. Expression of LDH and CK-MB in three groups of rats (U/L)

Index	Mock surgical group n=20	Myocardial infarction n=20	EPO group n=20	F	P
LDH	1618.79±125.34	3017.85±163.71	2016.46±131.38	521.7	<0.001
CK-MB	12.71±3.27	215.82±14.66	104.32±8.96	2029	<0.001

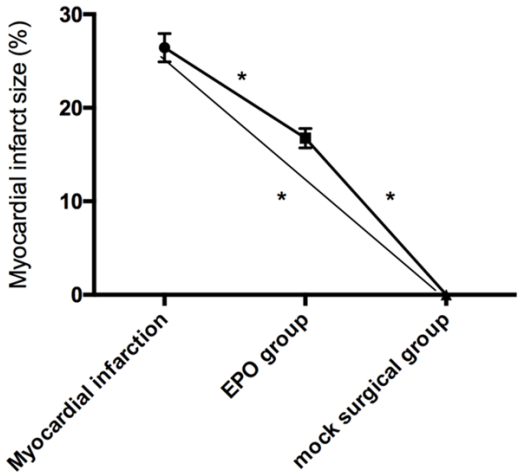

Figure 2. Comparison of myocardial infarct size in rats. The myocardial infarct size of MS group was smaller than that of the MI group and the EPO group. The myocardial infarct size of the EPO group was smaller than that of the MI group, and the difference was statistically significant (P<0.05). Note: *was compared with **, P<0.05; **meant P<0.05.

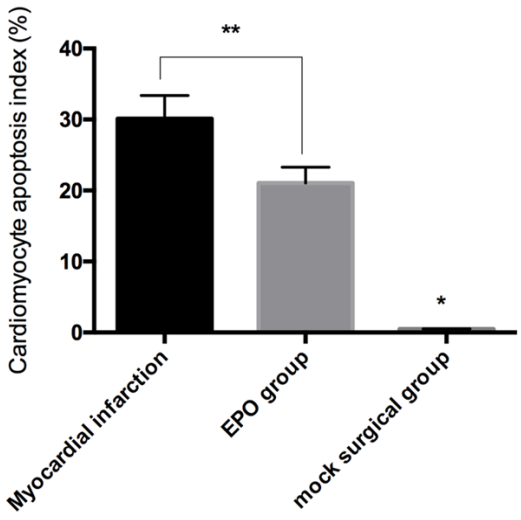

Figure 3. The cardiomyocyte apoptosis index of the MS group was lower than that of the MI group and the EPO group. The cardiomyocyte apoptosis index of EPO rats was significantly lower than that of the MI group, and the difference was statistically significant (P<0.05). Note: *was compared with **, P<0.05; **meant P<0.05.

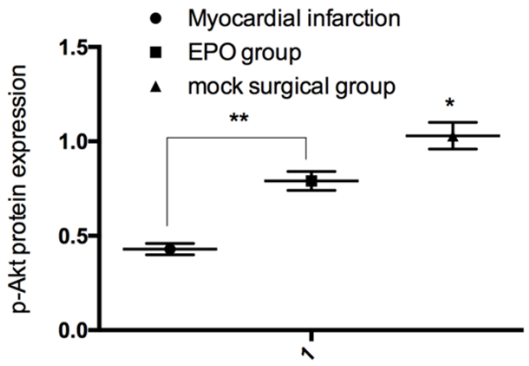

Figure 4. Comparison of p-Akt protein expression in myocardial tissue of rats in each group. The p-Akt protein in the MS group was higher than that in the MI group and the EPO group. The p-Akt protein in the EPO group was higher than that in the MI group, and the difference was statistically significant (P<0.05). Note: *was compared with **, P<0.05; **meant P<0.05.

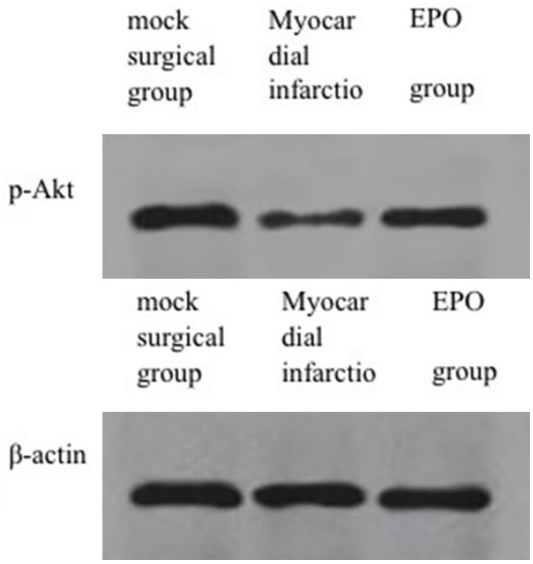

Figure 5. Protein map of p-Akt in myocardial tissue of each group of rats.

infarction showed that EPO can improve the cardiac function of the rabbits by myocardial injury inhibition and ventricular remodeling after ischemia. Our conclusions have also been confirmed and explained partially. Subsequ-

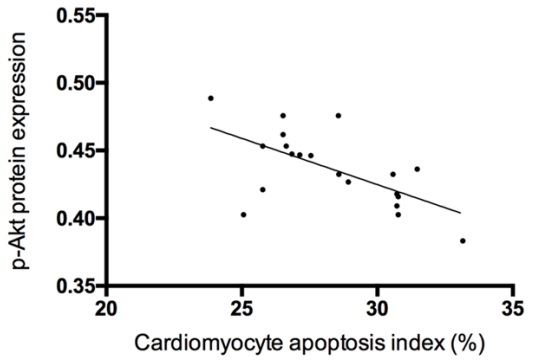

Figure 6. Correlation analysis between myocardial apoptosis index and p-Akt protein expression in myocardial tissue of rats with myocardial infarction. There was a negative correlation between cardiomyocyte apoptosis index and p-Akt protein expression in myocardial tissues of rats with myocardial infarction ($r=-0.623$, $P<0.05$).

ently, the effects of EPO on myocardial infarct size and myocardial apoptosis in rats were further explored. The results showed that the myocardial infarct size of the EPO group was smaller than that of the MI group. The cardiomyocyte apoptosis index of the EPO group was also lower than that of the MI group. This suggests that EPO has a significant effect on reducing myocardial infarct size and anti-cardiomyocyte apoptosis. Previous studies have analyzed the protective effect of EPO on the heart of rats with experimental myocardial infarction. The results of the study [20] indicated that when EPO was administered to the rats with myocardial infarction, the myocardial infarct size of rats can be significantly reduced. Moreover, bleeding can also be reduced. Other studies [21] also showed that for the myocardial infarction combined with heart failure, EPO can not only markedly improve cardiac function, but also reduce myocardial infarct size, which is consistent with our conclusions. Regarding the effect of EPO on cardiomyocyte apoptosis, previous studies [22] showed that when the EPO intervention was used to the myocardial ischemia-reperfusion injury model of rats, it was found that the cardiomyocyte apoptosis was significantly reduced. Akt acts as a liposome to phosphorylate downstream protein kinase B, which regulates cell growth, apoptosis, and metabolism through phosphorylation [23]. Previous studies [24] found that EPO in cardiomyocytes can protect the heart by regulation of phosphorylation of Aki. EPO has inhibited the apoptosis by inhibiting the activity of the pro-apoptotic factor caspase-3. Our conclusions also have been confirmed by that study. Subsequently, in order to further explore the anti-apoptotic mechanism of EPO in myocardial infarction, the p-Akt protein expression of myocardial tissue in each group was detected. The results showed that the p-Akt protein in the MS group was higher than that in the MI group. The p-Akt protein in the MS group was higher than that in the MI group and EPO group. The p-Akt protein in the EPO group was increased than that in the MI group ($P<0.05$). Among the myocardial infarction rats, there was a negative correlation between myocardial apoptosis index and p-Akt protein expression in myocardial tissues. This suggests that EPO may protect cardiomyocytes by regulating the Akt anti-apoptotic signaling pathway. Studies abroad [25] indicated that regulation on PI3K/Akt cell signaling pathway may be one of the mechanisms by which EPO protects cells. It is similar to our conclusion. However, some studies [26, 27] found that the cardiomyocytes apoptosis after EPO treatment can be reduced by 50% during the EPO intervention conducted on rat cardiomyocytes. It is also believed that the anti-apoptotic effect of EPO on cardiomyocytes is achieved through 3 signaling pathways of PI3K/Akt, ERK1/2-MAPK and Jak-STAT. This study suggests that PI3K/Akt may not be the only pathway for EPO to protect cardiomyocytes, which also provides a direction for our subsequent research on the cardiomyocytes protection mechanism of EPO.

In summary, for rats with myocardial infarction, EPO can effectively improve their cardiac function, and reduce myocardial infarct size and myocardial apoptosis. The mechanism of protective effect on cardiomyocytes may be relied on the regulation of Akt anti-apoptotic signaling pathway. However, there are certain deficiencies in this study. First, we only explored the possible cardiomyocytes protection mechanism of EPO in rats with myocardial infarction. The other regulatory pathways that may exist are not elaborated. Second, during the occurrence and development of myocardial infarction, there are many other factors such as HIF-1α and NF-κB that play a role in the cardiomyocytes apoptosis. The possible effect produced by EPO, and related factors to cardiomyocyte apoptosis were not elaborated in detail. Therefore, in order to provide detailed

elaboration, the cardiomyocytes protection mechanism of EPO will be further explored as much as possible.

Disclosure of conflict of interest

None.

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