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SHORT COMMUNICATIONS

Maintenance of viability and transport function after preservation of isolated rat hepatocytes in various simplified University of Wisconsin solutions

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Abstract—Rat hepatocytes were preserved for 24 hr with high recovery and good maintenance of viability and transport function both in University of Wisconsin (UW) solution and in various simplified UW solutions. Cell quality is somewhat affected after 48 hr of preservation in both the original UW solution and the simplified solutions. ATP content and uptake rate of taurocholic acid are more sensitive markers of cell viability than Trypan blue exclusion or the MTT test. A much less expensive solution than UW, containing only K^+-lactobionate, KH_2PO_4, $MgSO_4$ and raffinose, can be used successfully for preservation of rat hepatocytes for 24 hr for drug transport studies.

University of Wisconsin (UW*) solution has proved to be an excellent storage solution for solid organs such as the liver, kidney and pancreas in animal experimental transplantation [1–4] and in clinical transplantation [5–8]. In addition, isolated hepatocytes can be preserved with good maintenance of viability, morphology and transport functions in UW solution [9–14]. In previous studies it was investigated whether organ preservation was successful in simplified UW solutions [15–24]. At present, there is no agreement with regard to the most ideal composition of such a solution.

In the present study we used isolated rat hepatocytes to evaluate the benefit of some of the components of UW solution for cold preservation of hepatocytes. We were interested in whether less expensive versions of UW solution [in which in any case the expensive hydroxyethyl starch (HES) was omitted] were suitable for preserving isolated hepatocytes with similar (or even better) maintenance of viability and drug transport functions. The MTT test, Trypan blue (TB) exclusion and the ATP content were used as viability criteria and the uptake in and excretion from the hepatocytes of [^{3}H]taurocholic acid was taken as a measure of transport function [10]. In addition we investigated whether the storage time could be extended to 48 hr without appreciable loss of viability and transport functions.

Materials and Methods

Materials. UW solution was a kind gift from Du Pont Critical Care (Waukegan, IL, U.S.A.); lactobionic acid was obtained from Aldrich Chemie (Brussels, Belgium) and adenosine from Serva Feinbiochemica (Heidelberg, Germany). All other chemicals were from the same sources as described before [10].

Isolation, preservation and viability test of hepatocytes. Hepatocytes were isolated from non-fasted male Wistar rats (about 300 g) as described previously [25]. The cells were suspended in a modified Krebs–Henseleit buffer [KHB: 118 mM NaCl, 5.0 mM KCl, 1.1 mM $MgSO_4$, 2.5 mM $CaCl_2$, 1.2 mM KH_2PO_4, 25 mM $NaHCO_3$, 10 mM glucose, 10 mM HEPES, 1% (w/v) bovine serum albumin, saturated with O_2/CO_2 (95:5), pH 7.42]. The viability was measured by TB exclusion [10], the MTT test [10, 26] and cellular ATP content after 30 min of incubation at 37°, whereas drug transport function was measured by uptake of 21 μM [^{3}H]taurocholic acid for 20 min at 37° [10]. ATP was extracted from the cells as described before [10] and analysed by a modified HPLC method, according to Ryll and Wagner [27].

Hepatocytes were stored in air at 0–4°, by suspending 60×10^6 cells in 30 mL storage solution. The following preservation solutions were used: solution 1 consisted of the normal UW solution, but without penicillin [10]. Solution 2 was a greatly simplified UW solution, that contained only K^+-lactobionate (100 mM), KH_2PO_4 (25 mM), $MgSO_4$ (5 mM), raffinose (30 mM) and glutathione (3 mM). Solution 3 was a modification of solution 2, in which K^+-lactobionate was replaced by Na^+-lactobionate. Solution 4 was similar to solution 2 with omission of glutathione. After 24 or 48 hr the storage solution was discarded and the cells were washed twice with KHB. The cells were centrifuged in a 45% Percoll solution (10 min, 100 *g*) which has been shown [10] to separate the non-viable cells, (showing high TB uptake and swollen appearance with blebs) from the viable cells (showing intact morphology and high TB exclusion). The Percoll solution with non-viable cells on top was discarded and the cell pellet was washed twice and resuspended in KHB. The recovery, viability with TB and MTT, and cellular ATP content after 30 min of incubation at 37°, and the uptake and excretion rate of 21 μM [^{3}H]taurocholic acid were measured as in freshly isolated cells [10].

Calculation of transport parameters of taurocholic acid. The initial uptake rate (V_i) was calculated during the first 2.5 min, during which uptake was linear and the plateau value taken from 15–21 min. These V_i and plateau values were used for calculation of the transport parameters k_{in} and k_{out} (fractional rate constants for uptake and efflux respectively). k_{in} was calculated using the equation $V_i = Q_i \times k_{in}$, in which Q_i is the amount of taurocholic acid in the medium at $t = 0$. k_{in} was corrected for slight variations in the actual cell concentrations and is given for 1 mL containing 10^6 cells. k_{out} was calculated on the assumption that at plateau value the uptake rate equals the excretion rate: $Q_p \times k_{out} = Q_m \times k_{in}$, where Q_p is the amount in the cells at plateau and Q_m the amount in the medium at plateau.

Statistics. Results were compared statistically using the Student's *t*-test.

* Abbreviations: UW, University of Wisconsin; HES, hydroxyethyl starch; MTT, 3(4,5-dimethyl-thiazoyl-2-yl)2,5 diphenyltetrazolium bromide; TB, Trypan blue; KHB, modified Krebs–Henseleit buffer with 1% bovine serum albumin.

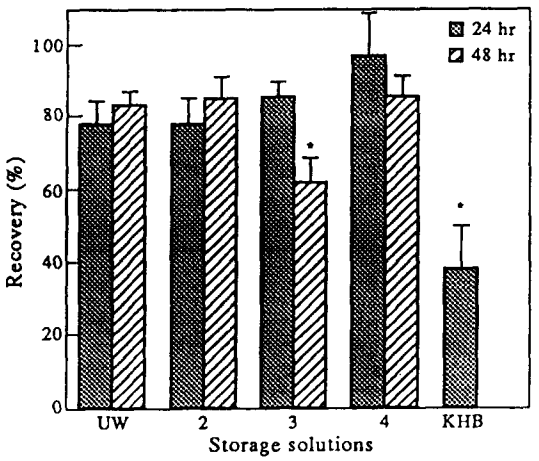

Fig. 1. Recovery of viable rat hepatocytes after preservation in either UW solution, three simplified UW solutions or KHB, and removal of non-viable cells by Percoll. Results are expressed as means ± SEM. N = 7 for UW solution and KHB, N = 3 for the other solutions. *P < 0.05 vs UW.

Table 1. Viability of hepatocytes after 24 and 48 hr of preservation

	TB* (%)	MTT† (%)	ATP‡ (μg/10^6 cells)
Control	83 ± 1 (8)	85 ± 1 (8)	14.0 ± 2.2 (5)
24 hr			
UW	85 ± 3 (8)	83 ± 2 (8)	13.4 ± 1.4 (6)
2	82 ± 2 (3)	88 ± 6 (3)	8.5 ± 2.4 (3)
3	82 ± 2 (3)	79 ± 7 (3)	9.4 ± 2.8 (3)
4	91 ± 2 (3)	91 ± 1 (3)	8.7 ± 2.9 (3)
KHB	81 ± 9 (3)	71 ± 20 (3)	10.8 ± 2.9 (3)
48 hr			
UW	82 ± 3 (8)	80 ± 2§ (8)	7.6 ± 1.9§ (3)
2	81 ± 5 (3)	85 ± 5 (3)	4.4 ± 2.3§ (3)
3	88 ± 3 (3)	71 ± 6§ (3)	4.4 ± 0.3§ (3)
4	84 ± 4 (3)	77 ± 6 (3)	3.4 ± 0.5§ (3)
KHB	ND	ND	ND

Viability tests were performed after removal of non-viable cells by Percoll centrifugation, and results are expressed as means ± SEM, with the number of experiments between brackets.

* TB exclusion after 50 min of incubation at 37°.

† MTT test after 50 min of incubation at 37°.

‡ ATP content in the cells after 30 min of incubation at 37°.

ND, not determined because of low recovery of cells (see Fig. 1).

§ Significantly different from control values at P < 0.05.

Results and Discussion

After 24 hr of preservation the mean recovery of cells after centrifugation in 45% Percoll was equally high for all four preservation solutions: at least 80% of the cells were recovered after washing the cell pellet, which indicates a very efficient recovery of the cells from the preservation solution considering the inevitable loss of cells during the two wash steps (Fig. 1). After 48 hr of preservation only in solution 3 was the recovery significantly decreased compared to UW. This indicates a negative effect of high Na^+ concentration. For comparison, the results of preservation for 24 hr in KHB [10] are given in Fig. 1, Fig. 2 and Table 1. After 24 hr of preservation of hepatocytes in KHB the recovery was much lower than after 24 or even 48 hr of preservation in UW or in one of the modified UW solutions. After 48 hr of preservation in KHB no viable cells were recovered in the pellet after Percoll centrifugation. The cells on top of the Percoll showed high TB uptake and appeared swollen in all of the preservation solutions tested.

The viability of the cells as measured by exclusion of TB *before* the start of the uptake experiments was equally high for all solutions tested (93% ± 1). The high recovery and viability indicate that only a small percentage of the cells lost their viability and appeared on top of the Percoll. Table 1 gives the viabilities, both exclusion of TB and reduction of MTT, as measured *after* the uptake experiments with 21 μM taurocholic acid (= after 50 min of incubation at 37°) indicating the vulnerability of the cells to incubation of 37°. After 24 hr of storage in solutions 1–4, the measured viability was not different from that in freshly isolated cells (control cells, see Table 1) but somewhat lower than before incubation. The reduction of MTT in the hepatocytes after preservation in KHB for 24 hr was somewhat lower than that in freshly isolated cells, but due to the large variation this decrease was not significant. After 48 hr of storage the reduction of MTT by cells preserved in UW solution and in solution 3 was slightly decreased. The lower value in solution 3 indicated again a slightly negative effect of the high Na^+ concentration. In general, the TB and MTT tests seem to give similar indications of cell viability.

During cold storage of isolated hepatocytes ATP may be broken down to its precursors ADP, AMP and adenosine [14]. The ATP content after rewarming and reoxygenation of the cells is considered a sensitive viability marker and gives a good estimate of the functional metabolic capacity of the cells [28–31]. ATP values after 30 min of incubation at 37° are also given in Table 1. After 24 hr preservation in solutions 1–4 or KHB, ATP values were similar to that in freshly isolated hepatocytes (14.0 ± 2.2 μg/10^6 cells). However, after 48 hr of preservation the cellular ATP content following 30 min incubation was decreased significantly for all four preservation solutions used. This apparent discrepancy between the various viability tests was also found by Page *et al.* [31] who showed that ATP content is a more sensitive viability marker than TB exclusion. In a study by Vreugdenhil *et al.* [13], the ATP content in hepatocytes that were rewarmed and reoxygenated during 4 hr, was not greatly affected even after 72 hr of preservation in cold (4°) UW solution. Possibly a longer (more than 30 min) rewarming (incubation) time is necessary in order to replenish the ATP concentration, especially after long storage periods.

The fractional rate constants for uptake (k_{in}) and excretion (k_{out}) of taurocholic acid are shown in Fig. 2. After 24 hr of preservation in either UW solution or the modified UW solutions, the k_{in} was similar to the k_{in} in freshly isolated hepatocytes, being 0.044 ± 0.006 min^{-1} per 10^6 cells. In contrast, storage in KHB for 24 hr lowered the k_{in} to about 25% of the fresh cell value. After 48 hr of preservation in solutions 1–4, the k_{in} values seemed to have decreased, but the difference did not reach statistical significance. The fractional rate constant for excretion (k_{out}) of taurocholic acid from the cells, being 0.167 ± 0.021 min^{-1} in control cells, was not altered significantly after 24 and 48 hr of hepatocyte preservation in solutions 1–4. In contrast the k_{out} was significantly decreased after 24 hr preservation in KHB. The observation that the uptake of taurocholic acid (k_{in}) can be affected while excretion (k_{out}) is still intact was also made by us in a study with human hepatocytes. After 24 hr of preservation in KHB both k_{in} and k_{out} were decreased significantly, showing that both uptake and excretion of taurocholic acid largely deteriorate during preservation in Krebs solution even though these cells show relatively high viability with TB, MTT and ATP.

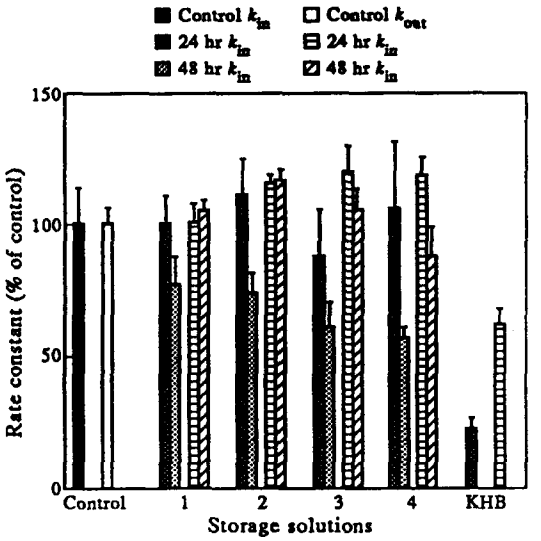

Fig. 2. Rate constants for uptake (k_{in}) and excretion (k_{out}) of taurocholic acid in isolated rat hepatocytes after preservation in either UW solution, three simplified UW solutions or KHB, and removal of non-viable cells by Percoll. Results are expressed as means ± SEM as percentage of control values (k_{in} = 0.044 ± 0.006 min^{-1} per 10^6 cells, k_{out} = 0.167 ± 0.021 min^{-1}). N = 7 for control (on the day of hepatocyte isolation), N = 8 for UW solution and N = 3 for the other solutions. *P < 0.05 vs control.

These results show that routine viability tests such as TB exclusion and the MTT test are not as sensitive parameters for cell viability as is the ATP content or drug transport rate. This indicates that in studies with hepatocytes viability tests should always include functional tests relevant to the particular study.

Considering the tested parameters together, we conclude that the capacities to preserve viability and drug transport in rat hepatocytes are similar for the four solutions tested. The more simple and less expensive solutions 2–4 appear to be good alternatives for the expensive UW solution. Thus adenosine, insulin, dexamethasone, allopurinol, glutathione and HES can be omitted from the UW solution for successful hepatocyte preservation at least for drug transport studies. Considering that HES is added to UW solution in order to prevent expansion of extracellular space [32] it is not unexpected that it can be omitted for preservation of isolated cells. No detrimental effects of the absence of glutathione in solution 4 on the cell quality after preservation was observed. Recently, Kerr-Conte *et al.* [33] showed that glutathione had a beneficial effect on hepatocyte viability only after more than 48 hr of preservation. As indicated by Marsh *et al.* [34] addition of glycine instead of glutathione is more effective for preserving cell viability. Recently, Poullain *et al.* [35] showed that rat hepatocytes completely lost their attachment property to plastic after 48 hr of preservation in UW solution. However, these results may have been influenced by the considerable proportion of non-viable cells in their seeding cell suspension, with release of proteolytic enzymes. In contrast to our study, they observed a considerable loss of cell viability after only 24 hr of preservation in UW solution, a discrepancy which remains to be explained.

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