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Experimental study on EPO treatment of model rats with infection-induced acute liver injury

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ABSTRACT

Objective: To study the effect of EPO therapy on liver injury, inflammation, oxidative stress and cell apoptosis in model rats with infection-induced acute liver injury.
Methods: SD rats were selected and randomly divided into Sham group, sepsis group (CLP group) and EPO intervention group (EPO group), the cecal ligation and puncture was adopted to establish sepsis models and 5000IU/kg human recombinant EPO was provided for intervention. 24 hours after intervention, serum levels of liver injury molecules, inflammatory factors and oxidative stress molecules as well as liver tissue levels of oxidative stress molecules and cell apoptosis molecules were detected.
Results: Serum ALT, AST, TNF-α, IFN-γ, IL-1β, IL-6, IL-8, MDA and AOPP levels, liver tissue Nrf2, ARE, MDA and AOPP levels as well as Bax, Caspase-9 and Caspase-3 mRNA expression of CLP group were significantly higher than those of Sham group while liver tissue HO-1 and SOD levels as well as Bcl-2 mRNA expression were significantly lower than those of Sham group; serum ALT, AST, TNF-α, IFN-γ, IL-1β, IL-6, IL-8, MDA and AOPP levels, liver tissue MDA and AOPP levels as well as Bax, Caspase-9 and Caspase-3 mRNA expression of EPO group were significantly lower than those of CLP group while liver tissue Nrf2, ARE, HO-1 and SOD levels as well as Bcl-2 mRNA expression were significantly higher than those of CLP group.
Conclusions: EPO therapy could reduce liver injury and inhibit inflammation, oxidative stress and cell apoptosis in model rats with infection-induced acute liver injury.

1. Introduction

Sepsis is the systemic inflammatory response syndrome (MODS) caused by severe infection, and the basic characteristics of MODS are the abnormal inflammation activation and immune response disorder caused by the excessive release of inflammatory mediators in the body. On the basis of continuous inflammatory reaction activation, the risk of multiple organ dysfunction syndrome (MODS) greatly increases, the prognosis is poor and the mortality is high[1-3]. Liver injury is a central part causing MODS development and change, and also an important factor that affects the prognosis of patients with sepsis[4-5]. Therefore, reducing the infection-induced acute liver injury is the important link to prevent the occurrence of MODS in patients with MODS and improve the disease prognosis and outcome[6].

Erythropoietin (EPO) is an endocrine hormone that has promoting effect on hematopoietic stem cell differentiation and red blood cell growth, and it is widely used in the treatment of anemia. Recent studies have found that EPO can not only promote hematopoietic function, but also has the biological effects such as inhibiting inflammation, resisting oxidative stress and inhibiting cell apoptosis, and it has protective effect on the damage to multiple organs[7-8]. In the following study, in order

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to define the EPO effect on liver injury in sepsis course, cecal ligation and puncture was adopted to establish rat models with infection-induced acute liver injury and the EPO protection on liver function was specifically analyzed.

2. Materials and methods

2.1 Experimental animals and groups

Adult SPF male SD rats with body mass 250-300g were selected as experimental animals and purchased from Sun Yat-Sen University laboratory animal center, and animal license was SCXK (Guangdong) 2011-0029. Animal experiments passed through the hospital ethical review, and animal experiments and animal processing after death were conducted according to the rules. The SD rats were randomly divided into Sham group, sepsis group (CLP group) and EPO intervention group (EPO group), 8 in each group.

2.2 Experimental reagents and instruments

Human recombinant EPO was bought from Shenyang Sunshine Pharmaceutical Company, enzyme-linked immunosorbent assay kits were bought from Nanjing Jiancheng Biotechnology Company, and RNA extraction, cDNA synthesis and PCR reaction kits were purchased from Beijing ComWin Company. Microplate reader and PCR reaction instrument were bought from Bio-rad Company.

2.3 Experimental methods

2.3.1 Model establishment and drug intervention methods

The CLP group and EPO group were established into sepsis models according to the following method: they received intraperitoneal injection of 3mg/kg 10% chloral hydrate for anesthesia, the median incision was made in hypogastrium to separate cecum and expose ileocecal valve, 3-0 silk thread was used to ligature the ileum at 15 cm from the ileocecal valve and the mesenteric vessels corresponding to 7-10 cm ileal loop, then the intestinal canal was put back in the abdominal cavity and the incision was sutured. For sham group, same methods were followed for intraperitoneal anesthesia and abdominal incision, the ileocecal valve was exposed, no ligation operation was performed, the intestinal canal was directly put back in the abdominal cavity and the incision was sutured. After model establishment, EPO group of rats were given 5000IU/kg human recombinant EPO injection via caudal vein, and the other two groups were given the same dose of saline injection via caudal vein.

2.3.2 Specimen collection methods

24 hours after model establishment, the three groups of rats were put to death, peripheral blood specimens were collected and centrifuged to separate serum, transfer it into 1.5ml EP tube and placed it in a -80℃ refrigerator; the liver tissue was anatomized, washed with saline for 3-5 times, then transferred into cryopreserved tube, shortly frozen in liquid nitrogen, then taken out and placed in a -80℃ refrigerator.

2.3.3 Index detection methods

Serum specimens were taken, automatic biochemical analyzer was used to detect the content of ALT and AST, enzyme-linked immunosorbent assay kits were used to detect TNF-α, IFN-γ, IL-1β, IL-6 and IL-8 levels, and the radioimmunoprecipitation kits were used to detect MDA and AOPP levels. Enzyme-linked immunosorbent assay kits were used to detect Nrf2 and ARE levels, radioimmunoprecipitation kits were used to detect HO-1, SOD, MDA and AOPP levels, BCA kits were used to detect total protein content, and the Nrf2, ARE, HO-1, SOD, MDA and AOPP levels per unit mass of total protein were calculated; RNA extraction kits and cDNA synthesis kits were used to extract the RNA in tissue and reversely transcribed it into cDNA, the PCR reaction kits were used to amplify the Bcl-2, Bax, Caspase-9 and Caspase-3, and the mRNA expression was calculated.

2.4 Statistical methods

SPSS19.0 software was used to input and analyze data, measurement data comparison among three groups was by variance analysis, pair-wise comparison was by LSD-t test and P<0.05 indicated statistical significance in differences.

3. Results

3.1 Serum liver injury molecule and inflammatory factor levels

Analysis of serum ALT and AST as well as inflammatory factors TNF-α, IFN-γ, IL-1β, IL-6 and IL-8 among three groups of rats was as follows: serum ALT, AST, TNF-α, IFN-γ, IL-1β, IL-6 and IL-8 levels of CLP group were significantly higher than those of Sham group, and serum ALT, AST, TNF-α, IFN-γ, IL-1β, IL-6 and IL-8 levels of EPO group were significantly lower than those of CLP group. Differences in pair-wise comparison of serum ALT, AST, TNF-α, IFN-γ, IL-1β, IL-6 and IL-8 levels were statistically significant among three groups of rats (P<0.05).

3.2 Oxidative stress molecule levels in serum and liver

Analysis of oxidative stress molecules MDA and AOPP in serum as well as oxidative stress molecules Nrf2, ARE, HO-1, SOD,

MDA and AOPP in liver tissue among three groups of rats was as follows: serum MDA and AOPP levels as well as liver tissue Nrf2, ARE, MDA and AOPP levels of CLP group were significantly higher than those of Sham group while liver tissue HO-1 and SOD levels were significantly lower than those of Sham group; serum MDA and AOPP levels as well as liver tissue MDA and AOPP levels of EPO group were significantly lower than those of CLP group while liver tissue Nrf2, ARE, HO-1 and SOD levels were significantly higher than those of CLP group. Differences in pair-wise comparison of serum MDA and AOPP levels as well as liver tissue Nrf2, ARE, HO-1, SOD, MDA and AOPP levels were statistically significant among three groups of rats ($P<0.05$).

Table 1

Comparison of serum liver injury molecules and inflammatory factors among three groups of rats

Parameters	Sham group	CLP group	EPO group	P value
Liver injury molecules				
ALT(U/L)	36.48±6.28	296.63±42.15*	126.74±18.92#	<0.05
AST(U/L)	29.12±4.95	164.21±20.19*	85.69±11.48#	<0.05
Inflammatory factors				
TNF-α(ng/ml)	4.29±0.72	14.29±1.95*	8.71±1.02#	<0.05
IFN-γ(ng/ml)	5.61±0.88	22.18±3.28*	9.52±1.15#	<0.05
IL-1β(ng/ml)	2.14±0.35	9.86±1.26*	4.28±0.68#	<0.05
IL-6(pg/ml)	126.74±17.38	368.71±66.28*	201.24±27.58#	<0.05
IL-8(pg/ml)	93.54±11.28	294.45±31.49*	152.29±22.15#	<0.05

*: compared with Sham group, differences were statistically significant, $P<0.05$; #: compared with CLP group, differences were statistically significant, $P<0.05$;

Table 2

Comparison of oxidative stress molecules in serum and liver among three groups of rats

Parameters	Sham group	CLP group	EPO group	P value
Serum samples				
MDA(μmol/L)	2.58±0.41	10.28±1.47*	4.41±0.62#	<0.05
AOPP(μmol/L)	1.03±0.15	6.58±0.93*	1.94±0.26#	<0.05
Liver samples				
Nrf2(ng/mg)	3.94±0.62	5.63±0.78*	9.47±1.15#	<0.05
ARE(ng/mg)	2.15±0.32	3.41±0.67*	7.03±0.94#	<0.05
HO-1(U/mg)	36.48±5.82	12.15±1.95*	27.58±4.96#	<0.05
SOD(U/mg)	72.58±9.38	30.28±5.82*	58.48±7.89#	<0.05
MDA(μmol/mg)	11.28±1.85	37.58±7.19*	20.38±4.85#	<0.05
AOPP(μmol/mg)	5.47±0.87	24.17±4.52*	10.29±1.76#	<0.05

*: compared with Sham group, differences were statistically significant, $P<0.05$; #: compared with CLP group, differences were statistically significant, $P<0.05$;

3.3 Apoptosis gene expression in liver

Analysis of apoptosis genes Bcl-2, Bax, Caspase-9 and Caspase-3 mRNA expression in liver tissue among three groups of rats was as follows: liver tissue Bcl-2 mRNA expression of CLP group was significantly lower than that of Sham group while Bax, Caspase-9 and Caspase-3 mRNA expression were significantly higher than those of Sham group; liver tissue Bcl-2 mRNA expression of EPO group was significantly higher than that of CLP group while Bax,

Caspase-9 and Caspase-3 mRNA expression were significantly lower than those of CLP group. Differences in pair-wise comparison of liver tissue Bcl-2, Bax, Caspase-9 and Caspase-3 mRNA expression were statistically significant among three groups of rats ($P<0.05$).

Table 3

Comparison of apoptosis gene expression in liver tissue among three groups of rats

Parameters	Sham group	CLP group	EPO group	P value
Bcl-2	1.00±0.17	0.45±0.09*	0.82±0.11#	<0.05
Bax	1.00±0.14	2.31±0.36*	1.42±0.18#	<0.05
Caspase-9	1.00±0.21	1.89±0.25*	1.33±0.16#	<0.05
Caspase-3	1.00±0.08	2.52±0.41*	1.50±0.21#	<0.05

*: compared with Sham group, differences were statistically significant, $P<0.05$; #: compared with CLP group, differences were statistically significant, $P<0.05$;

4. Discussion

Sepsis will develop into MODS on the basis of SIRS, liver injury is the important factor causing illness development and change in the process, and protecting the liver damage caused by sepsis is key link to prevent MODA[9-10]. In the research, the cecal ligation and puncture was adopted to establish the rat models with sepsis, and the analysis of serum liver injury molecule levels in model rats with sepsis showed that serum ALT and AST levels of CLP group were significantly higher than those of Sham group. It confirms that sepsis can cause significant liver damage. EPO is an endocrine hormone with anti-inflammatory, antioxidant, anti-apoptotic effects [11-12], and in order to further clarify EPO value for the treatment of acute liver injury induced by sepsis, serum liver damage molecule level change in EPO group was analyzed in the study, and the results showed that serum ALT and AST levels of EPO group were significantly lower than those of CLP group. It is confirms that the EPO has a protective effect on liver function injury caused by sepsis. The excessive activation of the inflammatory response is an important pathological feature of sepsis and also a key pathological link causing liver function injury, and the inflammation cascade activation mediated by TNF-α, IFN-γ, IL-1β, IL-6, IL-8 and a variety of other inflammatory factors is closely related to the occurrence of acute liver damage in the course of the sepsis[13-16]. In the study, analysis of serum inflammatory factor levels in rats with sepsis showed that serum TNF-α, IFN-γ, IL-1β, IL-6 and IL-8 levels of EPO group were significantly lower than those of CLP group. This means that EPO has significant inhibitory effect on the activation of the inflammatory response in the course of sepsis and can significantly reduce the synthesis and secretion of a variety of inflammatory factors.

Acute liver injury induced by sepsis is associated with the

activation of inflammatory response and the secretion of inflammatory factors, and the massive release of reactive oxygen species and the activation of oxidative stress reaction after inflammatory factor infiltration in local liver tissue are also involved in the initiation and progression of liver damage[17-18]. Liver is the body's largest reticuloendothelial system, energy metabolism disorder will appear in the development of sepsis and it will cause the increased formation of reactive oxygen species; locally accumulated reactive oxygen species will have oxidizing reaction with the lipid and protein in liver cells, which on the one hand, causes cell structure and function damage, and on the other hand, generate the corresponding products MDA and AOPP[19-20]. Nrf2 is an important oxidative stress receptor in liver tissue and the transcription regulator of endogenous antioxidant mechanism. In pathological condition of massive formation of reactive oxygen species, the Nrf2 will be activated, dissociate with Keap1and enter into the nucleus, and it is combined with reaction element ARE to start the compensatory expression of antioxidant enzymes such as HO-1 and SOD, and then scavenge the reactive oxygen species through the reduction reaction catalyzed by HO-1 and SOD[21-22]. In the study, analysis of the extent of oxidative stress reaction in rats with sepsis showed that serum MDA and AOPP levels as well as liver tissue Nrf2, ARE, MDA and AOPP levels of CLP group were significantly higher than those of Sham group while liver tissue HO-1 and SOD levels were significantly lower than those of Sham group. It means that the oxidative stress is significantly activated and the formation of oxidation products significantly increases in liver tissue of rats with sepsis, and there is also compensatory activation in Nrf2 antioxidant pathways, but the oxidative stress products will continue to consume HO-1 and SOD, so antioxidant enzyme levels decrease in local tissue. Further analysis of the EPO influence on oxidative stress injury showed that serum MDA and AOPP levels as well as liver tissue MDA and AOPP levels of EPO group were significantly lower than those of CLP group while liver tissue Nrf2, ARE, HO-1 and SOD levels were significantly higher than those of CLP group. This means that EPO can activate the Nrf2 antioxidant pathways in liver tissue of rats with sepsis and relieve oxidative stress reaction.

The inflammatory cytokines infiltrating and the reactive oxygen species accumulating in liver tissue can not only directly cause liver cell damage via inflammatory and oxidative stress reaction, but can also further activate apoptosis to cause liver damage. Mitochondrial pathway apoptosis is an important way to regulate cell apoptosis, and also the cell apoptosis-regulating way most closely related to the liver cell damage in the process of acute liver injury induced by sepsis[23-24]. Mitochondrial membrane proteins Bcl-2 and Bax are the important molecules that regulate mitochondrial pathway apoptosis the former is anti-apoptosis molecule, and the latter is pro-apoptosis molecule[25]. The Bcl-2 can form homodimer with each other to inhibit th release of apoptosis-related proteins from the mitochondria into the cytoplasm, and Bax and Bcl-2 can form heterodimer to antagonize the function of Bcl-2 and promote the release of apoptosis-related proteins from the mitochondria into the cytoplasm[26-27]. When the Bcl-2 and Bax balance is broken, the cytochrome C in mitochondria is released into the cytoplasm, activates caspase cascade amplification reactions, and then mediates apoptosis by Caspase-9 and Caspase-3[28-30]. In the study, analysis of above apoptosis molecule expression in liver tissue showed that liver tissue Bcl-2 mRNA expression in liver tissue of CLP group significantly decreased while Bax, Caspase-9 and Caspase-3 mRNA expression significantly increased; liver tissue Bcl-2 mRNA expression in liver tissue of EPO group significantly increased while Bax, Caspase-9 and Caspase-3 mRNA expression significantly decreased. It means that in the course of sepsis, the mitochondrial pathway apoptosis in liver tissue is significantly activated, and EPO intervention can inhibit the mitochondrial apoptosis in liver tissue.

Above all, it is believed that the activation of inflammation, oxidative stress and mitochondrial pathway apoptosis are closely related to the occurrence of acute liver injury induced by infection; EPO intervention can inhibit the inflammation, oxidative stress and mitochondrial apoptosis of liver tissue in the course of sepsis, and it has positive value for the treatment of acute liver injury caused by infection.

Conflict of interest statement

The authors report no conflict of interest.

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