The Effects of Cold Preservation Solutions Supplemented with UDCA and α-Lipoic Acid on the Viability and Function of Isolated Human Hepatocytes

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ABSTRACT

Background: Liver transplantation is the only treatment for end-stage and genetic liver diseases. The main burden of this treatment is the shortage of both living and cadaveric liver donors. An alternative treatment is using liver cell transplantation, which can be obtained from unused livers for transplantation. These hepatocytes should be kept ready in viable and functional situation in a frozen state to be instantly used when they would be needed. In our previous experience, we had isolated hepatocytes from unused livers.

Objective: To find a preserving solution for increasing viability and function of the isolated hepatocytes that are stored to be transplanted.

Methods: 9 cadaveric donor livers, which were not used for transplantation due to various causes such as severe steatosis, were selected to isolate hepatocytes. Various cold storage solutions were tried to find the best temperature for more viability and functionality for preservation of hepatocytes. University of Wisconsin (UW) solution and Williams E media were used as control media. 2 anti-apoptotic and anti-oxidative solutions, *i.e.*, α-lipoic acid and ursodeoxycholic acid (UDCA), were used as cold preservatives solutions. The numbers of viable hepatocytes were estimated by trypan blue method; the functionality was assessed by the cells ability to produce urea.

Results: The highest number of viable and functional hepatocytes was obtained from freshly isolated cells. However, after preservation, the number of these viable hepatocytes and their functionality were not significantly different in cold storage solutions comparing to the control media used. Functionality of the isolated hepatocytes stored in UW with and without UCDA solution was similar to freshly isolated hepatocytes.

Conclusion: Preservatives with anti-apoptotic and antioxidant activity could not increase the number of viable hepatocytes. Functionality of cold storing hepatocytes could be preserved similar to freshly isolated hepatocytes by UW solution with and without UCDA.

KEYWORDS: Transplantation; Viability; Cold storage; Preservation

INTRODUCTION

Orthotopic liver transplantation (OLT) is the standard treatment for end-stage liver diseases. However, shortage of transplant liver (cadaveric and living) has caused researchers to find alternative methods to substitute for liver transplant in chronic liver diseases and genetic or metabolic liver diseases [1]. Another problem is acute liver failure, which demands emergency liver transplantation that needs finding a liver within a very short period [1-3].

Transplantation of hepatocytes has been introduced as an alternative to whole organ trans-

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Table 1: Characteristics of nine cases of unused liver donors

No	Sex	Age (yrs)	Cause of death	Cold ischemic time (hrs)	Macrovesicular steatosis (%)	Other pathological findings
1	F	40	Cerebrovascular accident	43	—	Mild cholestasis
2	M	64	Intracranial hemorrhage	40	50	—
3	F	42	Intracranial hemorrhage	24	—	—
4	F	61	Brain edema	44	—	—
5	M	65	Cerebrovascular accident	41	—	—
6	F	29	Methadone poisoning	44.15	30–40	Moderate cholestasis
7	F	35	Brain tumor	43	—	Calcified aorta
8	M	24	Brain tumor	42	30	—
9	F	53	Coma with unknown cause	42	—	Necrosis and congestion

plantation in the above-mentioned situations [4, 5]. Transplantation of hepatocytes in acute liver failure (ALF) can be considered a bridge to recovery of the liver or until an appropriate donor could be found [6, 7].

We have tried to isolate the hepatocytes from unused fatty livers. However, after isolation, preservation of the isolated hepatocytes is very important, because incorrect preservation of these cells would decrease their functionality and vitality [8, 9].

Several methods have so far been used to increase the viability and functionality of the isolated hepatocytes after preservation. One of the agents that has rarely been used in previous reports is ursodeoxycholic acid (UDCA), a hydrophilic bile salt acting as an anti-apoptosis agent [12-14]. Another rarely used agent is α-lipoic acid, which possesses anti-oxidant properties through quenching a variety of reactive oxygen species [15, 16].

In this study, we tried to evaluate the effect of cold preservation solutions supplemented with UDCA and α-lipoic acid on the viability and functionality of isolated human hepatocytes.

MATERIALS AND METHODS

In this study, we isolated hepatocytes from the unused deceased-donor liver tissue samples. The samples were obtained after informed consent and approval by the local Ethical Committee of Shiraz University of Medical Sciences for donated livers. Hepatocytes were isolated from nine liver donors (six females and three males). The age ranged from 24 to 64 years. Table 1 shows the main characteristics of the nine livers.

The selected donor livers were biopsied and the prepared frozen sections were studied and reported by an expert pathologist (BG). Clinicopathologic evaluation and decision for not using the livers were made; unused livers were sent to our lab. The livers were sectioned immediately after transportation in the lab. Liver segments (II and III) were sectioned and flushed with cold 0.9% sodium chloride and 5% dextrose (1:1) followed by flushing with cold University of Wisconsin (UW, SPS-1, USA) solution. The tissue was kept in UW solution at 4 °C overnight.

Human Hepatocyte Isolation

After the above-mentioned procedures, hepatocytes were isolated by collagenase perfusion method as previously described [17]. Major hepatic vessels were cannulated by intravenous cannulae (12–18G, CalMed Laboratories, Medi Mark Europe) and the perfusion tubes were passed through the peristaltic pump heads (Cole-Parmer). The cannulated tissue was kept in sterile organ bag (3M Healthcare) and put in the water bath at 37 °C together with perfusion solutions. The flow rate was set at 60–80 mL/min. The first perfusion solu-

Table 2: Composition of 4 types of cold storage solutions used for preservation of hepatocytes in this study.

Solution	Medium	α-Lipoic acid	UDCA
1	Williams E medium (WEM)	5 mM/L	—
2	Williams E medium	—	5 Mm/L
3	University of Wisconsin solution (UWM)	5 Mm/L	—
4	University of Wisconsin solution	—	5 Mm/L

tion consisted of calcium and magnesium-free Hanks' Balanced salt solution (HBSS, Shelmax, H7015) containing 0.5 mM/L ethylene glycol tetra-acetic acid (EGTA, Sigma cat# E4378) and 5 mM N-acetyl cysteine (Sigma-Aldrich, cat#A9165). The second solution was HBSS without EGTA. In the third and final step, digestion of tissue was done with 0.5 g/L collagenase P in Williams E medium without phenol red (Lonza, cat#BE02-019F) and 50 mg/L DNase (DN25, Sigma-Alrich, CAS #9003-98-9). The digestion time was about 10–30 min; in the meantime, the solution was re-circulated. The digested tissue was then minced in cold high glucose DMEM (Biosera cat#1110). The resulting cell suspension filtered through sterile gauze and centrifuged at 50×g at 4 °C for 5 min. Three washing steps were performed to pellet hepatocytes. Hepatocytes were resuspended in Williams E medium supplemented with 10% FBS (Gibco, cat#10270-106), 32 U/L Insulin, 0.9 mg/L dexamethasone, penicillin-streptomycin (Bioidex, B11036), L-glutamine (Gibco, cat#35050-038) and 1M HEPES (Lonza, cat#BE17-737E). Viability and number of hepatocytes were assessed by trypan blue (Sigma-Aldrich) exclusion test using a hemacytometer (Neubauer, Germany). A sample of cell suspension was mixed with equal volume of 0.4% w/v trypan blue (Sigma, T6146) in phosphate buffered saline (PBS). Hepatocyte viability percentage was expressed in respect to the total number of cells.

Cold Storage Solutions' Composition

To evaluate the effect of α-lipoic acid (Sigma-Aldrich, cat#T1395) and UDCA (Sigma-Aldrich, U5127) on the viability and function of isolated human hepatocytes after storage at 4 °C, different cold storage solutions were made (Table 2). Williams E medium and UW solution were used as the control media.

Hepatocyte Cold Preservation

Immediately after isolation of hepatocytes, the cells were resuspended in different cold storage solutions in 15-mL tubes (Greiner Bio-one, cat#188271). Each experiment contained 4×10^6 viable hepatocytes per mL of cold storage solution (2×10^7 cells in 5 ml cold storage media).The experiments were done in duplicate.

Hepatocyte Culture

Freshly isolated viable hepatocytes (3×10^5 cells) were seeded in each well of 24 wells coated with collagen type I (BD Biocoat, cat#356408 BD Bioscience) in triplicate. The plates were kept at 37 °C, 96% humidity, 95% oxygen and 5% CO_2. After 24 hours, the supernatant of the cultured cells were collected and stored at -20 °C for further analysis.

Assessment of Viability and Plating Cold Storage Hepatocytes

After overnight cold storage, hepatocyte suspensions were centrifuged at 60×g for 5 min at 4 °C. The supernatant of the cells was then discarded; the cell pellets were resuspended in Williams E medium and centrifuged at 60×g for 5 min at 4 °C. The viability of the cells in different groups was determined by trypan blue method in duplicate and then the cells were plated (3×10^5 cells/well). The supernatant of the cultured cells were collected after 24 hours and stored at -20 °C for further analysis.

Assessment of Urea Production

Production and secretion of urea by hepatocytes were assessed by the quantitative colorimetric urea determination kit (Quantichrom Urea Assay kit, DIUR-500 BioAssay systems). The urea production rate was expressed as mg/dL.

Table 3: Hepatocyte viability after cold storage at 4 °C in various cold storage solutions

Groups	Mean number of viable hepatocytes*
Freshly isolated	66.29±1.82 (ref)
William's E medium (WEM)	34.33±2.58
WEM + α-lipoic acid	34.96±1.70
WEM+ Ursodeoxycholic acid	24.02±2.32
UWM (University of Wisconsin medium)	44.90±1.62
UWM + α-lipoic acid	42.65±2.19
UWM + Ursodeoxycholic acid	43.73±2.55

*Viability of hepatocytes after all other treatments was significantly ($p<0.001$) different from that of freshly isolated hepatocytes.

Statistical Analysis

For analysis and the comparison between two groups, *Student's paired t* test was used. Mann-Whitney U test was used for comparison of the effect of cold storage media with freshly isolated hepatocytes in each liver. One-way analysis of variance (ANOVA) and Dunnett test as the post hoc test were used for determination of statistical differences and comparing the groups. A p value <0.05 was considered statistically significant.

RESULTS

Hepatocytes were isolated from nine deceased donors (Table 1).

Viability of Hepatocytes

Freshly isolated hepatocytes showed a mean±SD viability of 66.3%±1.8%; the mean viability of hepatocytes in different groups of cold storage media decreased significantly ($p<0.05$). By using cold storage solutions made of Williams E medium a decrease from 31.3% to 42.3% ($p<0.001$) was observed. In cold storage solutions made of UW solution a decrease from 21.4% to 24.7% ($p<0.001$) was observed. Viability was also significantly ($p<0.001$) higher with UW-based solution (44.9%±1.6%) in comparison with Williams E-based solution (34.3%±2.6%) (Table 3).

Urea Production

The production of urea by hepatocytes (as a surrogate of functionality) was determined in different cold storage solutions at 4 °C to compare with the rate of urea production by freshly isolated hepatocytes. In UW and UW-UDCA, the decrease in urea production was similar to freshly isolated hepatocytes; no significant difference was found between these media and freshly isolated hepatocytes. In other groups, the urea production showed significant decrease comparing with freshly isolated hepatocytes ($p<0.05$) (Table 4).

Table 4: Influence of cold storage solutions on urea production by isolated human hepatocytes

Groups	Urea concentration (mg/dL)	p value (compared to freshly isolated cells)
Freshly isolated hepatocytes	11.33±0.95	
William's E medium (WEM)	6.99±0.72	0.001
WEM + α-lipoic acid	4.95±0.11	<0.001
WEM + Ursodeoxycholic acid	5.50±0.52	<0.001
UWM (University of Wisconsin medium)	9.21±1.14	0.330
UWM + α-lipoic acid	6.87±0.62	0.002
UWM + Ursodeoxycholic acid	8.44±0.91	0.085

DISCUSSION

OLT is currently the treatment of choice for hepatic failure and genetic metabolic disorders. This is however, an invasive procedure with high morbidity. In the meantime, OLT is limited by the shortage of donor liver. Using isolated hepatocytes from unused livers that are not suitable for OLT can be an alternative choice for patients in the waiting list [17]. Transplanting hepatocytes needs preparing sufficient and good quality cells available every time they are needed, so they should be kept cold until they are needed [10].

Cold preservation of the hepatocytes decreases viability and function. There have been many reported ways to help obtaining more functioning and viable hepatocytes during cold preservation [7].

In this study, we compared freshly isolated hepatocytes regarding the number and function with hepatocytes after cold preservation with UDCA and α-lipoic acid to find out if they can be helpful to obtain more functioning and viable hepatocytes, even after cold preservation. This cold preservation can be helpful to isolate hepatocytes, when the unused liver is available and then preserve them in cold until they would be needed in a patient with acute liver failure in emergency situation. However, cold preservation can be harmful to viability and function of hepatocytes [18]. Therefore, there have been many attempts in the previous studies to find an appropriate cold preservative solution to increase cell viability and function. Most of the previous research studies have been performed by using nutrient cold preservative solution media such as UW solution and William's E and an anti-oxidant or anti-apoptosis agent to preserve membrane integrity, and cytoskeletal structures to increase cell viability and function [19, 20].

The combination of anti-apoptotic and antioxidant solutions can be helpful to maintain viability and function of hepatocytes because apoptosis is the main cause of cell death of hepatocytes after cold preservation and anti-apoptotics such as UCDA can be helpful by preventing cell death [21-23].

Oxidative stress can also cause decreased liver cell function and viability [24-26]. Antioxidative agents such as α-lipoic acid can be helpful to increase viability and function of hepatocytes after storage in the cold environment [26].

Although the number of hepatocytes is lower than freshly isolated cells, they are better than preserved hepatocytes without using these solutions. It seems that the combination of UW solution with an anti-apoptotic such as UCDA, can be helpful for preservation of function of hepatocytes in cold environment before injection.

In conclusion, cold preservation of hepatocytes in different cold storage and anti-apoptotic or antioxidant agents cannot be helpful to maintain the number of viable hepatocytes as freshly isolated hepatocytes. However, UW solution with and without UCDA can be helpful to maintain functionality of hepatocytes as same as freshly isolated ones.

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