

## A Review on the Pretreatment Effect of EPO on Ischemic Tolerance in Different Tissues with an Approach to the Tissue Protection Mechanisms

Raheleh Gholamzadeh<sup>1</sup>, Mehdi Eskandari<sup>1</sup>, Hossein Mostafavi<sup>1</sup>, Mohammad Reza Bigdeli<sup>\*2</sup>

### Abstract

**Introduction:** Ischemia due to oxygen and nutrient deficit, as well as waste producing causes tissue damage or organ dysfunction. Ischemia can be achieved in various tissues during surgery, organ transplantation, and use of certain drugs, sickle cell anemia, congestive heart disease, stroke and other motives. Also reperfusion of ischemic tissue can cause more serious damage. Recently, research strategies have been concentrated on the preconditioning mechanisms, such as pharmacological preconditioning which is used to increase tolerance to ischemia. Erythropoietin has antioxidant and anti-inflammatory properties and can be effective in reducing the damage caused by ischemia.

**Methods:** In this paper, a review was performed on erythropoietin role in reducing the tissue damage related to ischemia in brain, heart, kidney, intestine, liver and lung. The review was done between 2003 up 2016 years in regard to preconditioning mechanisms and protective signaling pathway in tissues.

**Findings:** The results suggest that erythropoietin may be increase tolerance to ischemia in tissues through its effects on oxidative pathways, inhibition of inflammatory reactions and anti-apoptotic effects. The drug efficiency is related to dose and time of drug administration, and it appears that the greatest protective effect is related to its antioxidant property.

**Conclusion:** According to the studies, there is a hope that the drug could be used in the future as a preventive agent of ischemia, in ischemia-threatening conditions, especially during the surgeries. However, investigation of probable side effects of the drug use is mandatory for its final approval.

**Keywords:** Pretreatment, Erythropoietin, Ischemic Tolerance, Different Tissue.

1. Department of Physiology and Pharmacology, Faculty of Medicine, Zanzan University of Medical Sciences, Zanzan, Iran

2. Department of Physiology, Faculty of Biological Sciences, Shahid Beheshti University, Tehran, Iran.

#### \* Corresponding Author

Mohammad Reza Bigdeli, Department of Physiology, Faculty of Biological Sciences, Shahid Beheshti University, Tehran, Iran  
E-mail: Bigdelimohammadreza@yahoo.com

Submission Date: 12/12/2015

Accepted Date: 21/02/2016

### Introduction

In medicine, ischemia is known a restriction of blood flow to tissues that this leads to lack of cellular needed oxygen and glucose. Ischemia is generally caused by problems with the blood vessels resulting of damage or disruption in tissue [1]. Following ischemia reperfusion injury is more severe than the initial injury that is called the ischemia-reperfusion injury (IR) [2-3]. The damage could be looking at a stroke, sickle cell anemia, heart attack, blood pressure lowering, shock and bypass surgery which leads to dysfunction of distal organs such as renal tubular necrosis, impaired liver and intestinal ischemia. Also it occurs as a result of vascular disease tolls, stroke, and myocardial infarction. This damage can cause complications such as ischemic cardiomyopathy, vascular dementia and renal insufficiency [4].

In fact, ischemic-reperfusion injury is determined by production of the oxidant, active complement, leukocyte-endothelial cell adhesion, platelet-leukocyte accumulation, increased microvascular permeability and reduced endothelium-dependent dilation. In its most severe form, ischemic-reperfusion injury can cause dysfunction of several organs or death. The therapeutic managements of ischemic-reperfusion injury are complex due to the fact that the inhibition of inflammation resulting of ischemia-reperfusion injury may impair protective physiological

reactions that its result may be associated with immune suppression. So while restoring blood flow to the ischemic area at the risk region is the cornerstone of clinical practice, therapeutic strategies such as ischemic preconditioning, a controlled reperfusion, and perception of antioxidant medication, treatment of complements or neutrophils is significantly adequate to prevent or limit the damage caused by ischemia / reperfusion in humans [5].

Preconditioning is an endogenous phenomenon which is produced by harmful ischemic but in sub-lethal levels in cells so that it causes an induction of adaptive and protective responses to subsequent severe ischemia. The resistance against the occurred ischemic injury is called as ischemic tolerance, that it can be created in organs such as heart, kidney, liver, skeletal muscle, small intestine, brain, as well as lungs [6]. Various factors such as anoxia, seizures, heat, oxidative stress, oxidative phosphorylation inhibitor can create tolerance to ischemia [7]. Ischemic tolerance involves mediating mechanisms such as the synthesis of new protective proteins, antioxidant enzymes, growth factors, or anti-apoptotic products [8-9]. Preconditioning derived strategies include hematopoietic cytokines, immunological tolerance, and physical measures [10].

It is a fact that some pharmaceutical agents can create beneficial effects of ischemic preconditioning phenomenon called pharmacological preconditioning, and provide



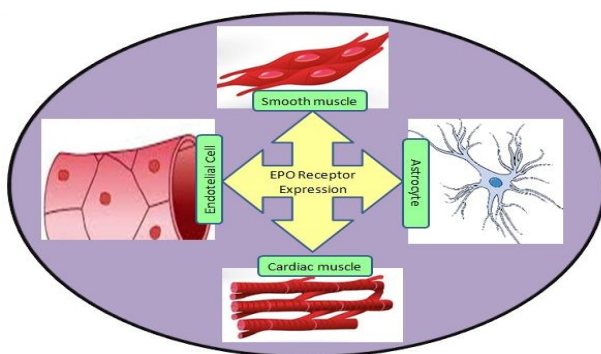
possibility that such a factor be taken in account to ensure the treatment of ischemic -reperfusion in the tissues [11]. It was recently reported that Erythropoietin (EPO) is a glycoprotein hormone of 165 amino acids with a molecular weight of 30 kDa [12], which may be used as a pharmacological preconditioning agent against ischemic /reperfusion damage in multiple tissues, including kidney, heart, lung and spinal cord [11]. EPO is produced primarily in the adult kidney, released into the blood circulation as well as has hematopoietic effects. It has been used in the last two decades for the treatment of anemia in patients [13]. The receptors of EPO as a cytokine are expressed by various cells, including endothelial cells, neuronal cells, cardiac myocytes, vascular smooth muscle cells, astrocytes, endothelial cells of the blood-brain barrier [12-14]. Erythropoietin receptor belongs to a family of cytokines that this family also includes receptors for many cytokines, including IL2, IL4, IL6, granulocyte macrophage colony-stimulating factor and growth hormone. EPO receptor is a dimer of two identical subunits. Erythropoietin binding to its homodimer receptor induces structural changes that lead to the activation of Janus tyrosine kinase 2, and thus the

phosphorylation of the remained tyrosine kinases of the erythropoietin receptor. This creates binding sites for several signaling pathways inside the cell [15].

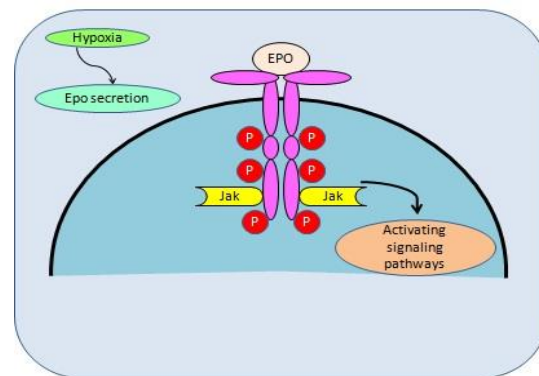
In addition to the known effects of EPO on red blood cell agglomeration in response to changes in tissue oxygenation, many studies have shown that it exerts a protective role against ischemic tissue.

It is believed by using the EPO, the direct effects via activating multiple biochemical mechanisms that provide anti-apoptotic effects, antioxidant and anti-inflammatory response to hypoxia/anoxia and also indirect effects through the systematic supply of oxygen to the tissues with potential angiogenesis in ischemic tissue is achieved. Erythropoietin has wide range of functions for protective effects on tissue [14].

Therefore, recent researches are concentrated on the effects of this drug for protection against an ischemic tissue. Hence, in this study was proposed to evaluate the effect of EPO on ischemic tolerance in the context of focusing on the mechanisms of preconditioning, cell receptors, intracellular signaling pathways that may play a role in tissue protection.



A



B

**Fig.1**, A, a different location of erythropoietin receptor expression (astrocytes, smooth muscle, endothelial cell, cardiac muscle). B, erythropoietin secretion by stimulating factors such as hypoxia, EPO binding to its receptor and activation signaling pathways.

## Methods

In the present study, according to research conducted by the laboratory researchers a review of the effects of EPO on ischemic tolerance in kidney, intestine, liver, brain and heart tissues was proposed in order to determine the drug's efficacy in preventing ischemic with approaching to protection mechanisms. Also according to the gathering results, the promising efficacy of the drug as a prophylactic agent in conditions of threats to ischemic is discussed.

### Effect of EPO in ischemic tolerance in heart

Shen et al. (2010) investigated the preconditioning effects of different doses of recombinant human erythropoietin on the ischemia/reperfusion injury in a model of ischemic/reperfusion in rats. In this study, descending coronary artery of a rat was ligated for 30 minutes, and then assessment of blood flow for 4 hours after ligation was performed. This group used dose of 100, 1000 and 5000 U/kg intravenous bolus administration of EPO for 30 minutes before the heart ischemic. The group examined

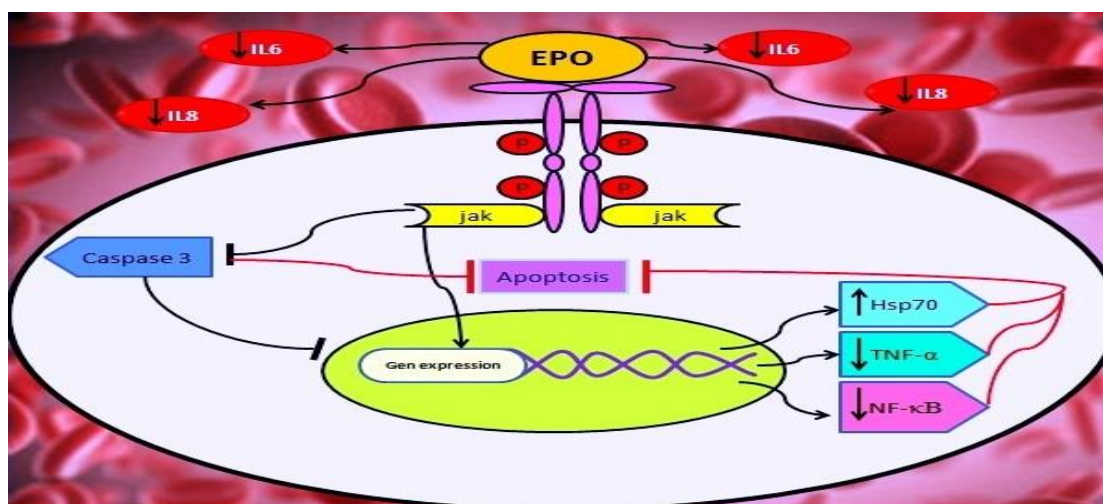
reperfusion-induced ventricular arrhythmia, including tachycardia and ventricular fibrillation and ventricular premature contractions using electrocardiography, as well as determined serum levels of inflammatory factor interleukin-8, interleukin-6 and tumor necrosis factor alpha at baseline, 2 and 4 hours after restoring the blood flow. They illustrated that in animals treated with EPO preconditioning as compared with the control group showed a significant decrease in ventricular arrhythmias and inflammatory markers of interleukin -8, IL-6 and alpha tumor necrosis factor which it was also due to the dose-dependent effects of EPO. The dose of 100 U/kg (dose anemia), EPO did not cause a protective effect on ischemic/reperfusion damage and the highest dose of EPO caused a lowest inflammatory response [16].

Xu et al. (2004) investigated effect of recombinant human erythropoietin therapy in the 24 hours before ischemia/reperfusion as a single intraperitoneal dose of 3000 U/kg which is to be used in the heart muscle in a model

of myocardial ischemia/reperfusion in rats. This study was done based on the left descending coronary artery ligation for 30 minutes and then reperfusion for 2 h with respect to the evaluation of heat shock protein 70 (Hsp70), kappa nuclear factor (NF- $\kappa$ B) and left ventricular infarct volume. Their findings showed that a single dose of EPO exactly 24 hours before ischemic/reperfusion damage increased the expression of Heat Shock Protein 70 in myocardium muscle, and decreased the amount of the nuclear kappa expression as well significantly reduced the volumetric rate of myocardial infarction compared with the control group [17].

Lipsic et al. (2004) studied effect of EPO (given as a single dose of 5000 U/kg /unit) on heart protection administrated at various time intervals during the 2 hours before the onset of the injury and at 45 minutes after the injury. In this study, the ratio of the surface area of the infarction, left ventricular hemodynamic function (left ventricular systolic pressure and its derivatives, left ventricular end diastolic pressure and heart rate) and apoptosis were examined. The results of these studies showed that administration of EPO at different time points, resulting in a 19 to 23 percent decrease in the myocardial infarction level to risk level (22.8% reduction in the onset of ischemia injury, 21.5 percent decline after the reperfusion attacks, 18.9 percent decline in pre-treatment) that it was consistent with the improvement of left ventricular hemodynamic parameters. Apoptosis and caspase-3 activity in positive cells in point of caspase-3 view in the group treated with EPO decreased significantly (29% reduction in the onset of ischemia, 38% reduction in blood flow after the attack and 16% reduction in pre-therapy) compared with myocardial ischemia. The comparison between the myocardial ischemia control group

and the treated with EPO experimental group at 24 hours post-ischemic/reperfusion were not observed significant differences in the mortality of the investigated groups [18]. Rong and Xijun (2015) evaluated the effects of EPO on myocardial IRI and its underlying mechanism, which exert these effects. 18 male Sprague Dawley rats were randomly divided into three groups, namely the sham, IRI-saline and IRI-EPO groups. Rats in the IRI-EPO group were administered 5,000 U/kg EPO intraperitoneally 24 h prior to the induction of IRI. IRI was induced by ligating of the left descending coronary artery for 30 min, followed by reperfusion for 3 h. Pathological changes in the myocardial tissue were observed and scored. The levels of the proinflammatory cytokines, interleukin (IL)-6, IL-1 $\beta$  and tumor necrosis factor (TNF)- $\alpha$ , were evaluated in the serum and myocardial tissue. Furthermore, the effects of EPO on phosphoinositide 3-kinase/protein kinase B (PI3K/Akt) signaling and EPO receptor (EPOR) phosphorylation were measured. Their result showed that pathological changes in the myocardial tissue increased expression levels of TNF- $\alpha$ , IL-6 and IL-1 $\beta$  in the myocardium and increased serum levels of these mediators so that IRI was significantly decreased by EPO pretreatment. The effects of EPO were found to be associated with the activation of PI3K/Akt signaling, which suppressed the inflammatory responses, following the initiation of EPOR activation by EPO. Therefore, EPO pretreatment was demonstrated to decrease myocardial IRI, which associated with activation of EPOR, subsequently increasing PI3K/Akt signaling to inhibit the production and release of inflammatory mediators. Thus, their results indicated that EPO might be useful for preventing myocardial IRI [19].



**Fig.2**, Erythropoietin binding to its receptor in the cardiac cells reduces the serum levels of inflammatory factors (such as IL6, IL8), and manages the process of apoptosis by inhibiting the activity of caspase-3, inhibits the expression of tumor necrosis factor alpha (TNF- $\alpha$ ), nuclear factor kappa and increased expression of heat shock protein 70 and induce cell protection.

#### Effect of EPO in ischemic tolerance in the brain

Givechian et al. (2009) evaluated global cerebral ischemia by deep hypothermic circulatory arrest procedures (DHCA) in an organism swine model for 1 h at a temperature of 20 ° C. These groups have examined how systemic treatment

with EPO can induce cerebral protection in deep hypothermic circulatory arrest. The animals were randomly divided into two groups that underwent a 60-minute DHCA. The animals treated with dose of 500 U/kg EPO during three consecutive days which started of 24 hours before surgery

to 24 hours after it. Intracerebral monitoring was performed by sub-cortical microdialysis, brain tissue oxygenation, measurement of the brain temperature and intracranial pressure. Neurological daily improvement was evaluated and serum level of protein S100 $\beta$  during the surgery was determined. At 6 days after surgery, the brain tissue was histologically examined to investigate the ischemic-hypoxic damage. The results of their work indicated that in both groups, brain sub-cortical microdialysis showed a significant increase in glycerol and lactate concentrations which were significantly higher in the brains of the investigated animals compared with the control group. However, there was no significant difference in neurological outcomes. Animals treated with EPO tended to have faster and more complete neurological recovery. In contrast, none of the animals in the control groups did not achieve complete remission of neurological outcomes. S100 $\beta$  protein as a putative marker of brain injury in the control group had a higher tendency and cerebral infarction was identifiable in all examined of the control group, while this phenomenon was detected in only 2 animals treated with EPO [20].

Li and colleagues in 2007 investigated the effect of EPO on the blood-brain barrier protection, and how this protection can influence vascular endothelial growth factor pathway in the rat. They administered a dose of 5000 U/kg which was intraperitoneally used at 30 min before ischemia, and once a day for three days after ischemia. Their findings showed that EPO dose of 5000 U/kg / unit decreased Evans Blue leakage and reduced brain edema after the ischemia. Expression of blood-brain barrier population markers of occludin, alpha-catenin and beta-catenin was maintained in animals receiving EPO. EPO increased expression of endothelial vascular growth factor. Although the expression of VEGF type-1 receptor (fatal hepatic kinase receptor, FIK1) significantly decreased in animals treated with EPO for three days after the ischemia, Li and his colleagues suggested that regardless of the increased level of endothelial vascular growth factor, EPO maintains the dam of the blood-brain against the blood-brain barrier damage induced by ischemia eventually partly by reducing the expression of FIK1 and response to signal of vascular endothelial growth factor in the acute phase after stroke [21].

Li and colleagues in 2012 investigated the effect of EPO dose of 5000 U/Kg as a single intraperitoneal injection immediately before ischemia on the blood-brain barrier integrity and expression of proteins associated with tight junctions including zonula occluden-1 (ZO-1), occludin and claudin-5 in rats after ischemia/reperfusion injury. In this study, rats were exposed for 2 hours of ischemia and then reperfusion injury 3 and 72 hours, respectively. Their findings showed that EPO could reduce infarct volume as well as blood-brain barrier damage induced by ischemia/reperfusion. Protein expression, m-RNA, ZO-1, claudin-5 and occludin significantly increased compared with ischemia/reperfusion at the same time. Treatment with EPO induced redistribution ZO-1, claudin-5 and occludin in brain microvascular. Compared with ischemia/reperfusion group, m-RNA levels of alpha tumor necrosis factor in the

small blood vessels of the brain were significantly decreased after treatment with EPO which was consistent with decreased levels of alpha tumor necrosis factor and nuclear factor kappa protein (NF- $\kappa$ B). The results of this study suggested that protective mechanisms of EPO on blood-brain barrier after brain ischemic/reperfusion damage was along with an increasing expression of proteins having tight junctions and also the decreasing expression of alpha tumor necrosis factor and kappa nuclear factor activity induced by the EPO may be involved in this process [22]. Yuanhua, et al in 2016, studied erythropoietin (EPO) preconditioning effects on the expression of glutamate transporter 1 (GLT-1) and glutamate aspartate transporter (GLAST) and its protection against rat cerebral ischemia-reperfusion injury. A total of 140 Sprague Dawley rats were randomly assigned to one of the following four groups: Sham, EPO-sham, middle cerebral artery occlusion (MCAO) and EPO-MCAO (the rats received an intravenous injection of EPO 15 min prior to the MCAO at a dosage of 5,000 U/kg body weight and blood perfusion was restored 2 h later). Neurological function were scored in 24, 36 and 72 h after reperfusion, the number of apoptotic neural cells, the cerebral infarct volume, the mRNA levels of GLT-1 and GLAST and the GLT-1 and GLAST protein levels in 72 h after reperfusion were measured too. Their result indicated that the infarct volume, neurological deficit score and the number of apoptotic cells of the EPO-MCAO group was significantly lower than that of the MCAO group ( $P < 0.01$ ). The GLT-1, GLAST mRNA and protein levels were increased significantly in the EPO-MCAO group relative to those in the MCAO group ( $P < 0.01$ ). In conclusion, EPO preconditioning could play a protection role against cerebral ischemia-reperfusion injury and upregulated the GLT-1 and GLAST expression [23].

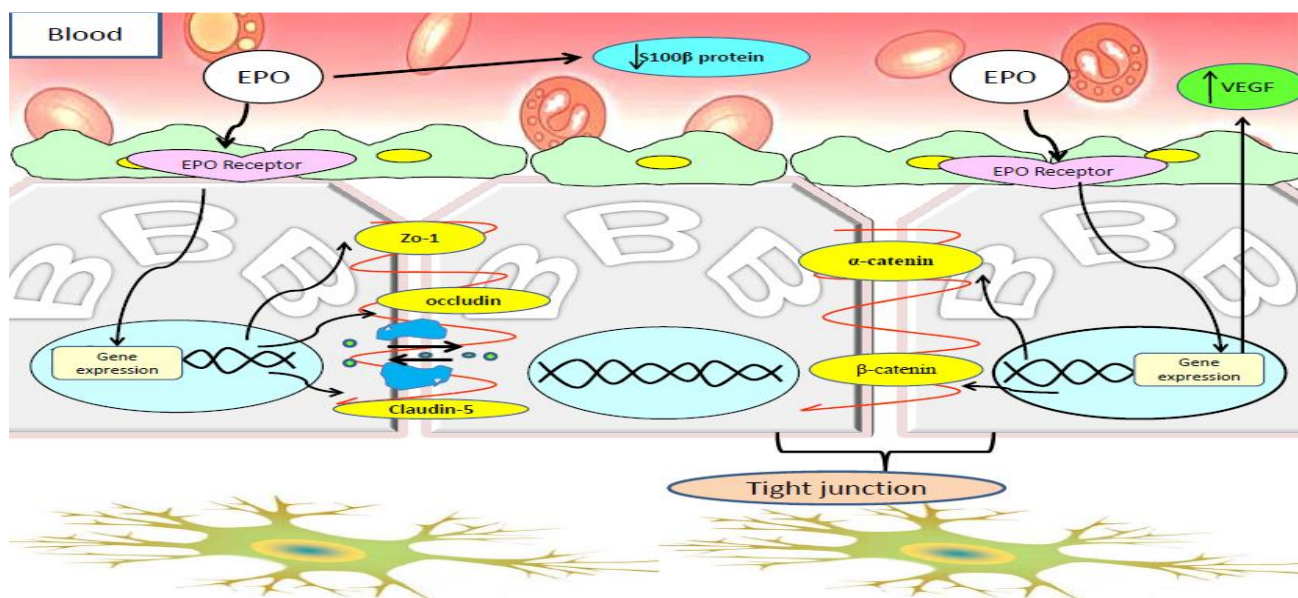
#### **Effect of EPO on ischemic tolerance in kidney**

Aortic cross-clamping during aneurysm surgery often causes ischemia/reperfusion in kidney. In renal transplantation, renal ischemia and reperfusion injury are associated with reduce in blood flow of the small blood vessels and loss glycocalyx, thus lead to a decrease in glomerular filtration rate, afferent arteriolar vasoconstriction and trans-cellular route leak back. In addition, ischemia /reperfusion causes propagation of inflammation due to an increasing expression of genes involved in deadly-like receptors, components of the complement pathway, chemokine and adhesion molecules which increases the transplantation rejection by the increased expression of genes involved in oxidative and apoptosis stress. In general, this process is responsible for the high rate of transplantation rejection, morbidity, and mortality during the transplant of kidney. Therefore, it is clinically important that the protective mechanism of kidney injury be understood to adjust this acute injury. Therefore, much research has been done to prevent kidney tissue ischemia.

In a study conducted by Matejkova and colleagues in 2012, the effects of the carbamylated- EPO (cEPO-FC) which connected to FC part of immunoglobulin G (IGg) protein and recombinant human EPO (rhEPO) on protection against

ischemia in kidneys of pigs with atherosclerotic disease was investigated. For assessing the protection of EPO, the animals were divided into three groups of control, receiving infusion of carbamylated EPO attached to proteins with dose of 50 mg / kg, and receiving infusions of recombinant human erythropoietin with dose of 5000 U/kg. The groups took the drug 120 minutes before occlusion of the aorta and 4 hours after reperfusion. In the work done by this group, blood creatinine levels and lipocalin, neutrophil gelatinase associated with (NGAL), creatinine clearance, fractional extraction of sodium (Na<sup>+</sup>) and HE coloring and PAS were used to evaluate histological damage and renal function. In the carried out research by them, as well as the plasma interleukin-6 (IL6), alpha tumor necrosis factor (TNF- $\alpha$ ), nitrates and nitrites and 8-isoprostane level were measured to assess systemic inflammation and oxidative and nitrosative stress. The results of this work showed ischemic / reperfusion injury causes an acute damage of kidney with decreased creatinine clearance, increased excretion of

sodium and levels of NGAL, impaired glomerular-tubular and apoptosis of moderate to severe, systemic inflammation and oxidative and nitrosative stress, but in these cases there was no difference between treatment groups. The experimental results showed that the nitrate and nitrite before ischemia and 8-isoprostane were respectively less and more than healthy animals. Body immunohistochemical histopathology showed a higher endothelial nitric oxide synthesis and a lower EPO receptor expression in biopsies of the examined kidneys before ischemic animals compared with control animals. The results of this study showed in pigs with atherosclerosis, recombinant human erythropoietin and carbamylated EPO connected to proteins could not successfully produce a long-term reduction of renal ischemia resulting of 8-hour reperfusion damage. This is probably due to the reduced expression of EPO receptors induced by pre-existing oxidative stress or reduced release of nitric oxide [24].



**Fig.3,** Protective effects of erythropoietin in the brain, erythropoietin binding to its receptor, increased gene expression of tight junction proteins (ZO-1, occludin, claudin-5,  $\alpha$ -catenin,  $\beta$ -catenin), decreased S100 $\beta$  protein serum level, maintain the strength of blood- brain barrier.

In a study by Lin et al. (2013), the protective effect of recombinant human analog erythropoietin on acute kidney injury induced by exhaustive exercise was reported. The purpose of this study was to investigate the protective effect of erythropoietin in chronic kidney damage from repeated exercise designed for four weeks. The animals were randomly allocated to one of four groups: control (C), exhaustive exercise test (ET), ET plus EPO pre-treatment (ET+EPO) and ET+EPO plus LY294002 pretreatment (ET+EPO+LY). The obtained results showed compared with the rats in control group, there was considerable damage in kidney cells of the ET group rats as revealed by histological and ultrastructural examinations. The pathological changes of kidney cell were much less compared with those of rats without EPO intervention. When LY294002, a specific inhibitor of phospholipids phthalocyanine inositol 3-kinase, was added to the EPO treated rats, the injury changes of renal cell were becoming

more pronounced. In the end, they concluded that the effect could be due to inhibiting the cell apoptosis and blocking the formation of interstitial fibrosis via activation of the PI3K/Akt pathway, thus it plays vital role in the endogenous protection of the kidney injury [25].

In a survey conducted by Muhammad and his colleagues in 2013, stated that despite cisplatin (CDDP), which is one of the most important factors useful in the treatment of solid tumors in chemotherapy, this drug creates the side effects such as renal nephrotoxicity. Hence, the effect of rhEPO on acute kidney injury induced by 9.0 mg/kg cisplatin injection to rats was studied by this group. Sixty male Wistar rats were divided into 3 groups of 20 each. First group (control): received saline. The second group of rats received cisplatin at a dose of 9 mg / kg, ip, once a week for 2 weeks, as the total dose. The third group of them received the same dose of cisplatin according to the second groups' daily intraperitoneal injections in addition to a recombinant

human erythropoietin administration with a dose of 100 U/kg, for two weeks. Twenty-four hours after the last dose of treatment, blood samples were collected for the measurement of creatinine, blood urea nitrogen, and total nitrite. Then, the kidneys were removed to assess the level of renal biochemical, molecular and histology. This result was observed by researchers the group treated with EPO significantly had lower rate on the amount and severity of kidney tissue symptoms compared to the untreated group. Creatinine, and blood urea nitrogen levels in the EPO-treated group was lower than that of cisplatin. The mortality rate in patients treated with EPO compared with cisplatin was reduced from 50% to 20%. As the obtained results illustrate, EPO treated group showed a significantly lower rate in the extent and severity of the histological signs of kidney injury than untreated one. This is attributed to (i) a decrease in the elevated oxidative and nitrosative stress markers, (ii) reduction of the expression of VEGF, HO-1 and iNOS as well as (iii) improvement of Bcl2 immunoreaction in most tubular cells. Thus, EPO may be one of the futures therapeutic possibilities to overcome the side effects of anti-cancer drugs induced acute renal injury through various mechanisms including down regulation of vascular endothelial growth factor (VEGF), hemeoxygenase-1 (HO-1) and inducible nitric oxide synthase (iNOS) expressions in addition to stimulation of tubular cell regeneration [26].

According to an assessment conducted by Hu and colleagues in 2012, it is stated that the tubular interstitial inflammation that is characteristic of kidney damage is inevitable in renal transplant. Regarding that the EPO has recently shown protective effect against ischemic/reperfusion damage of renal disease because of the fact it is associated with anti-apoptotic and antioxidant effects, the effect of EPO on the mechanism of ischemic/reperfusion damage of renal by focusing on tubular interstitial inflammation was examined by this group. So, male Sprague-Dawley rats were administrated with saline or EPO prior to IRI induced by bilateral renal pedicle clamping. Twenty four hours following reperfusion, the effects of EPO on renal IRI were assessed by determination of renal function and structure, tubulointerstitial myeloperoxidase (MPO) positive neutrophils, and proinflammatory mediator gene expression. The translocation and activity of NF- $\kappa$ B in renal tissues were also evaluated. In this work, the rats were randomly divided into three groups: sham group, ischemic/reperfusion damage treated with saline and treated with the EPO drug with a dose of 2000 U/kg, which the animals received intraperitoneally the drug 30 minutes before closing the renal pedicle. Bilateral renal obstruction was performed for 45 minutes using non-damaging vascular clamp and the obstruction was confirmed by observing the color change of the entire kidney surface. The achieved results showed in comparison with control groups, the EPO treated group exhibited lower serum urea and creatinine levels, limited tubular necrosis with a lower score of renal histological lesion. MPO positive cells in the tubulointerstitial area were greatly increased by IRI, while they significantly reduced by the treatment of EPO. The gene expression of proinflammatory cytokines (IL-1b, IL-

6, IL-10, and TNF- $\alpha$ ) and chemokine (MCP-1) was also significantly decreased by EPO. In addition, less activation of NF- $\kappa$ B nuclear-translocation was observed in the kidney treated by EPO. Overall, the results showed that EPO improved function and structure of the rats' kidney with ischemic/reperfusion lesion by reducing neutrophil from the tubular interstitial region, production of proinflammatory cytokine, chemokine and also NF- $\kappa$ B nuclear transfer activation. Hu and his colleagues also reported that EPO may have potential clinical applications as an anti-inflammatory agent for a wide range of physical injury [27]. In the research work done by Kiris et al. in 2007, the protective effect of EPO on renal ischemia/reperfusion damage in the abdominal aorta was examined. Twenty-four Wistar-Albino rats were randomized into 3 groups (8 per group). The control group underwent laparotomy and dissection of the infrarenal abdominal aorta without occlusion. The aortic IR group underwent clamping of the infrarenal abdominal aorta occlusion for 30 min followed by 60 min of reperfusion. The aortic IR-EPO group underwent the same aortic IR periods and was pretreated with 1000 U/kg subcutaneous erythropoietin 5 min before ischemia. In rat kidney specimens, tissue levels of malondialdehyde (MDA), superoxide dismutase, catalase, and glutathione peroxidase were measured. Histological evaluation of the rat kidney tissues was also done. The obtained results showed aortic IR significantly increased the levels of MDA and superoxide dismutase ( $P < 0.05$  versus control). Erythropoietin significantly decreased the levels of MDA, superoxide dismutase, and catalase ( $P < 0.05$  versus aortic IR). Histological evaluation showed that aortic IR significantly increased ( $P < 0.05$  versus control), whereas erythropoietin significantly decreased ( $P < 0.05$  versus aortic IR) the focal glomerular necrosis, dilation of Bowman's capsule, degeneration of tubular epithelium, necrosis in tubular epithelium, interstitial inflammatory infiltration, and congestion of blood vessels [28].

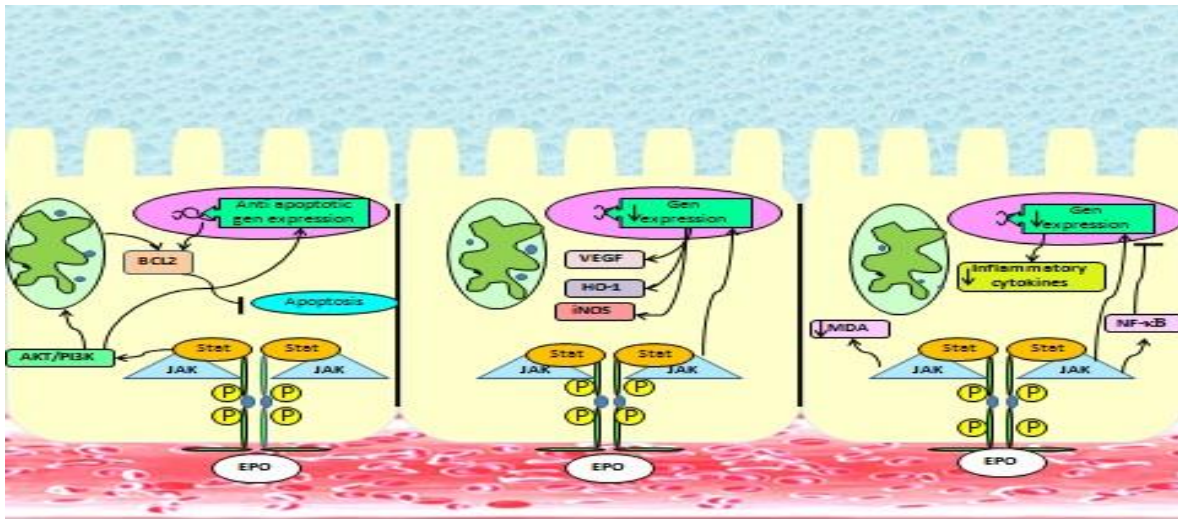
Zou, Zhang, Wang, et al in 2016 explored the effect and potential mechanism of EPO in renal IR injury. Kidney IR injury in rats was established by clamping the left renal artery for 30 minutes followed by 24 hours of reperfusion, along with contralateral nephrectomy. The EPO groups were treated with EPO (25, 50, or 100 mg/kg) by intraperitoneal injection at 1 hour before surgery. Renal function, renal histology, and expression of EPOR, p-EPOR, ERK, p-ERK, p-p53, p53, Bcl-2, Bcl-xl, Bad, and Bax were examined. Their results demonstrated that pretreatment with EPO significantly reduced renal dysfunction, pathologic change, and expression of Bad and Bax. Furthermore, EPO treatment enhanced the expression of p-ERK, p-p53, Bcl-2, and Bcl-xl with no influence on the expression of EPOR, ERK, and p53. Their findings demonstrated that EPO pretreatment could attenuate renal IR injury by inhibiting apoptosis through promoting activation of the ERK/p53 signaling [29].

#### **Effect of EPO on ischemic tolerance in intestine**

Intestine ischemic/ reperfusion damage is a serious disease which initiates with many clinical conditions including acute mesenteric ischemia, intestinal obstruction, incarcerated hernia, neonatal necrotizing intestinal

inflammation, trauma and shock. Although the exact mechanisms involved in the pathophysiology of ischemic/reperfusion injury are not fully understood, it

seems regulated apoptosis by extracellular and intracellular complex series play an important roles in the pathophysiology of intestinal ischemic/reperfusion.



**Fig.4.** Erythropoietin binding to its receptor on the surface kidney cells and affecting the signaling pathways leading to inflammation and apoptosis and decrease cell death.

The work done by Hashemzadeh et al. in 2012 noted that the protective effect of EPO on ischemic/reperfusion damage of intestine is less studied, so the aim of the research work is expressed to assess whether EPO has a protective effect on ischemic / reperfusion damage in rabbit intestine.

In tests conducted by the group, superior mesenteric artery of thirty male healthy rabbits was closed for 60 minutes. Then, the animals were randomly divided into two groups: a control group and the group treated with EPO which the group treated with EPO received subcutaneous 1000 U/kg 10 minutes before and 30 minutes after occlusion and immediately before the removal of the clamp. Similarly, saline as placebo was subcutaneously injected to the control group. Blood samples were collected before and at 2, 6 and 12 hours after ischemic / reperfusion injury for biochemical analysis such as interleukin-6 and tumor necrosis factor alpha measurement (TNF- $\alpha$ ). The results of this work showed that at 2 and 6 h after ischemic injury, the mean plasma levels of interleukin-6 in EPO-treated group was less than in the control group. However, the mean level of tumor necrosis factor alpha in the control group at 2 h after injury was lower. Also, it is observed that in the group treated with EPO, IL-6 levels at 6 h after ischemic / reperfusion damage was significantly higher than the values at 2 hours after the injury. In addition, IL-6 levels at 12 h after ischemic / reperfusion damage were significantly higher than those recorded during the first 2 hours after the injury. The overall results of this work suggest that EPO may have a protective effect against ischemic/reperfusion damage in rabbit model of intestinal ischemia [30].

In a study conducted by Bayomy et al. in 2010, the protective effect of EPO on histological, immunohistochemical and biochemical changes of ileal mucosal induced by ischemia /reperfusion in adult rat intestine was investigated. In this work, fifty adult albino rats were divided into three groups: control group,

ischemia/reperfusion and ischemia/reperfusion plus EPO. The superior mesenteric artery was occluded for 60-min to induce ischemia and then the clamp was removed for 120-min for reperfusion. EPO (5000 U/ kg) was administered intraperitoneally at the onset of reperfusion. At the end of the reperfusion period, terminal ileum was extracted for histological examination and immunohistochemical detection of the distribution of proliferating cell nuclear antigen (PCNA) from all animals. The levels of malondialdehyde (MDA, a biomarker of oxidative damage), myeloperoxidase (MPO, an index of the degree of neutrophil accumulation) and glutathione (GSH, a biomarker of protective oxidative injury) were also determined in all dissected tissues. The obtained results indicated in the ischemia/reperfusion group, the mucosa of the ileum showed shortening and distortion of the villi, erosion of the lining epithelium, degeneration of epithelial cells of the villi and the crypts of Lieberkuhn. Inflammatory cellular infiltration in mucosa was also observed. Ultrastructurally, degenerative changes were observed in the enterocytes in the form of cytoplasmic vacuolation, mitochondrial degeneration and dilatation of rER. The levels of MDA and MPO were significantly increased whereas those of GSH were significantly decreased. Immunohistochemical study revealed a non-significant increase in the number of PCNA - positive nuclei in the crypts. On the other hand, when erythropoietin was administrated at the onset of reperfusion at third group, intestinal morphological changes were alleviated and the levels of MDA and MPO were significantly decreased whereas those of GSH were significantly increased. Immunohistochemical study revealed a significant increase in the PCNA positive nuclei in the crypts of Lieberkuhn. The findings of this research suggest that EPO may offer a new protective strategy for ischemia/reperfusion injury in the field of intestinal surgery and transplantation [31]. Guneli et al. (2007) investigated the protective effect of

EPO on intestinal ischemia / reperfusion in rats. In this study, rats underwent intestine ischemia (30 min) and reperfusion (60 min). In this work, a single dose of EPO (5000 U/kg) was administered intraperitoneally at two different time points: either at five minutes before the onset of ischemia or at the onset of reperfusion. At the end of the reperfusion period, jejunum was removed for examinations. Myeloperoxidase (MPO), malondialdehyde (MDA), and antioxidant defense system were assessed by biochemical analyses. Histological evaluation was performed according to the Chiu scoring method. Endothelial nitric oxide synthase (eNOS) was demonstrated by immunohistochemistry. Apoptotic cells were determined by TUNEL staining. The results showed that compared with the sham, ischemia/reperfusion created intestinal tissue injury at the investigated group which the induced injury accompanied by increases in MDA levels, MPO activity, intensity of eNOS immunolabelling, the number of TUNEL-positive cells and a decrease in catalase activity. In comparison with the vehicle-treated ischemia/reperfusion, EPO improved tissue injury, decreased the intensity of eNOS immunolabelling, the number of TUNEL-positive cells, and the high histological scores, and increased catalase activity when given before ischemia, while it was found to have decreased the levels of MDA and MPO activity, intensity of eNOS immunolabelling, the number of TUNEL-positive cells, and the number of high histological scores when given at the onset of reperfusion. These results demonstrate that EPO protects against intestinal ischemia/reperfusion injury in rats by reducing oxidative stress and apoptosis; which the authors attributed this beneficial effect to the antioxidative properties of EPO [32]. In 2009, Sayan et al. studied EPO protective effect against ischemic / reperfusion damage. In this work (treatment with recombinant erythropoietin) the EPO intended to be as a medicinal pre-preparation factor and the objective of this study was expressed to determine whether the prescription has a preconditioning effect against ischemia and reperfusion injury in rat small intestine. In this work, intestinal ischemia was induced in male Wistar rats by clamping the superior mesenteric artery for 30 min, followed by reperfusion for 180 min. Recombinant human erythropoietin (1000 or 3000 U/kg) or vehicle was administered intraperitoneally 24 h prior to ischemia. After collection of ileal tissue, evaluation of damage was based on measurements of the accumulation of polymorphonuclear neutrophils by technetium-99m-labeled leukocyte uptake, content of malondialdehyde, reduced glutathione, contractile responses to agonists, and an evaluation of histopathological features in intestinal tissue. The work findings illustrate treatment with erythropoietin 24 h before ischemia significantly reduced the tissue content of malondialdehyde and increased that of reduced glutathione. Pretreatment also significantly suppressed leukocyte infiltration into the postischemic tissue; this was evidenced by the lower content of myeloperoxidase and technetium-99m-labeled leukocytes. Physiological and histopathological improvements were also significant with the rHuEpo treatment. Finally the authors concluded EPO might also be considered as a pharmacological

preconditioning agent, introducing a novel protective strategy in the setting of intestinal ischemia [11].

Kai-Lan and Si in 2015 studied the pretreatment effect of erythropoietin on intestine IRI by examining the change of intestinal histology; the expression of pro-inflammatory cytokines tumor necrosis factor  $\alpha$  (TNF- $\alpha$ ), interleukin 1 $\beta$  (IL-1 $\beta$ ), and interferon  $\gamma$  (IFN- $\gamma$ ); and the protein levels of EPOR, p-EPOR, p85, p-p85, Akt, p-Akt, I $\kappa$ B- $\alpha$ , p-p65, and p65. Rats were randomly divided into three groups: sham group (sham), IR-saline group (IRI), and the IR-EPO group (EPO). Rats were treated with EPO (5000 U/kg) at 1 hour before IR induction. A rat model of IR injury was established by ligating the superior mesenteric artery for 30 minutes, followed by reperfusion for 1 hour. The carried out work results showed EPO significantly decreased the pathologic changes of intestinal and reduced the elevation of pro-inflammatory cytokines TNF- $\alpha$ , IL-1 $\beta$ , and IFN- $\gamma$  in intestinal and serum caused by IR which was associated with suppressing NF- $\kappa$ B activation through further promoting activation of PI3K/Akt signaling. EPO ameliorated the acute intestinal injury caused by IR, which was associated with further activation of PI3K/Akt signaling to suppress NF- $\kappa$ B mediating inflammation [33].

#### **Effect of EPO on ischemic tolerance in liver**

In a study conducted by Sepodes et al. (2006), the protective effect of recombinant human erythropoietin against ischemic / reperfusion damage in rat liver using an intravenously dose of 1000 U/kg was investigated. The rats were subjected to 30 min of liver ischemia followed by 2 h of reperfusion. When compared with the sham-operated rats, ischemia/reperfusion resulted in significant rises in the serum levels of aspartate aminotransferase, alanine aminotransferase, lactate dehydrogenase, gamma-glutamyl transferase, tissue lipid peroxidation, caspase-3 activity and altered histology. The results of this work showed that in comparison with the control group, ischemia / reperfusion significantly increased serum levels of aspartate aminotransferase (AST), alanine aminotransferase (ALT), lactate dehydrogenase, glutamic Gamma transferase, tissue lipid peroxidation, caspase-3 and histological changes were observed. The findings showed injection of recombinant human erythropoietin at 5 minutes before ischemia reduced the evidence of biochemical signs of liver damage. Also, the levels of malondialdehyde in the group treated with EPO before the induction of ischemia has a reduction compared with the control group, and the liver structure was maintained. However, the protection was not observant at group that received EPO exactly 5 minutes before reperfusion. In terms of mechanism, early EPO injections could reduce oxidative stress and caspase-3 activity, which offers a further decrease in apoptosis [34].

In a study conducted by Yilmaz et al in 2004 to investigate the protective effect of EPO on liver ischemic/reperfusion damage, 48 rats were divided to three groups: ischemia / reperfusion, EPO + ischemia / reperfusion and controls. Ischemia was created by placing a microvascular clamp on liver pedicle for 45 min. EPO was given to EPO + ischemia / reperfusion group at a dose of 1000 U/kg 120 minutes before the onset of the ischemia. Blood samples and liver tissues were obtained after 45 minutes of reperfusion from



half of the rats in each group. The remaining rats were killed after a 24-hour observation period and blood and tissue samples were obtained. Blood alanine aminotransferase, tumor necrosis factor- $\alpha$  (TNF- $\alpha$ ), interleukin-2 (IL-2) and liver tissue malondialdehyde (MDA) levels were determined. Liver tissue histopathology was also evaluated by light microscopy. The findings illustrate in rats with hepatic ischemia, serum levels of ALT, TNF- $\alpha$ , IL-2 and liver tissue levels of MDA were reduced by the administration of erythropoietin and the histopathological score was also less severe. The authors concluded pre-ischemic administration of EPO has protective effects on hepatic ischemia/reperfusion injury [35].

Also Liu et al. (2009) investigated whether EPO pretreatment reduces liver ischemic/reperfusion damage. In this work, thirty Wistar male rats were divided into three groups: (1) to ten rats in the EPO group were given 1000 U/kg at the day before the surgery. (2) to ten rats in the control group was given normal saline as a placebo preoperatively and (3) Ten rats were used as controls. Hepatic ischemic/reperfusion damage was induced by occluding the hepatic arteries of the three cephalad hepatic segments and the portal vein for about 45 minutes, while in the sham-operated group only laparotomy was performed. The levels of ALT and AST were tested 24 hours pre- and post-operation. All rats were sacrificed 24 hours after the operation to assess the pathologic changes in the liver and measure the expression of hemeoxygenase-1 (HO-1) through Western blotting and RT-PCR. The results of this work showed that ischemic / reperfusion liver damage was significantly reduced in the treatment group compared with the control group. Additionally, the expression of HO-1 at the level of both transcription and protein increased prominently in the experimental group. These results demonstrate that EPO can regulate HO-1 in liver tissues and accordingly decrease hepatic injury through its anti-inflammatory property [36].

Liu, et al in 2015 examined the pretreatment effect of erythropoietin on the hepatic IRI by assessment of expression of tumor necrosis factor- $\alpha$  (TNF- $\alpha$ ), interleukin-1 $\beta$  (IL-1 $\beta$ ), and interleukin-6 (IL-6) and also the expression of nuclear translocation and phosphorylation of NF- $\kappa$ B p65, EPOR receptor (EPOR), p-EPOR, p-I $\kappa$ B-a, I $\kappa$ B-a, and TLR2. The warm IRI induced by clamping of the portal triad (hepatic artery, portal vein, and bile duct) to the left and median liver lobes for 45 minutes to introduce partial warm ischemia. The I/R-EPO group received the EPO (5000 U/kg, intraperitoneally) 0.5 hour before I/R induction. This work findings reveals EPO treatment significantly improved hepatic function and histology, as indicated by reduced transaminase levels and pathologic changes. The expression of TNF- $\alpha$ , IL-1 $\beta$ , IL-6, p-I $\kappa$ B-a, and TLR2 was significantly decreased with up-regulation of p-EPOR by EPO. Moreover, EPO pretreatment also reduced I/R-induced phosphorylation and nuclear translocation of NF- $\kappa$ B p65 subunits in liver tissue, but EPO had no influence on the expression of p65 and I $\kappa$ B-a. These results suggest that EPO pretreatment ameliorates hepatic I/R injury by suppressing TLR2/NF- $\kappa$ B mediated inflammation [37].

### **Effect of EPO on tolerance to ischemia in lung**

H. Wu et al in 2006 evaluated the effect of pretreatment with recombinant human erythropoietin (rhEPO) on I/R-induced lung injury. Left lungs of rats underwent 90 min of ischemia and then were reperfused for up to 2 h. Animals were randomly divided into three experimental groups as sham group, I/R group, and rhEPO + I/R group (a single dose of rhEPO was injected intraperitoneally 3000 U/kg 24 h prior to operation). Pathological change consists of the microscopic changes, tissue polymorphonuclear neutrophils (PMNs) accumulation, myeloperoxidase (MPO) activity, pulmonary microvascular permeability, blood-gas analysis and the serum concentration of tumor necrosis factor (TNF)- $\alpha$  were examined. Histological injury scoring revealed significantly lessened lung alveolus edema and neutrophils infiltration in the rhEPO pretreated group compared with I/R group ( $p < 0.05$ ). The rhEPO pretreated animals exhibited markedly decreased lung microvascular permeability ( $p < 0.05$ ) and myeloperoxidase activity ( $p < 0.05$ ). Blood-gas analysis demonstrated that the pretreated animals had significantly ameliorated pulmonary oxygenation function ( $p < 0.05$ ). The serum concentration of tumor necrosis factor- $\alpha$  in rhEPO pretreated group was markedly decreased compared with that of I/R group ( $p < 0.05$ ). Their finding showed that pretreatment with rhEPO appears to attenuate I/R-induced lung injury. This function is partly related with the capacity that rhEPO inhibits the accumulation of polymorphonuclear neutrophils in lung tissue and decreases the systematic expression of tumor necrosis factor- $\alpha$  [38].

H. Wu et al in 2009 examined the optimal dose and time-window of rhEPO timing administration for achieving minimal lung injury, and the mechanisms by which rhEPO attenuates lung I/R injury. The left lungs of rats underwent 90 min ischemia and 120 min reperfusion. Firstly, animals in different groups were intraperitoneally injected with various doses of recombinant human erythropoietin (3 KU/kg rhEPO) 24 h prior to operation, 2 h prior to operation, or after the onset of reperfusion. Ultrastructural changes of pneumocytes, pulmonary myeloperoxidase (MPO) activity, malondialdehyde (MDA) content, plasma concentrations of tumor necrosis factor- $\alpha$  (TNF- $\alpha$ ) and matrix metalloproteinase (MMP)-9 were measured. Their result showed that rhEPO maintained predominantly the pneumocyte ultrastructure and markedly reduced the pulmonary apoptosis index. Other than, in the treated animals the plasma concentrations of TNF- $\alpha$  and MMP-9 and their expression in lung tissue were significantly decreased. Treatment with rhEpo 2 h prior to operation was optimal for attenuating pulmonary MPO activity and MDA content [39].

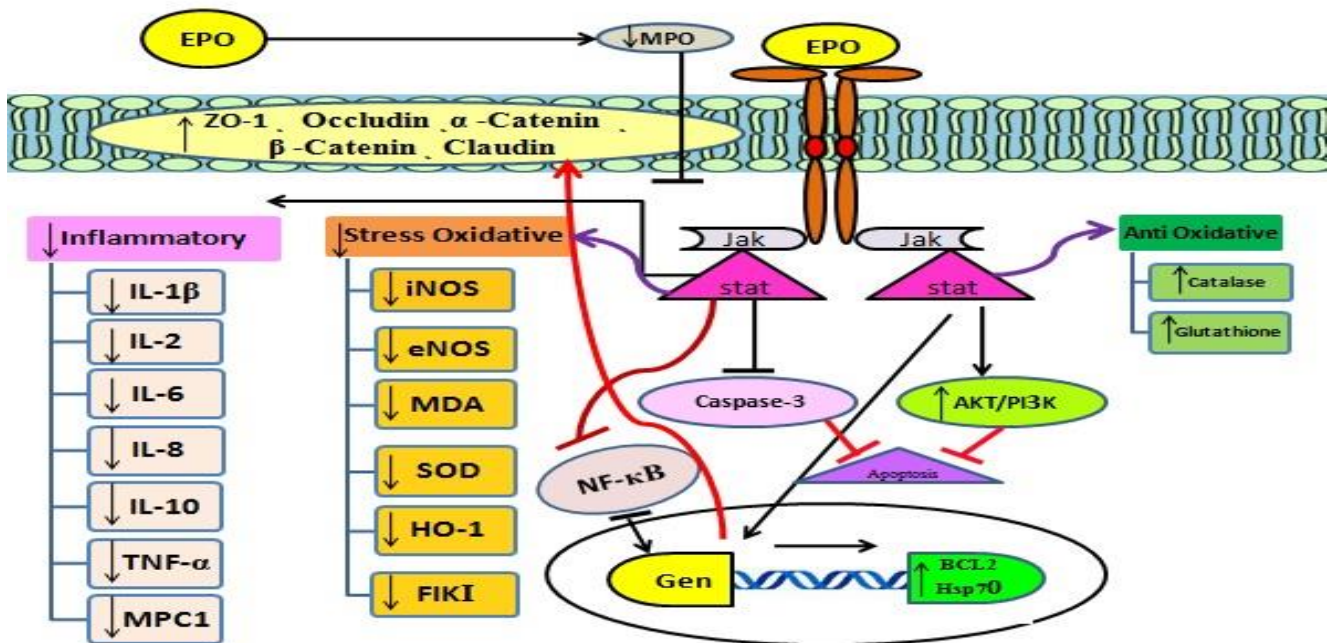
### **Summarization and Discussion**

Inhibit of injury and damage caused by ischemia-reperfusion is a clinical concern. Tissue ischemia is an important pathological agent which creates in association with various conditions including surgery, organ

transplants, use of certain drugs, congestive heart disease and bleeding can occur in various organs of the body. Tissue reperfusion after ischemia injury may be more severe than the initial injury caused by ischemia. During tissue ischemia, reduction of tissue perfusion causes hypoxia, a defect in energy production and reduction of the production of ATP.

Following the establishment of blood flow, a series of pathological events occur inside and outside the cell that are an increased release of glutamate receptor activation of AMPA / KA, increased intracellular sodium and calcium, mitochondrial stress, increased production of free radicals, peroxidation lipid creation, protein oxidation, mitochondrial defects, activation of caspases, complements systems, increased expression of inflammatory precursors, tumor necrosis factor alpha, apoptotic genes, decreased antioxidant defenses, increased vascular permeability that these factors ultimately causes cell swelling, DNA break down and cell death [6-40-41-42]. Understanding the signaling pathways and factors that control inflammatory pathways, oxidative stress and apoptosis can be effective to reduce the damage caused by ischemia and reperfusion. The results of the review on the studies conducted by different researchers on the effects of preconditioning of EPO in heart, brain ,kidney ,intestine liver and lung showed that the EPO may be increase tolerance to ischemia through effects on oxidative pathways including reduction of malondialdehyde levels [35-11], superoxide dismutase [28], nitrosative stress markers [24], endothelial nitric oxide synthase [32] and enhancement of glutathione [11], which is a marker of protection from oxidative damage. Also reduction of gene expression of proinflammatory cytokines

such as interleukins [24-27-35], necrosis tumor factor alpha [16-22-24-27-30-35], myeloperoxidase decline [27-31-32] (indicating neutrophil infiltration into the tissue), HO-1 [26-27-36] and the nuclear-kappa factor decline so that the inflammation is reduced as a result of the EPO preconditioning drug usage [17-22-27]. Also it could active AKT/PI3K pathway [6-25] and inhibit the activity of caspases, including caspase-3 [6-34] which inhibit apoptosis and increases expression of anti-apoptotic factors such as HSP70 [17] and BCL2 [26] in the cells and increases the cells' resistance by increasing the ratio of anti-apoptotic to apoptotic factors. Brain studies have also shown that EPO maintains or increases the protein expression levels of tight junctions and thereby increases the strength of the blood-brain barrier [16-22], which maybe a mechanism of ischemic preconditioning that retains the cell structure and reduces the rate of water penetration to cell and so the cell swelling. Also the drug has shown the dual effect of simvastatin on vascular endothelial growth factor; these factors showed an increased amount in brain tissue [21] and a reduction in renal tissue [26] therefore this suggests the behavior may result from different mechanisms in different tissues. But the drug decreases fatal vascular endothelial growth factor receptor expression in the brain, thereby reduces the effect of this factor [21]. The effect of EPO is depended to dose, the prescribed time and period of the drug administration in tissues [11-16-24]. By following the administration for a prolonged ischemia, tissue protective effects of EPO were not observable; this may be due to a decreased expression of EPO receptors in the presence of oxidative stress existed in the previous [24].



**Fig. 5.** An overview of the possible signaling pathways that the use of EPO drug as pretreatment therapy activates them in various ischemia tissues, causing the tissue conservation. Erythropoietin (EPO) • inducible nitric oxide synthase (iNOS) • endothelial nitric oxide synthase (eNOS), malondialdehyde (MDA) • superoxide dismutase (SOD) • hemeoxygenase 1 (HO-1) • fetal liver kinase receptor (FIK1) • interleukin (IL) • tubular necrosis factor α (TNFα) • nuclear factor-kappa (NF-kB) • heat shock protein 70 (Hsp70) • myeloperoxidase (MPO) • b cell cll/lymphoma 2 (BCL2)• activator threonine kinase/phosphatidil inositol 3 kinase (AKT/PI3K) ativator and transducer teranscription (Stat) • janus family protein (JAK) • zonula occluden-1 (ZO-1).

Also in cerebral ischemia, EPO decreased protein S100 $\beta$  (a marker of brain damage) which indicates that the drug is likely to affect the specific way of tissue damage in various organs [20]. Also most studies in this review showed that erythropoietin at a dose of 1000 to 1500 U/kg, could prevent damage caused by ischemia and reperfusion in most organs [11-16-20-26-28-30-34-35-36]. This result is conform with our study finding on the effect of pre-treatment with the same dose of erythropoietin for the brain edema reduction after IRI in wistar rats which was conducted in 2016 [43]. These findings suggest that erythropoietin pretreatment at a dose of 1000 to 1500 U/kg may be considered as at least pretreatment optimal dose for preventing the injury in most tissues in ischemia-threatening conditions. Although proof of this comment and also determination of the pretreatment duration and the administration method of erythropoietin requires further investigation.

### Conclusions

The obtained results of this review study suggest that EPO might be a promising therapy in the prevention of ischemia in various tissues, especially before the surgery. However, in these studies, side effects of prescribed medication have not been studied. Also there is needed a complementary investigation to determine the effectiveness of different doses of the medication in the prevention of ischemia.

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