Bilirubin, a Physiological Antioxidant, Can Improve Cryopreservation of Human Hepatocytes

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ABSTRACT

The availability of cryopreserved hepatocytes is required for a more widespread use of hepatocyte transplantation, but human hepatocytes are easily damaged during freezing-thawing. Here, preincubation with unconjugated bilirubin, a physiological antioxidant, resulted in increased viability and function of hepatocytes (as determined by trypan blue exclusion, mitochondrial succinate dehydrogenases activity, urea synthesis, and cytochrome P450 1A/2) compared with cells incubated without the pigment. These findings suggest that unconjugated bilirubin may be used as cryoprotectant in clinical hepatocyte transplantation.

Key Words: bilirubin, freeze-thaw, hepatocyte transplantation, liver metabolism

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Hepatocyte transplantation is a promising therapeutic tool for a variety of liver diseases (1). A major unresolved issue is the availability of livers for cell isolation, especially with patients requiring treatment in an emergency setting, such as children with urea cycle disorders (2). This problem could be partially solved by establishing banks of cryopreserved hepatocytes. Unfortunately, the freezing-thawing procedures result in poor viability and function of liver cells. The mechanisms underlying freezing-thawing damage are poorly understood but include intracellular formation of ice crystals affecting ultrastructural integrity of the cell, osmotic and mechanical stress, and generation of free radicals (3). The addition of antioxidants, which prevents accumulation of free radicals during the freezing-thawing procedure, can reduce cell damage (4). However, such antioxidants are often not suitable for use in humans. Bilirubin, a yellow tetrapyrrole derived from the enzymatic degradation of heme, is a physiological antioxidant that can act as an efficient free-radical scavenger in vitro at micromolar concentrations (5). Moreover, we have previously shown that bilirubin effectively inhibits bile acid–induced apoptosis in freshly isolated hepatocytes (6). Therefore, in the present study, we investigated whether preincubation of human hepatocytes with unconjugated bilirubin (UCB) can improve postthaw cell viability and function.

MATERIALS AND METHODS

Human liver tissue (n = 7) was collected from donor livers rejected or unused for transplantation and from liver resections after informed consent, in accordance with the research ethics committee of King's College Hospital. Hepatocytes were isolated using a collagenase perfusion technique (7). The freshly isolated human hepatocytes were incubated at 4°C for 2 hours in 10 mL of Williams medium E containing 5% fetal calf serum (control group) or in the same medium supplemented with 50, 100, and 200 μmol/L UCB (UCB 50 μmol/L, UCB 100 μmol/L, and UCB 200 μmol/L groups) (4). UCB (catalogue no. B4126 Sigma-Aldrich) was recrystallized before use (8) and dissolved as described previously (6) to obtain the required pigment concentrations (50, 100, and 200 μmol/L) corresponding to a UCB/bovine serum albumin molar ratio of 10.4, 5.2, and 2.6, respectively. The hepatocytes were pelleted at 50*g* at 4°C for 5 minutes and resuspended at a density of 3×10^6 viable cells per milliliter of cryopreservation medium (University of Wisconsin solution/10% [vol/vol] dimethyl sulfoxide), frozen using a controlled-rate freezer and stored at −140°C for 2 weeks (4). Samples of the freshly isolated hepatocytes were immediately cryopreserved using the same procedure (standard cryopreservation group). The cryopreserved hepatocyte suspensions were thawed quickly by gentle shaking in a 37°C water bath, and the dimethyl sulfoxide was slowly diluted by adding ice-cold Williams medium E (9). After thawing, the hepatocyte pellets were checked for cell number and viability using trypan blue exclusion test, and assayed for mitochondrial succinate dehydrogenases activity (3-[4,5-dimethylthiazol-2-y -2,5-diphenyl tetrazolium [MTT] assay), protein synthesis, albumin synthesis, urea production, and cytochrome P450 1A1/2 activity (7). Statistical analysis of data was performed by comparing means using analysis of variance with repeated measurements and Tukey post hoc test. A P value <0.05 was considered statistically significant.

RESULTS AND DISCUSSION

Hepatocytes are particularly sensitive to cryopreservation (10). Several authors report a remarkable decrease in postthaw viability of human hepatocytes when compared with freshly isolated cells, as well as alterations in cell structure, attachment efficiency, and metabolic functions (11). However, off-the-shelf availability of cryopreserved hepatocytes is critical to allow a more widespread use of the procedure for the treatment of liver disease, especially in emergency settings (7). In the present study, simple preincubation of hepatocytes at 4°C had no effect on postthaw viability, whereas preincubation with UCB improved viability from 43.5% to 66.7%, a value more similar to that for freshly isolated hepatocytes (74.5%). A linear relation was observed between the

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TABLE 1. Effect of incubation of human hepatocytes with or without UCB before cryopreservation on cell viability and function

Experimental groups	Viability, %	MTT assay (OD units)	Urea, mg/mg cell protein	Protein synthesis, counts/min	Albumin synthesis, μg/mg protein	CYP1A2 activity, $pmol \cdot min^{-1} \cdot mg^{-1}$)
Fresh	74.5 ± 2.8	0.155 ± 0.015	6.80 ± 0.900	110.6 ± 32	5.4 ± 0.7	7.72 ± 0.70
Standard	$43.5 \pm 5.7^{\ddagger}$	$0.088 \pm 0.009^{\ddagger}$	$5.00 \pm 0.720^{\ddagger}$	$56.7 \pm 20^{\ddagger}$	$2.95 \pm 0.3^{\ddagger}$	$5.40 \pm 1.18^{\ddagger}$
Control	49.1 ± 2.8	0.089 ± 0.007	5.90 ± 1.800	68 ± 34.5	3.14 ± 0.3	3.75 ± 0.84
50 μmol/L UCB	49.0 ± 3.9	0.092 ± 0.006	6.30 ± 1.900	71.6 ± 37	2.76 ± 0.4	4.41 ± 1.12
100 μmol/L UCB	$58.1 \pm 5.2^{*,\#}$	0.104 ± 0.007	5.81 ± 1.90	68.9 ± 35.3	2.88 ± 0.6	4.62 ± 0.92
200 μmol/L UCB	$66.8 \pm 1.7^{\dagger,\#}$	$0.110 \pm 0.006^{*,\S}$	$6.80 \pm 1.90^{*,\S}$	64.7 ± 33.7	2.89 ± 0.4	$5.09 \pm 1.23^{\dagger}$

Data are expressed as mean ± SE. CYP1A2 = cytochrome P450 1A/2; MTT = methylthiazol tetrazolium; OD = optical density; UCB = unconjugated bilirubin; Fresh = cells after isolation; Standard = cells cryopreserved immediately after isolation; Control = cells preincubated in media alone before cryopreservation.

* Different from control, $P \leq 0.05$.
† Different from control, $P \leq 0.01$.
‡ Different from fresh group, $P < 0.05$.
§ Different from standard, $P \leq 0.05$.
# Different from standard, $P \leq 0.01$.

UCB concentration and cell viability in the groups incubated at 4°C with 50, 100, and 200 μmol/L of UCB ($r = 0.9797$, $P = 0.037$).

Temperature changes were shown to increase reactive oxygen species production in isolated cells (12,13). Both in vitro and in vivo studies have indicated that bilirubin inhibits lipid oxidation and protects against oxygen radical formation that could damage membrane lipids (14). It has also been shown that bilirubin could prevent hepatocyte apoptosis (6), a mechanism that seems to be partially responsible for the postthaw loss of cell viability. However, further investigation is needed to determine the molecular mechanisms underlying the cryoprotective action of bilirubin. The minimal amount of UCB taken up by the cells following incubation at 4°C (0.8 mg/10^9 cells, data not shown) is unlikely to have any impact on blood bilirubin levels following cell transplantation because the infusion of 5×10^9 UCB-treated hepatocytes would correspond to about a 1% increase over the daily bilirubin production (250–350 mg). This intracellular bilirubin is likely to be responsible for the protective effects observed during freezing and thawing. Preincubation of hepatocytes with ascorbate did not affect viability significantly, whereas 30.4% and 35.5% improvement in postthaw viability, respectively, was reported with the addition of trehalose to the cryopreservation medium (15) and by preincubation of hepatocytes in the presence of α-lipoic acid (4). Therefore, the improvement in cell viability observed with UCB supplementation is similar to that described with previously tested antioxidants.

UCB is a physiological compound suitable for clinical use differently from trehalose and has a positive effect on mitochondrial dehydrogenases activity, unlike α-lipoic acid (4). In vitro cell viability may not reflect cell function after transplantation in vivo. Recently, Stéphenne et al (16) demonstrated that cryopreservation of hepatocytes induced alteration of the mitochondrial machinery and suggested that protection of the respiratory chain could be critical to improving the quality of cryopreserved hepatocytes. Interestingly, the improvement in cell viability (Table 1) was paralleled by an enhanced mitochondrial dehydrogenase activity as determined by the MTT assay (Table 1), which directly detects the activity of mitochondrial succinate dehydrogenases present in the inner mitochondrial membrane of viable cells. UCB supplementation improved urea synthesis and cytochrome P450 1A1/2 (Table 1), a typical liver-specific function, but it did not ameliorate protein and albumin synthesis (Table 1), suggesting that the results of the metabolic tests, even when significant, should be interpreted with caution. A similar discrepancy among different metabolic parameters was also previously reported (4,17). A linear relation was observed between the UCB concentration and cytochrome P450 1A1/2 in the groups incubated at 4°C with 50, 100, and 200 μmol/L of UCB ($r = 0.9814$). Interestingly, it was recently shown that bilirubin mainly protects intracellular lipids from oxidation, whereas GSH primarily protects water-soluble proteins, suggesting that the 2 classes of antioxidants may have complementary roles (14). These observations suggest that different antioxidants could be combined to improve postthaw cell viability. As new and promising cryoprotective protocols are being described (11), it has become apparent that the mechanisms of cryopreservation injury are complex, involving mechanical stress (ice crystal formation), osmotic imbalance, and free-radical formation (18). Therefore, optimal cryoprotection will likely require the association of multiple remedies active on the different factors involved in cell injury. The finding that a physiological antioxidant such as bilirubin can act as cryoprotectant could contribute to provide good-quality cryopreserved hepatocytes fully compatible with clinical transplantation.

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