

Original Article

The protective effects of estrogen against ischemia-reperfusion injury in rat liver transplantation

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Abstract: Objective: To investigate the protective effects of estrogen (E2) against cold ischemia-reperfusion (I/R) injury in rat liver transplantation. Methods: The Sprague-Dawley male rats were randomly divided into Sham operation group (Sham group), control group (I/R group) and experimental group (I/R+E2 group). The receptor of experimental group received intraperitoneal injection of 17 β -estradiol at 500 ug/kg/day for 7 days, the sham operation group and control group were injected with equal volume of saline. Both control and experimental groups used a modified two-cuff technique of liver transplantation with 4 h for cold ischemia. After 6 h reperfusion, serum ALT, AST, LDH, TNF- α and liver tissue MDA, MPO, SOD, Bcl-2 were detected. Survival rate of each group were investigated. Results: (1) Serum ALT, AST, LDH, TNF- α content in I/R+E2 group were significantly lower compared with those in I/R group ($P < 0.05$). (2) Liver tissue MDA concentration, MPO activity in I/R+E2 group were significantly decreased than those in I/R group ($P < 0.05$). (3) Liver tissue Bcl-2 expression and SOD activity in I/R+E2 group were higher than those in I/R group ($P < 0.05$). (4) E2 pretreatment prolonged the survival time of hepatic graft after I/R. Conclusion: Estrogen has significant protective effects against I/R injury during rat's liver transplantation, and the mechanism of this might be reducing the levels of inflammatory cytokines, improving tissue's antioxidant capacity and slowing down liver cell apoptosis.

Keywords: Reperfusion injury, estrogen, liver transplantation, rat

Introduction

Treatment of liver disease in the terminal stage was once a challenge for the clinician, while the survival time of those patients was short due to the lack of effective treatment. Since successfully transferred from experiment to clinic, liver transplantation has become the most effective treatment also the only way to cure the liver disease in the terminal stage. But the cold ischemia-reperfusion (I/R) injury, an inevitable damaging process during liver resection and transplantation [1], often influences the surgical outcome and causes many complications [2] such as primary dysfunction of transplanted liver, acute and chronic graft rejection. Particularly, as shortage of organs, the uses of marginal liver donors means higher risk of graft dysfunction [3]. Gender difference was reported to affect the outcome of I/R injury, Previous study demonstrated the protective effect against I/R injury observed in females was related with endogenous 17 β -estradiol (E2) [4, 5]. During the development of murine

model of reduced-size liver I/R, female mice exhibited less hepatocellular injury and longer survival time than male mice [4]. In addition, female patients who taken hepatectomy because of liver tumor survive a significantly longer time than male patients [6]. Other researches also found that 17 β -estradiol had protective effect against myocardial, cerebral I/R injury [7, 8], and estrogen can accelerate recovery of myocardial cell function after I/R [9]. Based on the above study, we hypothesize that estrogen may play an important role as the protector against injury during liver transplantation. In this research we used the 17 β -estradiol which has the strongest activity in the estrogen family to study its protective effects on cold I/R injury in rat liver transplantation and its related mechanism.

Material and methods

Experimental animal and reagent

This experiment selected 36 healthy male SD rats weighing 200~240 g for each and provid-

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Table 1. Comparison of serum concentration of ALT, AST, LDH between every two groups (U/L, $\bar{x} \pm s$)

Group	Sham	I/R	I/R+E2
ALT	81.36±12.67	1206.66±231.38*	513.39±98.17*.#
AST	59.98±9.16	2461.60±302.19*	1442.31±168.16*.#
LDH	318.86±46.72	2939.82±418.26*	1813.47±268.89*.#

*P < 0.05 versus sham group; #P < 0.05 versus I/R group.

Table 2. Comparison of serum TNF- α content between every two groups (pg/ml, $\bar{x} \pm s$)

Group	Sham	I/R	I/R+E2
TNF- α	12.1±1.9	48.2±9.9*	21.6±4.6*.#

*P < 0.05 versus sham group; #P < 0.05 versus I/R group.

ed by Soochow University Animal Center while the receptor's weight slightly heavier than the donor's. MDA, MPO, SOD, TNF- α kit were purchased from NanJingJianCheng Bioengineering Institute, Bcl-2 and GAPDH antibody were purchased from Santa Cruz Company and 17 β -estradiol were purchased from Sigma company.

Experimental design and surgical procedure

The receptors fasted but drunk without restriction, while donors didn't fast and drunk freely 12 h before operation. The animals were randomly divided into 3 groups with 12 for each. I/R+E2 group received intraperitoneal injection of 17 β -estradiol with 500 μ g/kg/day for 7 days, while sham operation group and control group were equally injected with the same volume of saline. Ketamine was intraperitoneal injected at the dose of 80-100 mg/kg for anesthesia before surgery. Sham group only received opening and closing abdomen operation to dissect ligament around the liver. I/R+E2 group used a two-cuff technique for orthotopic liver transplantation referring to Kamada. Portal vein and liver's inferior vena cava were anastomosed by using the self-made medical plastic sleeve. Liver's superior vena cava was closed by continuously suturing with 6/0 polypropylene sutures. Common bile duct was anastomosed by inserting pediatric epidural catheter stent, and hepatic artery was ligated. Donor liver is preserved in sodium lactate Ringer's solution at 0~4°C for 4 h after removal, and the receptor's anhepatic phase was about 15 min. After the operation, receptors were kept on electric blanket for insulation. When awake, the rats were raised in a single

cage for each and supplied with food. 10 rats in each group were used for survival research.

Specimen collection

The rats in I/R+E2 group and I/R group were executed 6h after portal vein reperfusion and the same procedure was performed for sham group 6 h after closing abdomen as described below. 5~6 ml blood sample was drawn from the inferior vena cava, one part of which was put in anticoagulant tube for detection of liver function, while another part was centrifuged at 4000 r/min for 10 min at 4°C, then kept at -20°C for detection of TNF- α . 300 mg liver tissues was added with tissue protein lysate and protease inhibitor, then the total cellular protein was extracted and stored at -20°C for detection of the expression of Bcl-2 by Western blot. 2 g of liver tissues were made to be 10% tissue homogenate and kept at -20°C for test of MDA content, MPO and SOD activity.

Detection of liver enzyme level, antioxidant capacity and apoptosis

1. Liver function test: Blood samples were sent to the biochemistry laboratory of the institute immediately and the level of alanine aminotransferase (ALT), aspartate aminotransferase (AST), lactate dehydrogenase (LDH) was determined by OLYMPUS-Au2700 full-automatic biochemical analyzer.
2. Serum TNF- α test: Enzyme-linked immunosorbent assay (ELISA) was performed according to the instructions in the kit to measure the absorbance values and standard curve to calculate the content of TNF- α in the specimen.
3. Detection of MDA, MPO, SOD: Enzyme-linked immunosorbent assay (ELISA) was performed according to the instructions in the kit to test the MDA content, MPO and SOD activity in the tissue.
4. Western blot analysis: To test the expression of Bcl-2 in liver tissue, the protein of liver tissue was extracted by lysis buffer, separated by 10% polyacrylamide gel for electrophoresis, then transferred to the polyvinylidene fluoride (PVDF) membrane. The membrane was blocked by 5% milk, then incubated with primary and secondary antibody, and the band density was analyzed by ImageJ.

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Table 3. Comparison of MDA content, MPO and SOD activity in liver tissue between every two groups ($\bar{x} \pm s$)

Group	Sham	I/R	I/R+E2
MDA (nmol/mg)	0.28±0.04	0.71±0.05*	0.54±0.03*#
MPO (U/g)	0.26±0.04	0.64±0.03*	0.47±0.02*#
SOD (U/mg)	16.9±2.1	8.0±0.9*	12.2±1.8*#

*P < 0.05 versus sham group; #P < 0.05 versus I/R group.

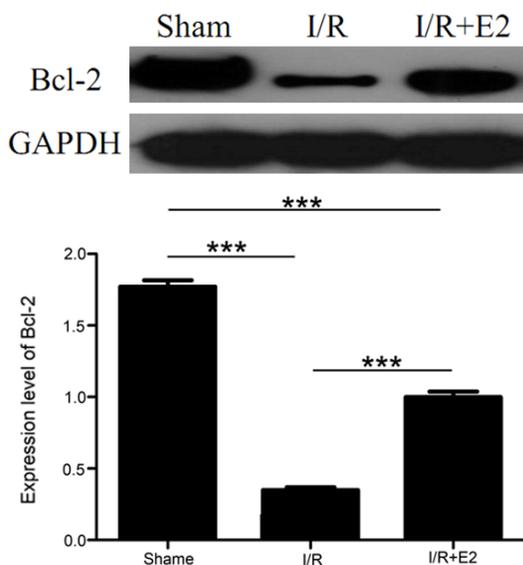


Figure 1. Western blot analysis of Bcl-2 in liver tissue in all groups. The Bcl-2 expression in liver tissue in I/R+E2 group was significantly higher than that in I/R group, while the Bcl-2 levels in I/R+E2 group was lower than that in sham group.

Statistical analysis

All data are represented as the mean \pm standard deviation ($\bar{x} \pm s$), the comparison between every two groups were analyzed using Student's t-test. Graft survival among the groups was evaluated using the log-rank test and Kaplan-Meier survival curves. P < 0.05 was statistically significant.

Results

E2 pretreatment protected liver function after I/R

ALT, AST, LDH levels in I/R+E2 group were significantly lower than those in I/R group after 6 hours of donor liver reperfusion (P < 0.05). ALT, AST, LDH levels in the I/R+E2 and I/R groups were higher than those in sham group (P < 0.05, **Table 1**).

E2 pretreatment reduced serum TNF- α content after I/R

The serum TNF- α content in the I/R+E2 group was significantly lower than that in I/R group (P < 0.05). The serum TNF- α content in the I/R+E2 group and I/R group was higher than that in sham group (P < 0.05, **Table 2**).

E2 pretreatment decreased lipid peroxidation products while increased antioxidant enzymes expression in liver tissue after I/R

The content of MDA, activity of MPO in liver tissue in I/R+E2, I/R groups were higher than those in sham group (P < 0.05), while MDA, MPO in I/R+E2 group were lower than those in I/R group (P < 0.05). The activity of SOD in liver tissue in I/R+E2, I/R groups were lower than that in sham group (P < 0.05), while the SOD activity in I/R+E2 group was higher than that in I/R group (P < 0.05, **Table 3**).

E2 pretreatment alleviate hepatic apoptosis after I/R

The Bcl-2 expression in liver tissue in I/R+E2 group was higher than that in I/R group, while the Bcl-2 levels in I/R+E2 group was lower than that in sham group (**Figure 1**).

E2 pretreatment prolonged the survival time of hepatic graft after I/R

Survival rate of all groups after operation was assessed. 60% of the rats in IR+E2 group survived at 21 days, while only 20% of the rats in the I/R group survived at the same time (**Figure 2**). All rats were alive at 21 days in sham group.

Discussion

Ischemia-reperfusion injury is an important non-immune factor which has significant influence on the result of liver transplantation [10]. This process is considered as the trigger of acute inflammatory reaction, explosion of oxygen radicals, apoptosis and necrosis of cells which commonly cause the damage and dysfunction of transplanted liver [11, 12]. Researches have be focused on how to minimize the damage caused by I/R in order to protect the transplanted liver function. It has been reported that estrogen had significant protective effect on hepatic warm ischemia injury

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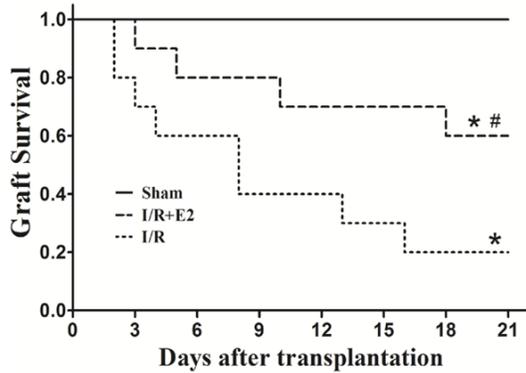


Figure 2. E2 pretreatment prolonged hepatic graft survival after IR. In I/R+E2 group, rat survival rate was significantly increased compared with that in the I/R group. *P < 0.05 versus sham group; # P < 0.05 versus I/R group.

through activating mitogen-activated protein kinase (MAPK) and nitric oxide synthase (eNOS) [13, 14]. However there is few study about the protective effect of estrogen on cold I/R injury. In this study, by pretreatment with exogenous estrogen and usage of rat liver transplantation model, we revealed estrogen could protect transplanted liver function after cold I/R injury.

Our results showed that administration of E2 significantly decrease the concentration of AST, ALT, LDH after I/R, meanwhile, prolonged the survival time of hepatic graft, compared with I/R group without E2 treatment. Concerning liver protection, E2 alleviate hepatic damage through reduced neutrophil infiltration and oxidative stress as decreased TNF- α , MDA, MPO, enhanced antioxidant enzyme activities as increased SOD, and reduced apoptosis and necrosis as increased Bcl-2 in liver tissue.

The Kupffer cells in liver were activated and release a large amount of TNF- α after I/R [15]. There is report showed that TNF- α cause liver endothelial cells produce excessive adhesion molecules and neutrophil accumulation in the liver, which mediated hepatic cell apoptosis and necrosis [16, 17]. Conversely, anti-TNF- α monoclonal antibodies could reduce hepatocyte injury after I/R in an isolated rat liver I/R model [18]. We found E2 pretreatment for rats significantly decreased the expression of TNF- α in transplanted liver after cold I/R and thus attenuate liver injury.

Another possible mechanism to cause I/R injury is oxidative stress, referred as toxic effects

through the generation of peroxides and oxygen-derived free radicals, such as MDA, MPO, the marker of oxidative stress and neutrophil activation, could damage cell components and cause disturbance to cell metabolism and function, eventually leading to cell death. SOD serves key antioxidant enzymes which serves as an oxygen radical scavenger produced by body and provide protection from damage [19]. Treatment with SOD could reduce oxidative stress thus attenuate hepatic I/R injury [20, 21]. We observed that estrogen pretreatment can increase the SOD activity while reduce the MDA content and MPO activity, which relieves the degree of hepatic oxidative stress and inhibits neutrophil infiltration, as a result, significantly alleviates liver injury in cold I/R.

Bcl-2 protein is the founding member of Bcl-2 family and a primary anti-apoptotic protein. Bcl-2 can prevent the releasing of cytochrome c from mitochondria into the cytoplasm, which contributes to the inhibition of cell apoptosis. In this experiment, the Bcl-2 protein expression level in I/R group was significantly decreased compared with that in sham group, correspondingly, liver injury in I/R group was more severe than that in sham group. However, administration of E2 increased Bcl-2 expression compared with I/R group, which partly eased the hepatic injury during rat's liver transplantation.

Above all, estrogen has a significant protective effect against cold I/R injury and prolonged the survival time of hepatic graft after rat's liver transplantation, the mechanism might be due to improvement of tissue's antioxidant capacity, reduction of inflammatory cytokines and inhibition of liver cell apoptosis.

Acknowledgements

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Disclosure of conflict of interest

None.

Abbreviations

E2, 17 β -estradiol; I/R, ischemia-reperfusion; ALT, alanine aminotransferase; AST, aspartate aminotransferase; LDH, lactate dehydrogenase; TNF- α , tumor necrosis factor α ; MDA, malondialdehyde; MPO, myeloperoxidase; SOD, superoxide dismutase.

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References

- [1] Casillas-Ramirez A, Mosbah IB, Ramalho F, Rosello-Catafau J and Peralta C. Past and future approaches to ischemia-reperfusion lesion associated with liver transplantation. *Life Sci* 2006; 79: 1881-1894.
- [2] Pirenne J, Gunson B, Khaleef H, Hubscher S, Afford S, McMaster P and Adams D. Influence of ischemia-reperfusion injury on rejection after liver transplantation. *Transplant Proc* 1997; 29: 366-367.
- [3] Busuttill RW and Tanaka K. The utility of marginal donors in liver transplantation. *Liver Transpl* 2003; 9: 651-663.
- [4] Harada H, Pavlick KP, Hines IN, Hoffman JM, Bharwani S, Gray L, Wolf RE and Grisham MB. Selected contribution: effects of gender on reduced-size liver ischemia and reperfusion injury. *J Appl Physiol* (1985) 2001; 91: 2816-2822.
- [5] Gabel SA, Walker VR, London RE, Steenbergen C, Korach KS and Murphy E. Estrogen receptor beta mediates gender differences in ischemia/reperfusion injury. *J Mol Cell Cardiol* 2005; 38: 289-297.
- [6] Lee CC, Chau GY, Lui WY, Tsay SH, King KL, Loong CC, Hshia CY and Wu CW. Better post-resectional survival in female cirrhotic patients with hepatocellular carcinoma. *Hepato-gastroenterology* 2000; 47: 446-449.
- [7] Fraser H, Davidge ST and Clanachan AS. Activation of Ca(2+)-independent nitric oxide synthase by 17beta-estradiol in post-ischemic rat heart. *Cardiovasc Res* 2000; 46: 111-118.
- [8] Wise PM, Dubal DB, Wilson ME, Rau SW, Bottner M and Rosewell KL. Estradiol is a protective factor in the adult and aging brain: understanding of mechanisms derived from in vivo and in vitro studies. *Brain Res Brain Res Rev* 2001; 37: 313-319.
- [9] Xu Y, Arenas IA, Armstrong SJ, Plahta WC, Xu H and Davidge ST. Estrogen improves cardiac recovery after ischemia/reperfusion by decreasing tumor necrosis factor-alpha. *Cardiovasc Res* 2006; 69: 836-844.
- [10] Lemasters JJ and Thurman RG. Reperfusion injury after liver preservation for transplantation. *Annu Rev Pharmacol Toxicol* 1997; 37: 327-338.
- [11] Walsh KB, Toledo AH, Rivera-Chavez FA, Lopez-Neblina F and Toledo-Pereyra LH. Inflammatory mediators of liver ischemia-reperfusion injury. *Exp Clin Transplant* 2009; 7: 78-93.
- [12] Shen SQ, Zhang Y and Xiong CL. The protective effects of 17beta-estradiol on hepatic ischemia-reperfusion injury in rat model, associated with regulation of heat-shock protein expression. *J Surg Res* 2007; 140: 67-76.
- [13] Harada H, Bharwani S, Pavlick KP, Korach KS and Grisham MB. Estrogen receptor-alpha, sexual dimorphism and reduced-size liver ischemia and reperfusion injury in mice. *Pediatr Res* 2004; 55: 450-456.
- [14] Vilatoba M, Eckstein C, Bilbao G, Frennete L, Eckhoff DE and Contreras JL. 17beta-estradiol differentially activates mitogen-activated protein-kinases and improves survival following reperfusion injury of reduced-size liver in mice. *Transplant Proc* 2005; 37: 399-403.
- [15] Wanner GA, Ertel W, Muller P, Hofer Y, Leiderer R, Menger MD and Messmer K. Liver ischemia and reperfusion induces a systemic inflammatory response through Kupffer cell activation. *Shock* 1996; 5: 34-40.
- [16] Jaeschke H and Farhood A. Neutrophil and Kupffer cell-induced oxidant stress and ischemia-reperfusion injury in rat liver. *Am J Physiol* 1991; 260: G355-362.
- [17] Marubayashi S, Oshiro Y, Fukuma A, Okada K, Maeda T and Dohi K. Protective effect of monoclonal antibodies to adhesion molecules on rat liver ischemia-reperfusion injury. *Transplant Proc* 1999; 31: 1054.
- [18] Ben-Ari Z, Hochhauser E, Burstein I, Papo O, Kaganovsky E, Krasnov T, Vamichkim A and Vidne BA. Role of anti-tumor necrosis factor-alpha in ischemia/reperfusion injury in isolated rat liver in a blood-free environment. *Transplantation* 2002; 73: 1875-1880.
- [19] Shen SQ, Zhang Y, Xiang JJ and Xiong CL. Protective effect of curcumin against liver warm ischemia/reperfusion injury in rat model is associated with regulation of heat shock protein and antioxidant enzymes. *World J Gastroenterol* 2007; 13: 1953-1961.
- [20] Luedde T and Trautwein C. The role of oxidative stress and antioxidant treatment in liver surgery and transplantation. *Liver Transpl* 2006; 12: 1733-1735.
- [21] Minor T, Isselhard W, Yamamoto Y, Obara M and Saad S. The effects of allopurinol and SOD on lipid peroxidation and energy metabolism in the liver after ischemia in an aerobic/anaerobic persufflation. *Surg Today* 1993; 23: 728-732.