Does Erythropoietin Protect the Intestine against Ischemic/Reperfusion Injury in Rabbits?

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Abstract: The protective effect of erythropoietin (EPO) on intestinal ischemic/reperfusion injury has been less studied. Therefore, the aim of the present study was to evaluate whether EPO has protective effects on the intestinal ischemic/reperfusion injury in rabbits. Thirty healthy male New Zealand white rabbits underwent clamping of the superior mesenteric artery for 60 minutes. Then, the animals were randomly divided into two groups: the control group (n=15) and the EPO-treated group (n=15). In the EPO-treated group, subcutaneous EPO (1000 IU/kg) was given 10 minutes before clamping, 30 minutes after clamping and immediately before declamping. Likewise, subcutaneous saline was injected as placebo in the control group. Blood sampling was performed before, at 2, 6 and 12 h after ischemic/reperfusion injury for biochemical analysis including interleukin-6 (IL-6), and tumor necrosis factor-alpha (TNF-α) measurements. At 2, 6 and 12 hours after ischemic/reperfusion injury, a segment of distal part of the terminal ileum was surgically resected from the ischemic intestine for light microscopic study. At 2 and 6 hours after the ischemic/reperfusion injury, the mean plasma levels of IL-6 in the EPO-treated group were lower than those in the controls (P<0.05). However, the mean TNF-α levels were lower in the control group at 2 hours after the injury (P=0.01). In the EPO-treated group, the mean levels of IL-6 at 6 hours after the ischemic/reperfusion injury were significantly higher than those at 2 hours after the injury (P=0.02). Furthermore, the mean levels of IL-6 at 12 hours after the ischemic/reperfusion injury were significantly higher compared with those at 2 hours after the injury (P=0.04). Histopathological assessment revealed that Park's score at 12 hours after the ischemic/reperfusion injury was significantly lower in the EPO-treated group compared with the control group (P=0.001). In conclusion, EPO might exert a protective effect against ischemic/reperfusion injury in the rabbit model of intestinal ischemia.
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1. Introduction

Ischemic/reperfusion injury of the intestine is a serious disease which can be caused by many clinical conditions including acute mesenteric ischemia, intestinal obstruction, incarcerated hernia, small intestine transplantation, neonatal necrotizing enterocolitis, trauma, and shock (Yuan et al., 2011). It is believed that the injury caused by restoration of blood flow into the tissue is markedly more destructive than the ischemic damage itself (Guneli et al., 2007). This condition is extremely dangerous and is associated with a high risk of mortality and morbidity (Granger and Korthuis, 1995). Small intestinal mucosa is one of the tissues being most sensitive to ischemia-reperfusion injury. Although the exact mechanisms involved in the pathophysiology of ischemic/reperfusion injury have not been fully understood, apoptosis regulated by series of complicated intracellular and extracellular pathways seems to play an important role in the pathophysiology of intestinal ischemic/reperfusion (Brath eta l., 2011; Ortiz et al., 2003). In the process of apoptosis, cytokines belonging to the tumor necrosing factor (TNF) superfamily bind to and activate cell membrane death receptors (Ortiz et al., 1999).

Recent studies show that erythropoietin (EPO), a glycoprotein hormone essential for normal erythropoiesis, has a protective role in prevention of programmed cell death (Hashemzadeh et al., 2012). EPO binds to a surface receptor and begins a series of mechanisms leading to production and activation of certain anti-apoptotic proteins (Oda et al., 1998; Silva et al., 1999). The protective effect of EPO on apoptosis has been studied on different organs including intestine. Apart from its anti-apoptotic

features, protective effects of EPO on ischemic/reperfusion injury in tissues have been attributed to anti-oxidative, anti-inflammatory, and angiogenic characteristics of EPO (Hu et al., 2012; Wang et al., 2010; Fan et al., 2010; Manzoni et al., 2005). Although numerous investigations have targeted at the effects of EPO on ischemic/reperfusion injury in different tissues (Sharples et al., 2004; Solaroglu et al., 2004, 2003; Junk et al., 2002; Ergur et al., 2008; Karaca et al., 2009; Hashemzadeh et al., 2012), the efficacy of EPO on intestinal ischemic/reperfusion injury has been less studied. Therefore, the aim of the present study was to evaluate whether EPO has protective effects on the intestinal ischemic/reperfusion injury in rabbits.

2. Material and Methods

Thirty healthy male New Zealand white rabbits were included in the study in accordance with the NIH Guide for the Care and Use of Laboratory Animals and local institutional guidelines for humane use of animals in research. All rabbits were kept under standard laboratory conditions and examined by a veterinarian. The animals had free access to food and water and were kept in room temperature (Hashemzadeh et al., 2012; Somi et al., 2011; Jouyban et al., 2011; Ashrafi et al., 2013). The study protocol was approved by the ethic committee for experimental research at Tabriz University of Medical Sciences.

Feeding of the animals was stopped 24 hours prior to the induction of the intestinal ischemic/reperfusion injury and they received only water. Animals were kept under general anesthesia with intraperitoneal pentobarbital (50 mg/kg) and placed on temperature controlled surgery tables. The anesthetized animals were maintained on heating pads throughout the procedure. Following a midline laparatomy, superior mesenteric artery was occluded with an atraumatic microvessel clamp for 60 minutes. Then, the animals were randomly divided into two groups: the control group (n=15) and the EPO-treated group (n=15). In the EPO-treated group, subcutaneous EPO (1000 IU/kg) was given 10 minutes before clamping, 30 minutes after clamping and immediately before declamping. Likewise, subcutaneous saline was injected as placebo in the control group. All other conditions were similar in the two groups.

Blood sampling from the ear-vein catheter was performed before, at 2, 6 and 12 h after ischemic/reperfusion injury. Stored blood samples at -70°C were used for biochemical analysis including aspartate aminotransferase (AST), alanine aminotransferase (ALT), interleukin-6 (IL-6), tumor necrosis factor-alpha (TNF-α), blood urea nitrogen (BUN), and creatinine (Cr) measurements.

For histopathological evaluation, each studied group was subdivided into 3 groups of five. The animals in these groups underwent resection of a 3-cm segment of distal part of the terminal ileum from the ischemic intestine at 2, 6 and 12 hours after ischemic/reperfusion injury, respectively. All sections were stained with hematoxylin and eosin, and were assessed by a blinded pathologist using light microscopy. A histopathology score was recorded for each section based on Park's score: grade 0, no damage to villi; grade 1, occasional tips affected; grade 2, majority of tips affected; grade 3, majority of tips and some villi affected; grade 4, tips, mid and lower portions of the majority of villi affected (Mori et al., 2008).

Data were presented as mean ± standard deviation (SD). Statistical analysis was performed with statistical package for social sciences (SPSS) for windows version 16.0 using Independent Samples T Test. A P value <0.05 was considered statistically significant.

3. Results

Before the ischemic/reperfusion injury, the mean creatinine, LDH and AST values were higher in the EPO-treated group, while the control group had higher mean ALT values (P<0.05, Table 1). At 2 and 6 hours after the ischemic/reperfusion injury, the mean plasma levels of IL-6 in the EPO-treated group were lower than those in the controls (P<0.05, Tables 2 and 3). However, the mean TNF-α levels were lower in the control group at 2 hours after the injury (P=0.01, Table 2). In addition, the mean BUN and LDH levels were lower in the EPO-treated group at 2, 6, and 12 hours after the ischemic/reperfusion injury (P<0.05, Tables 2 and 3). In contrast, the mean creatinine levels were higher in the EPO-treated group at 2, 6, and 12 hours after the ischemic/reperfusion injury (P<0.05, Tables 2-4).

Table 1. The biochemical parameters before the ischemic/reperfusion injury (CRP, C-Reactive Protein; IL-6, Interleukin-6; TNF-α, Tumor Necrosis Factor-Alpha; BUN, Blood Urea Nitrogen; Cr, Creatinine; LDH, Lactate Dehydrogenase; AST, Aspartate Aminotransferase; ALT, Alanine Aminotransferase)

	EPO-treated group	Control group	P value
CRP	0.06±0.25	0.00±0.02	0.37
IL-6	0.54±0.18	0.48±0.23	0.49
TNF-α	3.26±1.25	3.53±0.75	0.48
BUN	50.96±8.26	54.06±14.77	0.48
Cr	2.12±0.5	0.94±0.21	<0.001
LDH	198.02±73.75	127.78±21.02	0.001
AST	20.72±7.61	11.63±6.42	0.001
ALT	20.24±5.13	23.86±4.51	0.04

Table 2. The biochemical parameters 2 hours after the ischemic/reperfusion injury (CRP, C-Reactive Protein; IL-6, Interleukin-6; TNF-α, Tumor Necrosis Factor-Alpha; BUN, Blood Urea Nitrogen; Cr, Creatinine; LDH, Lactate Dehydrogenase; AST, Aspartate Aminotransferase; ALT, Alanine Aminotransferase)

	EPO-treated group	Control group	*P* value
CRP	0	0.03±0.12	0.32
IL-6	0.4±0.12	0.79±0.53	0.01
TNF-α	6.78±5.89	2.52±0.82	0.01
BUN	60.74±6.99	81.27±10.37	<0.001
Cr	2.46±0.6	1.35±0.18	<0.001
LDH	901.25±187.85	1165.5±362.78	0.01
AST	33.17±7.13	80.04±107.67	0.1
ALT	30.71±7.13	36.04±11.14	0.13

In the EPO-treated group, the mean levels of IL-6 (*P*=0.02), LDH (*P*<0.001), AST (*P*=0.03), and ALT (*P*=0.02) at 6 hours after the ischemic/reperfusion injury were significantly higher than those at 2 hours after the ischemic/reperfusion injury. Furthermore, the mean levels of IL-6 (*P*=0.04), creatinine (*P*=0.003), BUN (*P*=0.04), LDH (*P*<0.001), AST (*P*<0.001), and ALT (*P*<0.001) at 12 hours after the ischemic/reperfusion injury were significantly higher compared with those at 2 hours after the ischemic/reperfusion injury. Between 6 and 12 hours after the ischemic/reperfusion injury, only the mean LDH (*P*=0.003), AST (*P*<0.001), and ALT (*P*<0.001) levels significantly increased in the EPO-treated group.

Table 3. The biochemical parameters 6 hours after the ischemic/reperfusion injury (NA, Not Available; CRP, C-Reactive Protein; IL-6, Interleukin-6; TNF-α, Tumor Necrosis Factor-Alpha; BUN, Blood Urea Nitrogen; Cr, Creatinine; LDH, Lactate Dehydrogenase; AST, Aspartate Aminotransferase; ALT, Alanine Aminotransferase)

	EPO-treated group	Control group	*P* value
CRP	0	0	NA
IL-6	0.58±0.25	1.31±0.82	0.003
TNF-α	3.76±2.48	3.34±1.49	0.57
BUN	63.74±3.56	83.23±13.03	<0.001
Cr	2.74±0.57	1.61±0.18	<0.001
LDH	1222.1±150.8	1918.8±473.87	<0.001
AST	40.83±11.13	76.42±29.46	<0.001
ALT	36.86±7.32	38.7±11.13	0.59

Histopathological evaluation results of the present study are shown in Table 5. Histopathological assessment revealed that Park's score at 12 hours after the ischemic/reperfusion injury was significantly lower in the EPO-treated group compared with the control group (*P*=0.001, Table 5).

Table 4. The biochemical parameters 12 hours after the ischemic/reperfusion injury (CRP, C-Reactive Protein; IL-6, Interleukin-6; TNF-α, Tumor Necrosis Factor-Alpha; BUN, Blood Urea Nitrogen; Cr, Creatinine; LDH, Lactate Dehydrogenase; AST, Aspartate Aminotransferase; ALT, Alanine Aminotransferase)

	EPO-treated group	Control group	P value
CRP	0.06±0.25	0.04±0.15	0.73
IL-6	1±1.08	1.84±1.97	0.21
TNF-α	4.09±3.67	6.44±8.26	0.32
BUN	68.65±12.58	79.8±16.46	0.04
Cr	3.1±0.48	2.23±0.3	<0.001
LDH	1553.2±359.73	2021.1±613.61	0.01
AST	70.62±19.97	81.54±9.6	0.06
ALT	65.36±13	40.91±12.47	<0.001

Table 5. Histopathology scores in both studied groups

	EPO-treated group	Control group	P value
Score at 2 h	2±1.87	2.4±1.81	0.74
Score at 6 h	2±1.22	3±1.22	0.23
Score at 12 h	1.6±0.57	3.4±0.54	0.001

4. Discussions

The present study revealed that EPO might have protective effect on the intestinal ischemic/reperfusion injury probably through suppression of IL-6 production. This finding is parallel to that of the similar investigation by Mori and colleagues (2008). Mori et al. (2008) induced intestinal ischemic/reperfusion injury in rats followed by subcutaneous administration of EPO (1000 U/kg) and its nonhematopoietic derivative (asialoEPO). They concluded that EPO and asialoEPO had protective effects against intestinal ischemic/reperfusion injury through inhibiting apoptosis, and release of IL-6 and TNF-α (Mori et al., 2008). Likewise, other similar studies confirmed this protective effect of EPO on intestines. However, these studies differ from our and Mori and colleagues' investigations in terms EPO dosage and route of administration, and biochemical analyses. In a study on rats, Guneli and colleagues (2007) found that intraperitoneal administration of EPO (5000 U/kg) either at five minutes before the ischemia or at the onset of reperfusion resulted in protection against intestinal ischemic/reperfusion injury. They reached such a conclusion based on tissue indicators of decrease in oxidative stress and apoptosis, and improvement in tissue (jejunum) injury (Guneli et al., 2007). In another study by Sayan et al. (2009), intraperitoneal administration of recombinant human EPO (1000 or 3000 U/kg) 24 hours before the intestinal ischemic/reperfusion injury in rats led to significant physiological and histopathological improvements. The former was determined according

to the inhibition of oxidative stress and leukocyte infiltration following EPO injection (Sayan et al., 2009).

The results of the present study are consistent with those of the previous investigations highlighting the protective effect of EPO on ischemic/reperfusion injury in different tissues. Protection against the ischemic/reperfusion injury following EPO administration has been reported in retina (Junk et al., 2002), central nervous system (Solaroglu et al., 2003; Smith et al., 2011; Teng et al., 2012), myocardium (Calvillo et al., 2003), kidney (Yang et al., 2003), liver (Solaroglu et al., 2004), lung (Wu et al., 2006), testicle (Ergur et al., 2008), and ovary (Karaca et al., 2009). In contrast, our previous investigation on the limb ischemic/reperfusion injury failed to result in similar findings (Hashemzadeh et al., 2012). Based on the plasma IL-1β, IL-6, and TNF-α levels as well as the histopathological assessment, EPO had no protective effect on ischemic/reperfusion injury of the limbs in rabbits (Hashemzadeh et al., 2012).

In conclusion, EPO might exert a protective effect against ischemic/reperfusion injury in the rabbit model of intestinal ischemia. This protective effect might be attributed to the anti-inflammatory and antiapoptotic characteristics of EPO through IL-6 suppression.

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