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Maintenance of xenobiotic metabolism and toxicity in rat hepatocyte cultures after cell preservation at 4°

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Abstract—Isolated rat hepatocytes were stored at 4° in modified University of Wisconsin solution for 22–24 hr and then placed into culture. Satisfactory viabilities were obtained for all preparations. No loss of alcohol dehydrogenase or 7-ethoxy-3,4-dimethylcoumarin *O*-deethylase activity was observed in the cold stored relative to the control cultures. The protein and glutathione contents, allyl alcohol and precocene II (PII) toxicities were also similar, as was the PII-mediated glutathione depletion. For the parameters measured, cultures of cold stored hepatocytes were identical to normal cultures.

In order to make maximal use of scarce liver samples or high yields of isolated hepatocytes a storage method that preserves the function of the cells is required. It has recently been reported that isolated hepatocytes can be successfully stored at 4° in University of Wisconsin (UW*) solution, which was developed for the preservation of organs for transplantation [1]. This solution is based on phosphate buffer containing magnesium and sodium (or potassium) ions, plus impermeants to control cell swelling. It has been shown that viability, morphological appearance, ATP content [1] and transport functions [2] of isolated cells are maintained when hepatocytes are stored for 22 hr in UW solution. Culture of hepatocytes after isolation from cold preserved liver has also been reported [3]. Maintenance of cytochrome P450 (P450) in these cells was comparable to that in control cultures but when isolated hepatocytes were cold stored for 4 hr prior to culture, poor attachment was observed. It has also been reported that the essential components of UW solution for the preservation of rabbit livers were lactobionate, raffinose and glutathione; omission of hydroxyethyl starch (which is not commercially available), adenosine, allopurinol and insulin did not significantly alter the efficacy of the solution [4]. It was therefore suggested that simpler solutions may be adequate for cell storage as opposed to organ preservation. In this study, hepatocytes were successfully cultured for 24 hr after 22–24 hr of cold storage, with maintenance of drug metabolizing activities, in a simplified UW solution.

Materials and Methods

The supplies of plasticware, chemicals, culture media and sera used in these studies have been reported in detail elsewhere [5], as has the source and maintenance of the Wistar rats [6]. Lactobionic acid was obtained from Fluka (Glossop, U.K.). The 7-ethoxy-3,4-dimethylcoumarin was synthesized by alkylation of 7-hydroxy-3,4-dimethylcoumarin with methyl iodide [7], the 7-hydroxy-3,4-dimethylcoumarin itself being synthesized by condensation of resorcinol with ethyl α-methylacetoacetate. Isolation and culture of hepatocytes, and determination of toxicity using 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) have been described previously [8]. Plasma membrane integrity of preparations, before and after cold storage was determined using Trypan blue exclusion. Hepatocytes were seeded onto Falcon *Primaria* 24- and 6-well plates in modified Williams' Medium E (WEC) containing 10% v/v foetal calf serum at 1.2×10^5 cells/cm^2. After allowing 2 hr for attachment, cultures were washed and maintained for 24 hr in serum-free medium (WEX). The toxicities of precocene II (PII) and allyl alcohol (24 hr exposure) were then determined using MTT. Neutral red (NR) uptake was also used to determine PII toxicity: cultures were incubated with medium containing NR at 0.1 mg/mL for 2 hr at 37°, and then washed with saline and treated with 0.5 mL/well destain solution (1% v/v glacial acetic acid, 49% v/v ethanol and 50% v/v water). Samples from each well were then transferred to 96-well plates and read on a Bio-rad microplate reader at 540 nm, with a 405 nm reference filter. For both MTT and NR the percentage survival at each dose was calculated relative to the solvent control for each culture. The TC_{50} values (toxic concentration causing 50% decrease in cell survival) were then determined from interpolation from the survival curve. Protein content and non-protein sulphydryls (NPS; mostly glutathione) were measured after a 6 hr exposure to PII [8]. Homogenates of 24 hr cultures were prepared by sonication [9] and alcohol dehydrogenase (ADH) and 7-ethoxy-3,4-dimethylcoumarin *O*-deethylase (EDCOD) (100 μM substrate concentration) activities determined [8], EDCOD by the method described for 7-methoxycoumarin *O*-dealkylase.

Isolated cells for cold storage were resuspended in 20 mL modified UW solution (100 mM lactobionic acid, 30 mM raffinose, 25 mM KH_2PO_4, 50 mM Na_2HPO_4, 5 mM $MgSO_4$, 3 mM glutathione; pH 7.4) and kept at 4° for 22–24 hr. The cells were then resuspended in WEC + 90% Percoll (1:1, v/v), centrifuged for 10 min and then washed once more with medium before being plated out. Statistical analysis was performed using paired *t*-tests or ANOVA, as appropriate.

Results and Discussion

Recovery of hepatocytes after cold storage, ranged widely from 27 to 78% (Table 1). Only one quote of cell recovery after cold storage could be found in the literature: 85% recovery after Percoll [2], which is clearly better than the 78% maximum obtained in this study. The reason for this loss is not known, however a more complex UW solution may prove to be beneficial with respect to recovery. However, the membrane integrity of all suspensions exceeded 85%, despite the prevalence of numerous small blebs visible on the stored cells (not shown), and all preparations were successfully used to initiate cultures. There were no observable differences in either the attachment or flattening of the stored hepatocytes compared to the controls. The protein data (Tables 2 and 3) also suggest that attachment was not markedly affected by storage at 4°.

* Abbreviations: ADH, alcohol dehydrogenase; EDCOD, 7-ethoxy-3,4-dimethylcoumarin *O*-deethylase; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; NPS, non-protein sulphydryls; NR, neutral red; P450, cytochrome P450; PII, precocene II; TC_{50}, toxic concentration causing 50% decrease in cell survival; UW, University of Wisconsin solution; WEC, serum-containing Williams' medium E; WEX, serum-free Williams' E.

Table 1. Effect of cold storage on recovery and plasma membrane integrity of isolated hepatocytes

% Integrity		% Recovery
Before storage	After storage	After storage
95.5, 92–98	92.0, 86–99	54, 27–78

Plasma membrane integrity and % recovery were determined using Trypan blue exclusion after Percoll separation of viable and dead cells. Median with range, N = 12.

Table 2. Effect of cold storage on enzyme activities in 24 hr cultures of rat hepatocytes

Activity	Control	Cold stored
Protein content, mg/mL	5.08 ± 0.43	4.11 ± 0.36
EDCOD, pmol/min/mg	36 ± 3	42 ± 3
ADH, nmol/min/mg	6.59 ± 0.50	8.28 ± 0.86

Protein content and enzyme activities were determined in homogenates of 24 hr cultures of control and cold stored hepatocytes. Mean ± SEM (8); $P > 0.05$, paired t-test.

ADH, and P450-dependent EDCOD activity were measured in homogenates of 24 hr cultures of stored and unstored hepatocytes (Table 2). Cold storage had no effect on either enzyme activity, suggesting that these cells were still capable of both P450- and non-P450-dependent drug metabolism. In addition, the P450-dependent toxicity of PII [10] and the ADH-dependent toxicity of allyl alcohol [11], as determined by TC_{50} using MTT, were similar in cultures from cold stored and control cells (Table 4a). The MTT absorbances of both control and cold stored untreated cultures were also similar (data not shown), suggesting that mitochondrial function was not impaired in the cultures from cold stored cells. The toxicity of PII was also determined using NR, a supravital dye which accumulates in the lysosomes of cells with intact plasma and lysosomal membranes [12]. The TC_{50} values using NR were similar to those determined using MTT (Table 4b), in both the cultures from control and cold stored cells. The similarity of the toxicity of PII using NR to that determined using MTT provides assurance that the MTT assay is a measure of cell death rather than non-lethal mitochondrial impairment.

Table 4. Effect of cold storage on toxicity in 24 hr cultures of rat hepatocytes

Toxin	Test	TC_{50} μM Control	TC_{50} μM Cold stored
(a) Allyl alcohol	MTT	8.0 ± 3.5 (4)	5.8 ± 1.2 (4)
PII	MTT	46 ± 6 (6)	62 ± 17 (6)
(b) PII	MTT	53 ± 8 (8)	62 ± 17 (6)
PII	NR	41 ± 5 (8)	61 ± 11 (6)

Twenty-four hour cultures of control and cold stored hepatocytes were exposed to PII or allyl alcohol for 24 hr and then the toxicity was determined. Toxicity is expressed as TC_{50}, mean ± SEM (N), calculated as described in Materials and Methods. (a) MTT: $P > 0.05$, paired t-test, control vs cold stored (b) PII: $P > 0.05$ paired t-test, TC_{50} MTT vs TC_{50} NR.

These results indicate that at least two independent pathways of drug metabolism are active in these cultures after cold storage, and also that the mechanism of PII toxicity in cultures from cold stored cells is similar to that in the control cultures. This was further investigated by determination of concentration-dependent NPS depletion after a 6 hr exposure to PII (Table 3). For the solvent controls there was no significant difference in either the proteins or NPS levels between the cultures from cold stored cells and the controls. At this time point there was also no significant loss of cell protein, in cultures from either stored or control cells, at any of the concentrations of PII tested relative to the solvent controls. Depletion of NPS at each concentration of PII was very similar in both sets of cultures, confirming the similarity in the mechanism of toxicity.

This preliminary data indicates that hepatocytes cold stored for 24 hr in a modified UW solution can be used in drug metabolism studies. They retain at least some P450 activity, as judged by the maintenance of EDCOD and the toxicity of PII. ADH activity and allyl alcohol toxicity were also maintained. In addition, the NPS content was similar, as was the concentration-dependent depletion of NPS in the presence of PII. As well as avoiding waste of scarce cellular material, e.g. human liver, this method of storing cells will enable maximal utilization of hepatocytes from a single animal, allowing many parameters to be measured in the same cell preparation, which is not always possible with the time span of one day. In itself, this could help to reduce the numbers of animals required for certain studies.

Table 3. NPS and protein content of 24 hr cultures after a 6 hr exposure to PII

PII (μM)	NPS: nmol/mg (% of 0 μM) Unstored	NPS: nmol/mg (% of 0 μM) Stored	Protein: μg/well (% of 0 μM) Unstored	Protein: μg/well (% of 0 μM) Stored
0	24.2 ± 5.8 (100)	20.4 ± 3.4 (100)	133 ± 12 (100)	108 ± 17 (100)
20	20.2 ± 4.8 (83)	15.4 ± 2.6 (76)	125 ± 11 (94)	107 ± 17 (99)
75	14.8 ± 4.1 (61)	12.7 ± 2.3 (62)	118 ± 10 (89)	109 ± 18 (101)
300	7.00 ± 2.2 (29)	7.00 ± 1.2 (34)	110 ± 10 (83)	107 ± 16 (99)

Twenty-four hour cultures from stored and unstored hepatocytes were exposed to PII for 6 hr and then the NPS and protein contents were determined. Data shown are means ± SEM, N = 7. Stored vs unstored at 0 μM: $P > 0.05$, paired t-test. No significant differences in protein due to PII in either stored or unstored: ANOVA.

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