Original Article

Erythropoietin Prevention effect on Induced Apoptosis by Ischemia-Reperfusion in Myocytes of Rat

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Abstract

Background: Ischemia-reperfusion is the common cause of apoptosis in most of cells specially myocytes. Prevention and reduction of apoptosis in myocardium can be one of the main medical goal before surgical operation, angioplasty and after infarction. Erythropoietin receiving effect 24 hours before hypoxia beginning on myocytes apoptosis rate and inflammatory process following half an hour hypoxia and 1.5 hour reperfusion are aims of this study.

Methods: 40 Rats were divided randomly into two groups. 24 hours before surgical operation, 5000 IU/Kg erythropoietin was injected to experimental group. During operation 12 rats from experimental group and 11 rats from control group were lost. After anesthesia, using ligation in left coronary artery for 30 minutes hypoxia and 1.5 hours reperfusion were applied. Then Thorax was opened and after bleeding, the animal’s heart was isolated and two tissue samples of infarct and non infarct area were separated and fixed. Then blood serum samples separated and incubated in -76°C. Apoptosis intensity in heart tissue was measured by tunel method, CK-MB level by method and DGKC, hsCRP by Elisa using Immunodiagnostic kit. The results were calculated Mean± SD. Then using paired student’s t-test their difference were shown. Level of statistical significant was considered P< 0.05.

Results: Activity level of CK-MB (1550U/L to 340) in experimental group was less than control group (P<0.000). hs- CRP serum rate was 450 and 225 ng/ml in control and experimental group respectively (P<0.000). Apoptosis index in infarct area was 11.5 and 4.8% in control and experimental group respectively (P<0.000). This difference was seen in non infarct area.

Conclusion: 5000 IU/Kg injection of erythropoietin before ischemia reperfusion, reduce cell injury and control inflammation process in hypoxia and control myocytes apoptosis in infarct and non infarct area.

Keywords: Ischemia- reperfusion " Erythropoietin " Apoptosis

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Introduction

It is true that reperfusion in ischemia tissue after infarction is necessary for finishing ischemia injury; however, reperfusion can cause ionic and metabolic disorders that finally lead to tissue injury and myocardial cells’ death\(^1\). There are different observational experiments that show the unreturnable cell damages following reperfusion\(^2\). As reperfusion in many conditions such as angioplasty, coronary artery bypass and infarction occur, and then it is necessary to do medical actions to prevent cell damages\(^3\). The protective role of erythropoietin: against cell death process and apoptosis in variety of tissue have been studied recently, such as brains\(^4\), spinal cord\(^5\), retina\(^6, 7\), kidneys, endothelial cells\(^9, 10\) and heart myocard\(^11-15\). In animal model, erythropoietin injection has decreased neuronal damage following Ischemia shock\(^16\). Adult Rat treatment using erythropoietin, 24 hours before hypoxia by 5000 IU/Kg dose, increases heart activity following reperfusion\(^17\). Shie and co-workers stated that remedial dose decrease apoptosis\(^18\). Treatment by erythropoietin decrease inflammation reaction after heart hypoxia\(^19\). But there is not agreement on protective dose of erythropoietin for specific time of hypoxia and drug injection time distance from hypoxia beginning. We injected 5000 IU/Kg erythropoietin 24 hours before Ischemia to animals and studied its effect after 30 minutes from left coronary artery obstruction and 1.5 hours reperfusion.

Methods

1. Animals
40 heads wistar Rats, 250-280 gr (weight) and 4-5 month (age) that were prepared from animal center of Tabriz Medical Sciences University were selected and keeping place was animal center of Tabriz Medical Sciences University. Keeping condition was standard and 12 hours light and 12 hours darkness in 22 ±2°C. Animals were divided into two groups. 20 heads Rat were in control group and 20 heads Rat were in experimental group. Experimental Rat group was injected by 5000 IU/Kg erythropoietin\(^20\). During operation 12 Rat in experimental and 11 Rat in control group were lost.

2. Erythropoietin injection method to experimental group:
Firstly, Rats were measured and recorded. Then experimental group Rats were put in glass container including ether for erythropoietin injection, were anesthetized proportionally. Tail of animal was scraped by scalper and then was rubbed by hot water, so that tail vessels were observed for injection and necessary erythropoietin was injected by insulin syringe from tail vessels.

3. Rat’s preparing for Ischemia – Reperfusion
Wistar mail Rats, 250-280 gr Weight, were anesthetized by injection of 50 mg/Kg sodium thiopental plus 6.6 mg/kg kralvz in peritoneum. Then cannulated trache and artificial respiration was done using palmer Respirator (54 respiration in one minute). Body temperature of animal was kept in normal position by heating lamp. Then one cannula including DMSO %5 in saline was put into left jugular vein for bleeding from Rats to measure heart isoenzyme, creatine kinase enzyme and C Reactive protein. Then carotid artery of head was cannulated for measuring blood pressure. A 15 minutes stabilization period was given to each Rat after these actions. Systemic artery blood pressure using a cannula including heparin saline that was sent to head carotid artery was recorded on Narco Bio- Systems (USA) Mk-III- S physiograph by Narco transducer and systole artery blood pressure and diastole were calculated. Conventional limb lead I was used by putting electrodes beneath the skin (hypoderm) for ECG monitoring on Narco physiograph that by ECG, heart beat and arrhythmia were measured. The thorax was splinted in left side of wishbone, by cutting 4 and 5 ribs, and after cutting pericardium, the heart was pull out by a soft force on ribs. The left coronary artery was isolated and suture thread (silk suture) braided around it and the heart was returned its place. Then animal was stabilized 15 mintues. During stablized, the Rats that had artery pressure less than 70mm and permanent arrhythmia were thrown off. Then ligation was done and animals were in ligation for half an hour. In this period, every one minute, arrhythmia resulted from heart ischemia was measured. In the first 15 minutes of ligation, the heart had typic arrhythmia and after 15 minutes heart gained its normal rhythm. 30 minutes after ligation, silk suture was separated and ligation
ended. Reperfusion stage lasted 1.5 hours. Then Thorax was opened and after bleeding from jugular vein animal heart isolated and then two samples were separated from infarct and non infarct regions were fixed in fixator for next studies. Blood samples were centrifuged an incubated in -76°C after separating from serum.

4. Evaluation of Apoptosis in tissues by tunel method:
After providing paraffin block from rats’ tissue, 5 micron thickness slices created. Then these sics were put on one lam. For each Rat, two lams were prepared that one of them was colored by hematoxilin and the second by tunel method for apoptosis examining on Roch Kit 22. For images, Japan Olymous/ 3H-2 and ASA 400 Kodak ultra optical microscope were used. The lams were examined under optical microscope.

5. Biochemical factors measuring:
For measuring hs- CRP, Rat CRP Immunodiagnostic Elisa kit was used that is an enzyme Immuno Assay (ELISA) method (23). For measuring creatine – kinase enzyme activity and heart isoenzyme (CK- MB), they used DGKC immunologic method.

Results

The effect of Ischemia – Reperfusion on serum Biochemical parameters: The results of serum creatine phosphokinase analysis in control and experimental groups showed in Table 1. On the basis of results, CK serum level in experimental group has significant reduction than control group (P<0.000). CK-MB reduction in experimental group was significant than control group and this reduction is prominent that CPK reduction in two cases.

Table 1 - Mean (±SD) of serum levels of CPK and CK- MB in control and experimental groups (receiver of 5000 IU/kg erythropoietin) after 45 minutes Ischemia and 90 minutes reperfusion

<table>
<thead>
<tr>
<th>Groups (n=9)</th>
<th>CPK (U/L)</th>
<th>CK-MB (U/L)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control group</td>
<td>2500 ± 600</td>
<td>1550 ± 280</td>
</tr>
<tr>
<td>Experimental group</td>
<td>1500 ± 350</td>
<td>340 ± 95</td>
</tr>
<tr>
<td>P</td>
<td>0.00</td>
<td>0.00</td>
</tr>
</tbody>
</table>

In control group, hs- CRP level is more than Experimental group after Ischemia – Reperfusion. Statistical analysis shows this difference is significant. The results were shown comparatively in Table 2.

Table 2- Mean (±SD) of serum levels of hs-CRP in control and experimental group (receiving 5000 IU/ kg erythropoietin) after 45 minutes Ischemia and 90 minutes reperfusion

<table>
<thead>
<tr>
<th>Groups (n=9)</th>
<th>hs- CRP (ng/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>450 ± 85</td>
</tr>
<tr>
<td>Experimental</td>
<td>255 ± 48</td>
</tr>
<tr>
<td>P</td>
<td>0.000</td>
</tr>
</tbody>
</table>

The Effect of Ischemia – Reperfusion on apoptosis of heart cells in Rats that received Erythropoietin.
In experimental group that was used erythropoietin for 24 hours before Ischemia start, the number of apoptotic cells in Ischemia zone of the heart reduced than control group (P<0.000). For statistical analysis, two microscopical field were selected randomly from each slice and number of brown cells recorded and all of the cells counted.

Apoptosis index \( \frac{\text{positive cells}}{\text{all cells}} \times 100\% \) for each sample was calculated and mean (±SD) for each groups identified (Table 3). Apoptosis index in infarct area was high. There is significant difference between two groups. In non infarct area, apoptosis is affected, and the difference between two groups is significant.

Table 3- Effects of Erythropoietin on index of myocytes apoptosis in infarct and non infarct heart area in Rat underwent Ischemia reperfusion

<table>
<thead>
<tr>
<th>Groups (n=9)</th>
<th>index of apoptosis in infarct area</th>
<th>index of apoptosis in non infarct area</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control group</td>
<td>11.5 ± 2.5</td>
<td>1.5 ± 0.35</td>
</tr>
<tr>
<td>Experimental group</td>
<td>4.8 ± 1.2</td>
<td>0.5 ± 0.2</td>
</tr>
<tr>
<td>P</td>
<td>&lt;0.001</td>
<td>&lt;0.05</td>
</tr>
</tbody>
</table>
Discussion

In Ischemia heart, reperfusion is necessary for cells’ live and heart activity. Reperfusion is essential for hear but is dangerous for tissues and Ischemia cells. Free radicals production, phosphate reduction of cells, myocardial injury and endothelial and calcium increase are the most causes of cell damage in reperfusion. It was shown that primary cells’ apoptosis erythropoietin, control blood and increase half life of them. Cells that have cytokine receptors for erythropoietin (EPO-R), cell stimulation by apoptosis control affect on special protein kinases. EPO-R gene appearance in different cells of heart and vascular system has been shown. The role of anti apoptic in vitro condition on Rat myocyte cells against hypoxy has been experienced that its mechanism is like endotheliyal cells. It has increased coronary blood flow in isolated heart of Rat. The results of this study shows that erythropoietin injection 24 hours before Ischemia – reperfusion, decrease tissue damage, control inflammation and prevent apoptosis of myocardial cells. This finding is similar to Moon.C and co-workers report. Dai Suke N and co-workers showed and studied erythropoietin effect on heart recovery after infarction and stated that treatment with Epo prevent heart necrotic impairing and decrease apoptosis by blood supply correction and angiogenesis increase. Myocardial enzymes free after myocytes damage by Ischemia-Reperfusion. Then enzyme examination from valid factors is for heart infarction distinguish. The change of special myocardial enzymes such as CK, LDH is one of the myocardial damage markers. CK-MD enzyme of heart CK enzyme is important. It was shown that after Ischemia, CK and Ck-MB was increased and maximize before others. CK-MB has direct relation with myocyte’s necrosis. CK-MB increase to peak without Q wave shows the disease high risk. CPK serum level after Ischemia- reperfusion in control group increase, and CK-MB increase is predominant. Enzyme increasing control by erythropoietin in Rats shows the protective, apoptosis and necrosis prevention role of this complex A CPK rate has direct relation with infarct, we can conclude that erythropoietin has protective role against reperfusion damage by...
reduction of area size. The results of this study are similar to TV opol EJ reports. They have shown that heart’s long protection of Ischemia, increase its ability against interferences. The results of this study also confirms Gregg W report that myonecrosis is a serious risk for patients after heart interventions, that can increase death and CK- MB serum level increase can be proper index for interventions. Inflammation has important role in extending heart and vascular disease and inflammation processes have relationship with myocardial ischemia and necrosis. It has been confirmed that serum level increase of CRP is a forecasting factor for myocardium damages. Studies have proven CRP role in atherosclerosis progress in vessels. It has been shown that CRP measurement for predicting inflammation process start after reperfusion has high value. In this study hs- CRP significant difference in experimental group shows erythropoietin role in inflammation control and cell damage reduction than control group. The role of erythropoietin on inflammation after heart attack especially after CABG was not reported. This shows that erythropoietin control inflammation process. Inflammation has important role in cardiovascular disease development and inflammation processes has relationship with ischemia myocardium and necrosis. It has been proven that increasing of serum level CRP is a predicting factor to myocardial damages. Studying CRP roles has proved Atherosclerosis development in veins. It has been shown that CRP measurement has great value for predicting inflammation process beginning after reperfusion. In this study, meaningful difference of serum hs-CRP than control group shows erythropoietin role in inflammation control and reduction of cell damage. Erythropoietin role on inflammation after heart surgery especially after CABG was reported less. This finding shows that erythropoietin control inflammation. Rifai N and his coworkers stated that hs-CRP is a reliable biochemical user for coronary disorders start or return of atrium. Future studies in Europe and America have proven hs-CRP role in CHD risk prediction in two sexes. They have proposed medical actions in the case of increasing hs-CRP. hs-CRP increasing control by erythropoietin proves this compound's controlling role. Ischemia reperfusion is the common cause of apopthis in most cells specially myocytes. The common causes of myocytes ischemia are vein involvement, Heart surgery, angioplasty and heart infarction. Cell damage and myocytes reduction by apoptosis reduce heart ability and reduction of its recovery ability in operations. Then preventing and apoptosis level reduction in myocardium can be one of the important aims before surgery, angioplasty, and after MI. On the basis of coloring infarct and non infarct area of myocardium by TUNEL method, positive Tunel cells in control group are more than experimental group. This finding shows controlling role of erythropoietin in preventing apoptosis cells of infarct area and whole of heart.

Conclusion

The results of this study show that 5000 IU/kg usage of erythropoietin and 24 hours before ischemia reperfusion can reduce infarct area and minimize cell damage, control inflammation process start and reduce myocytes apoptosis and prevent myocardial disability after reperfusion. Suggestion: Epo protective role after 3 days, one month and 45 days after ischemia in laboratory animals be studied so that controlling role of this composition be manifested in longtime.

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