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• BRIEF REPORTS •

Protective effect of doxorubicin induced heat shock protein 72 on cold preservation injury of rat livers

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Abstract

AIM: To observe the protective effect of heat shock protein 72 (HSP 72) induced by pretreatment of doxorubicin (DXR) on long-term cold preservation injury of rat livers.

METHODS: Sprague-Dawley rats were administered intravenously DXR at a dose of 1 mg/kg body mass in DXR group and saline in control group. After 48 h, the rat liver was perfused with cold Linger's and University of Wisconsin (UW) solutions and then was preserved in UW solution at 4 ℃ for 24, 36 and 48 h. AST, ALT, LDH and hyaluronic acid in preservative solution were determined. Routine HE, immunohistochemical staining for HSP 72 and electron microscopic examination of hepatic tissues were performed.

RESULTS: After 24, 36 and 48 h, the levels of AST, ALT and hyaluronic acid in preservative solution were significantly higher in control group than in DXR group ($P<0.05$), while LDH level was not significantly different between the 2 groups ($P>0.05$). Hepatic tissues in DXR group were morphologically normal and significantly injured in control group. HSP 72 was expressed in hepatocytes and sinusoidal endothelial cells in DXR group but not in control group.

CONCLUSION: Pretreatment of DXR may extend the time of rat liver cold preservation and keep liver alive. The expression of HSP 72 in liver can prevent hepatocytes and sinusoidal endothelial cells from long-term cold preservation injury.

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INTRODUCTION

The term of organ preservation has been extended enormously since Belzer invented University of Wisconsin (UW) solution in 1988[1]. In liver transplantation, the use of UW solution doubles the time of liver cold preservation compared with the use of Euro-Collins solution. But hepatic cold preservation reperfusion injury remains the important reason for the failure of liver transplantation[2-5]. It was reported that cold preservation reperfusion injury in liver, kidney and lung could be prevented by the expression of heat shock protein, which was induced by heat shock reaction (HSR)[6-8]. Kume *et al*[9] reported that doxorubicin (DXR) could induce HSR, which attenuated hepatic warm ischemia reperfusion injury. DXR is a kind of cytotoxic agent and may induce HSR[10]. Being administered intravenously, DXR was accumulated and metabolized in liver. As a result, semiquinone free radicals were produced, which could induce stress oxidative reaction and expression of HSP 72[11]. Hela cells and cardiac cells could express HSP 72 by pretreatment of DXR *in vitro* in experiments[12,13]. The aim of this study was to observe the protective effect of HSR induced by DXR on long-term hepatic cold preservation injury.

MATERIALS AND METHODS

Reagents

Doxorubicin was purchased from Shenzhen Main Luck Pharmaceuticals. Inc., China-Merian Corp., Japan. University of Wisconsin solution was purchased from Dupan Co., USA. HA RIA kit was purchased from Shanghai Ocean Research Biomedical Technology Center, China. Monoclonal antibody of HSP 72 was purchased from Oncogene Co. DAKO Envision™ kit was from Dako Co.

Animals

Male Sprague-Dawley rats weighing 200-300 g were purchased from Sino-British Sippr/BK Lab Animal Ltd. All rats were kept in animal quarters with controlled temperature (18-25 ℃) and light (light on from 7 a.m. to 7 p.m.) and were fed with a standard laboratory chow and water in accordance with institutional animal care policies. Prior to the experimental procedure, the rats were fasted for 12 hours but allowed free access to water.

Experimental design

Thirty-six rats were randomly assigned into DXR group and control group. The rats in DXR group were injected intravenously DXR at a dose of 1 mg/kg body mass 48 h before the operation[9], while those in control group were treated with 1 mL normal saline (NS). After being cold perfused orthotopically, the livers were preserved in UW solution for 24, 36 and 48 h.

Liver procurement and cold storage

Rat orthotopic liver cold perfusion was used according to the method described by Kamada and Calne[14]. Under ether anesthesia, 2 mL of heparinized saline solution (250 units/mL) was injected intravenously. Laparotomy was performed via a midline incision. After the abdominal aorta was cannulated with a 14-gauge angiocatheter, the liver was flushed with 10 mL of Linger's solution at 4 ℃. Simultaneously, the supra and infrahepatic vena cava were cut to enhance flushing of the liver. Then, 5 mL of Linger's solution, followed by 10 mL of

UW solution at 4 ℃ was progressively injected into portal vein. Finally the liver was excised and preserved in 40 mL of UW solution at 4 ℃.

Hepatic enzyme assays of preservative solution

Alanine aminotransferase (ALT), aspartate aminotransferase (AST) and lactic dehydrogenase level (LDH) in the preservative solutions, collected at 24, 36 and 48 h after cold preservation, were determined by a standard biochemistry autoanalyzer (Backman DU 640).

Assessment of hyaluronic acid (HA) of preservative solution

HA in the preservative solutions collected at 24, 36 and 48 h after cold preservation was determined using a HA RIA kit according to the manufacturer' s protocols.

Histology and immunohistochemical analysis for HSP 72

The liver specimens collected at 24, 36 and 48 h after cold preservation were fixed in buffered 10% formalin and embedded in paraffin. Tissue sections (4-μm thick) were stained with hematoxylin-eosin.

Immunohistochemical analysis was performed by using a mouse anti-HSP 72 monoclonal antibody at a dilution of 1:100, and rabbit anti-mouse IgG as a secondary antibody. Then the specimens were incubated with horseradish peroxidase-conjugated streptavidin complex. Diaminobenzidine was used as the substrate. The specimens were counterstained with hematoxylin. All specimens were examined under an optical microscope (Olympus BX51).

Transmission electron microscopical analysis

Liver fragments of approximately 1 mm×1 mm×2 mm were fixed in 20 g/L glutaraldehyde at 4 ℃. After washed in phosphate buffer solution, fragments were postfixed with 10 g/L osmium tetroxide, dehydrated in graded alcohols, replaced in epoxy ethane and stained with uranyl acetate. All specimens were examined under an electron microscope (Hitachi 500).

Statistical analysis

Values are expressed as mean±SE. Intragroup analysis was performed by paired Student' s *t* test. *P* value less than 0.05 was considered statistically significant.

RESULTS

ALT, AST and LDH level

ALT and AST levels were significantly higher in control group after preservation for 24, 36 and 48 h than in DXR group ($P<0.05$), but LDH level was not different between the 2 groups ($P>0.05$) (Table 1).

Table 1 Comparison of ALT, AST and LDH levels between two groups

Group	*n*	Term of storage	ALT (IU/L)	AST (IU/L)	LDH (IU/L)
Control	6	24 h	337.3±163.4[b]	417.2±247.5[a]	3 369.1±851.9[c]
	6	36 h	589.8±237.3[a]	744.5±317.6 [a]	3 623.2±907.1[c]
	6	48 h	713.3±191.8[a]	1 160.2±321.3 [a]	3 771.7±733.5[c]
DXR	6	24 h	140.5±58.5	148.8±110.6	3 162.5±1442
	6	36 h	347.3±89	340.5±139.4	3 468.7±815.7
	6	48 h	406.0±79	733.3±394.4	3 549.8±894.1

[a]$P<0.05$, [b]$P<0.01$, [c]$P>0.05$ *vs* DXR.

HA level

HA level was significantly higher in control group after preservation for 24, 36 and 48 h than in DXR group ($P<0.05$) (Table 2).

Table 2 Comparison of HA levels between two groups

Group	*n*	Term of storage	HA (ng/mL)
Control	6	24 h	43.1±17.0[a]
	6	36 h	91.2±19.8[b]
	6	48 h	143.7±23.2[a]
DXR	6	24 h	26.2±6.0
	6	36 h	44.2±7.8
	6	48 h	90.2±13.5

[a]$P<0.05$, [b]$P<0.01$ *vs* DXR.

Histology and immunohistochemistry for HSP 72

After cold storage for 24 to 48 h, hepatocytes in control group were swelling, ballooning, vacuolizing and partly autolyzing while the majority of sinusoidal endothelial cells (SECs) also became swollen, rounded and detached from basement membrane and hepatic sinusoids were narrowed. In DXR group, hepatocytes remained normal and few SECs appeared swollen, rounded or detached.

After cold storage for 24 to 48 h, some hepatocytes and SECs expressed HSP 72 in DXR group but not in control group.

Transmission electron microscopical analysis

After cold storage for 24 to 48 h, hepatocytes in control group showed swollen mitochondria, fatty dots, and while cellular swelling, rounding, detachment from the matrix and nuclear condensation were seen in part of SECs. In DXR group, hepatocytes remained normal and few SECs appeared swollen, rounded or detached.

DISCUSSION

The use of University of Wisconsin solution extended the time of organ cold preservation to 24 h in theory[15]. UW solution was designed to attenuate cell swelling, inhibit reactive oxygen intermediates (ROIs), offer substrates for synthesizing adenosine triphosphates (ATP) and prevent cells from absorbing ions of sodium and calcium[16,17]. But in the course of cold storage, cells remained to be injured in different degrees with the extension of preservation time. In the period of liver cold storage, hepatocytes and SECs were all injured, especially the latter[18,19].

During warm ischemia hepatocytes were the most vulnerable cells whereas SECs were less sensitive[20]. But hepatocytes were less damaged during cold preservation[21]. However, along with the extension of the time of cold storage, hepatocytes were also injured in different degrees. The mechanisms underlying hepatocyte injury during hypothermia storage were intracellular acidosis due to anaerobic glycolysis and lactate accumulation, drawback of protein synthesis and exhaustion of hepatin and ATP which lessen the ability to resist rewarm ischemia and reperfusion damage[21]. In morphology, changes of hepatocytes included swelling, ballooning and vacuolization. Although little loss of cell viability occurred after 24 h of storage in UW solution, the cellular functions were already injured[22]. In this study, histological results showed that hepatocytes were apparently damaged in control group after cold storage for 36 to 48 h. In contrast, the shapes of hepatocytes were normal in DXR group. In preservation solution, ALT and AST levels in DXR group were significantly lower than in control group ($P<0.05$). These data indicated that pretreatment with DXR could evidently protect hepatocytes.

During hepatic cold ischemia, SECs were most susceptible

to hypothermia injury. Injuries of SECs were due to angiogenic factors and proteases, resulting in digestion of fibronectin, a key molecule linking collagen to the sinusoidal lining cell membrane integrin[23]. In morphology, SECs became rounded, detached, and sloughed into the sinusoidal lumen[24-26]. These changes were more distinct along with the prolongation of cold storage[27]. But little loss of viability of SECs occurred after 24 h of storage in UW solution[28]. This study indicated that SECs in both groups were injured after 24 to 48 h of cold storage in UW solution, but the degree of injury was significantly attenuated in DXR group than in control group. The shape of SECs was normal after 24 h and a small quantity of SECs became rounded, and detached after 36 to 48 h in DXR group. But in control group, SECs were obviously rounded and detached after 24 h, and the morphological changes became more significant after 36 to 48 h. On the other hand, hyaluronic acid level was significantly higher in control group than in DXR group ($P<0.05$). Hyaluronic acid is a high-molecular-weight polysaccharide that could be rapidly and specifically degraded by SEC[29]. Hyaluronic acid level was a sensitive index of SECs' injury[30]. These results showed that pretreatment with DXR could protect SECs.

Cold storage of livers for more than 12 to 18 h carried the risk for graft failure[31-34] and post-transplantation infection[35]. In recent years, preconditioning has been used to prolong hepatic cold ischemia[6,36-38]. The liver graft was subjected to an acute sublethal stress and this preconditioning could offer resistance to subsequent lethal injury by inducing the expression of heat shock proteins (HSPs). One of the important functions of HSPs was to protect cells and organs from a wide variety of damages such as hypothermia, ischemia, and ROIs[39-41]. Cheng[42] reported that pretreated by Zinc and heat shock, the expression of HSP 72 was markedly elevated after rat livers were stored in UW solution for 24 h. It was also reported that rat kidneys were preserved well successively for 45 h in cold UW solution[43]. In our study, the protective effect of pretreatment of DXR on hepatic long-term cold storage injury might be attributed to induction of HSR and expression of HSP 72 in rat livers. HSR induced by DXR extended the time of hepatic cold storage to 48 h while morphological shape and functions of both hepatocytes and SECs remained commendable. The expressions of HSP 72 in hepatocytes and SECs were confirmed by immunohistochemical staining. It was clear that induction of HSP 72 in advance could make rat livers enter into stress state ahead of schedule and endure long-term cold ischemia injury afterwards.

In conclusion, these results demonstrate that pretreatment with doxorubicin could induce the expression of HSP 72 in rat livers. Thus pharmacological induction of HSR may be a successful method to prevent liver from long-term hypothermia preservation injury.

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