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Effects of cold preservation and warm reperfusion on rat fatty liver

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INTRODUCTION

Although liver transplantation for irreversible liver diseases is increasingly prevalent worldwide, patient die while waiting for donors because of organ short ages. One important problem commonly encountered is that fatty livers often affect the outcome of liver transplantation. It is reported that the incidence of abnormal fatty livers in autopsies after accidental death ranged from 15% to 24%. Since fatty livers may result in a primary nonfunction (PNF) liver graft, which contributes to an increased risk of mortality[1], they are usually out of consideration in liver transplantation. However, some fatty livers can be successfully transplanted. Therefore, how to choose fatty livers as donor organs correctly is the crux of success in liver transplantation.

In this study, we preserved fatty livers of rats fed with a choline-deficient diet in cold Lactate Ringer's (LR) solution for various periods, and evaluated the effects of cold preservation on fatty liver in terms of portal perfusion pressure, endothelin-1, enzyme release in the effluent and mortality of sinusoid lining cell (SLC) using isolated perfused rat liver model.

MATERIALS AND METHODS

Animals and induction of fatty livers

Male Wistar rats, weighing 240 g - 260 g, were obtained from the Experimental Animal Center of Harbin Medical University. To induce fatty deposion in the livers, experimental rats were fed with a chline deficent diet (CDD) for 14, 28 or 42 days. The composition of this diet is shown in Table 1[2].

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Table 1 Components of choline-deficient diet (CDD)

Components	Percentage
Casein	8.0
Lard	47.32
Sucrose	39.275
Minerals	4.0
Vitamins	0.65
L-cystine	0.625
$Mg(OH)_2MgCO_3$	0.1
Vitamin D_3	0.02
Vitamin E	0.01
Total	100.0

Surgical procedures and experimental groups

We used isolated nonrecirculating perfused rat liver as preservation-reperfusion model[3]. Animals were anesthetized with pentobarbital (30mg/kg. ip). After cannulation of the bile duct and portal vein, the liver was flushed with 20 mL 0 ℃ LR solution via portal vein. The liver was removed immediately and stored in 0 ℃ LR solution. After cold storage for various periods, the liver was reperfused for 30 min via the portal vein at 3 $mL \cdot g^{-1} \cdot m$ in^{-1} with Kreb-Henseleit bicarbonate buffer (pH 7.4, 37℃) saturated with a 95% O_2:5% CO_2 mixture in a nonrecirculatory system. Animals were divided randomly into four groups: ① control group (n = 21) fed with a standard diet including three subgroups containing 0 h (n = 7), 6 h (n = 7) and 12 h (n = 7) cold storage; ② mildly fatty liver group (n = 7) fed with a CDD for 14 days was preserved for 12 hours; ③ moderately fatty liver group (n = 14) fed with a CDD for 28 days including two subgroups containing 6 h (n = 7) and 12 h (n = 7) cold storage; and ④ severely fatty liver group (n = 14) fed with a CDD for 42 days consisting of two subgroups containing 0 h (n = 7) and 6 h cold preservation.

Macroscopy and histology of livers before storage

The morphology of livers before cold storage was assessed by macroscopy and light microscopy. Liver biopsy specimens taken from the right lobes of the same site were stained with hematoxylin and eosin.

Portal perfusion pressure

The portal perfusion pressure was detected at the 30th min of reperfusion period when livers were reperfused at 3 $mL \cdot g^{-1} \cdot min^{-1}$ constantly.

ET-1 assay

Two ml effuent was taken at the 30th min of reperfusion period for ET-1 assay. ET-1 values were detected by standard radioimmunoassay methods using a commerci al radioimmunoassay kit (General Hospital of PLA)[4]. Standard curves were obtained with known concentrations of ET-1.

Bile volume

The total bile volume was collected within 30 min reperfusion period. Bile volume was expressed as bile secretion $\mu L \cdot min^{-1} \cdot g^{-1} \cdot$wet weight.

Enzymes in the effluent

Two ml effluent at the 30th min of reperfusion period was collected for detection of aspartate aminotransferase (AST), alanine aminotransferase (ALT) and lactic dehydrogenase (LDH) with an automated GEMSTAR Biochemistry Analyzer (USA).

Mortality of SLC

Trypan blue staining is indicative of loss of cell viability. After 30 min reperfusion, the liver was perfused with trypan blue (200 μm) for 5 min and fixed with a 2% paraformaldehyde: 2% glutaraldehyde solution in the perfusion buffer. Livers were sectioned at the same level in the left lobes and were paraffin-embedded and stained in two sets. One staining with hematoxylin and eosin allowed quantitating of SLC and the other staining with eosin alone permitted determination of the number of trypan blue positive (nonviable) SLC. Five pericentral and five periportal regions within a field measuring 325 μm×325 μm were examined under high power (×400). Mortality of SLC was expressed as the ratio of the number of trypan blue positive SLC to the total amount of SLC.

Statistics

Statistical evaluation was done by Student's *t* test. The results were expressed as $\bar{x}\pm s$. Means were considered significantly different when $P<0.05$.

RESULTS

Macroscopy and histology of livers before storage

In the control group, the morphology of livers before cold storage was normal under macroscopy and light microscopy. The livers of the rats fed with a CDD for 14 days had no abnormality in the appearance, but their specimens contained fatty vacuoles in less than one third of the liver cells. They belonged to mildly fatty livers. The livers of rats fed with a CDD for 28 days were a little larger than normal and appeared slightly yellow. The specimens of these livers contained fatty vacuoles in more than one third of the liver cells but less than two thirds of the cells. They pertained to moderately fatty livers. The livers of rats fed with a CDD for 42 days were obviously larger than normal and appeared grossly yellow. The specimens contained fatty vacuoles in two thirds or more of the liver cells and scattered hepatocyte necrosis occasionally, which were considered as severely fatty livers.

Portal perfusion pressure and ET-1 values

There was significant increase of portal perfusion pressures in each group in parallel with the duration of preservation (Table 2). No remarkable difference of portal perfusion pressures was found between mildly fatty liver group and control group after 12 h preservation, between moderately liver group and control group after 6 h preservation, between severely fatty liver group and control group without preservation. Portal perfusion pressures were significantly higher in moderately fatty liver group than in control group after 12 h preservation ($P<0.01$) and in severely fatty liver group than in control group after 6h preservation ($P<0.01$). The changes of ET-1 values in the effluent were consistent with those of portal perfusion pressures (Table 2).

Bile secretory volume

Bile secretory volume in each group decreased significantly as the preservation time prolonged (Table 2). There was no obvious difference between mildly fatty liver group and control group after 12 h preservation, between moderately liver group and control group after 6 h preservation, and between severely fatty liver group and control group without preservation. Bile production was markedly lower in moderately fatty liver group than in control group after 12 h preservation ($P<0.05$) and in severely fatty liver group than in control group after 6 h preservation ($P<0.01$).

Enzymes in the effluent and mortality of SLC

The changes of enzymatic levels (AST, ALT and LDH) in the effluent and mortality of SLC were consistent with those of portal perfusion pressures (Table 3).

Table 2 Portal perfusion pressure, ET-1 and bile production ($\bar{x}\pm s$, $n=7$)

Group	Preservation time (hours)	Portal perfusion pressure (cmH_2O)	ET-1 (ng/L)	Bile secretory volume ($\mu L \cdot min^{-1} \cdot g^{-1} \cdot wt$)
Control	0	9.5±0.8	35.6±5.8	0.40±0.09
	6	12.8±1.3	60.2±8.4	0.28±0.07
	12	16.0±1.7	83.7±11.9	0.15±0.05
Mildly fatty	12	16.5±2.1	85.9±13.4	0.16±0.05
Moderately fatty	6	13.9±1.7	65.8±10.1	0.26±0.08
	12	20.1±2.3[b]	124.5±27.6[b]	0.11±0.03[a]
Severely fatty	0	9.8±1.0	32.7±4.9	0.38±0.08
	6	18.5±2.1[c]	90.4±15.9[c]	0.12±0.03[c]

[a]$P < 0.05$ *vs* control (12 h); [b]$P < 0.01$ *vs* control (12 h); [c]$P<0.01$ *vs* control (6 h).

Table 3 AST, ALT, LDH in the effluent and mortality of SLC ($\bar{x}\pm s$, $n = 7$)

Group	Preservation time (hours)	AST ($u \cdot l^{-1} \cdot g^{-1} \cdot wt$)	ALT ($u \cdot l^{-1} \cdot g^{-1} \cdot wt$)	LDH ($u \cdot l^{-1} \cdot g^{-1} \cdot wt$)	Mortality of SLE (%)
Control	0	2.42±0.08	8.75±1.40	23.64±4.52	<0.5
	6	2.58±0.12	8.90±1.51	25.72±4.79	5.9±1.4
	12	9.65±1.82	16.75±3.10	54.74±9.70	13.8±2.8
Mildly fatty	12	10.20±2.10	18.20±4.20	58.26±12.15	15.1±3.2
Moderately fatty	6	2.63±0.21	9.01±1.72	28.40±5.10	7.0±1.8
	12	13.50±2.74[b]	24.10±5.86[b]	74.48±19.5 3[b]	18.7±4.3[a]
Severely fatty	0	2.10±0.06	8.60±1.25	21.70±4.30	<0.5
	6	11.82±1.97[c]	19.45±4.76[c]	62.75±17.54[c]	17.4±3.5[c]

[a]$P<0.05$ *vs* control (12 h); [b]$P<0.01$ *vs* control (12 h); [c]$P<0.01$ *vs* control (6 h).

DISCUSSION

Although progress in organ retrieval, preservation, recipient implantation and the rarity of hyperacute rejection, has improved patient survival after orthotopic liver transplantation (OLT), PNF still occurs in 2%-23% of transplanted livers. Transplantation of a fatty liver may lead to PNF. Some researchers hold that fatty liver grafts are unsuitable for elective OLT, since clinical experience evidenced that such grafts may lead to PNF more frequently than nonfatty ones. But others disagree about this because of successful OLT cases with fatty liver grafts. In order to increase the usage of donor livers on the premise of the unaffected outcome of OLT, it is vital to decide whether to choose fatty livers as do nor organs and how to choose them properly.

The CDD-induced fatty liver was produced according to methods described elsewhere. Free fatty acid (FAA) synthesized in the liver bind to phospholipid apoprote in B complex, which is excreted into the blood as very-low-density lipoprotein (VLDL). Choline is a precursor of phosphoryl choline and is important in lipoprotein pro duction. Choline deficiency suppresses the synthesis of the phospholipid-apoprotein B complex, and inhibits VLDL-secretion from the liver. Furthermore, sucrose-rich diet elevates the triglyceride concentration in the liver. These fact ors may cause fatty deposition in the rat liver after 14 days of CDD.

In clinical transplantation, fatty livers are generally graded to three scales depending on the degree of fatty infiltration: mildly (<30%), moderately (30% to 60%) and severely (>60%)[5]. According to these criteria, the liver of rats fed with a CDD for 14, 28 and 42 days should be classified as mildly, moderately and severely fatty livers, respectively. Anchony reported that the overall incidence of fatty infiltration in 124 liver donor biopsies was 24.4%, with 12.3% of biopsies exhibiting mild changes, 8.9% moderate changes, and 3.2% severe changes. Fatty infiltration of the liver can occur in a variety of conditions. Common causes of fatty infiltration include alcohol intake, obesity, nutritional disorders (particularly malnutrition), drug therapy and diabetes although the reason diabetic patients may develop fatty deposion could be related to obesity.

Our experiment showed that there was no obvious difference in the preservative effects between mildly fatty liver group and control group after 12 h preservation and between moderately fatty liver group and control group after 6 h preservation. In view of this, if we gave up using all the fatty livers as donor organs, a lot of available donor livers would be wasted. Meanwhile, the present study demonstrated that preservation reperfusion injury was more severe in moderately fatty liver group than in control group after 12 h preservation and in severely fatty liver group than in control group after 6 h preservation. The increase in the preservation reperfusion injury was manifested as significantly higher portal perfusion pressure, higher ET-1 values, lower bile production, higher enzymatic levels in the effluent and increased mortality of SLC. Therefore, in order to lower the occurrence of PNF, some fatty livers, such as severely fatty livers, should be discarded resolutely although their function was normal or basically normal before storage.

The etiology of increased preservation reperfusion injury of fatty livers has not been clarified[6,7]. To explain the loss of viability in fatty liver grafts after cold preservation, four underlying mechanisms are suggested: ① The solidification of triglycerides during cold storage causes the rupture of the hepacytes containing fat upon rewarming. The rupture of these cells results in the release of fat glubules into the hepatic microcirculation with disruption of sinusoidal architecture, focal hemorrhage and hepatocellular necrosis. ② The increase in Kupffer cell activation was possibly caused by the increased number of Kupffer cells in fatty livers. Activated Kupffer cells can produce

many types of chemical substance and peptide mediators, which may play a significant role in microcirculatory disturbance and reperfusion injury. ③ FFA accumulation in the hepatic mitochondria cause inhibition electron transport in the respiratory chain, affects oxidative phosphorylation activity, reduces the production of ATP, leading to disturbance of energy metabolism. ④ Cellular disruption and release of triglycerides and free fatty acids activate phospholipases and lipid peroxidation, with free radical formation, thereby causing further cellular damage.

In summary, the present study confirms that moderately and severely fatty livers are highly susceptible to cold preservation reperfusion injury and are likely to lose their viability after cold storage more easily than the nonfatty livers, while no obvious difference of the preservative effects is found between mildly fatty livers and nonfatty livers. Fatty livers should not be discarded blindly only for its high incidence of PNF. However, fatty livers should not be used arbitrarily only for the shortage of donor organs either. To use only hepatic function test to assess donor liver is not enough, since the functions of fatty livers are always within normal range. The use of preoperative donor liver biopsies in many sites is considered the most valuable means for the assessment of abnormal hepatic pathology and the correct selection of donor livers. We proposed the following criteria for the use of a fatty liver as a graft: ① a mildly fatty liver can be used in the same way as a nonfatty liver; ② a moderately fatty liver can be used depending on the time of preservation and the balance of the emergent needs of recipient and the donor organ supply; and ③ a severely fatty liver should be discarded without hesitation.

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