In Vitro Cell. Dev. Biol. 26:931-932, October 1990

0883-8364/90 $01.50+0.00

Letter to the Editor

CULTURE OF ISOLATED RAT HEPATOCYTES AFTER PROLONGED STORAGE ON ICE—A BETTER USE OF CELL RESOURCES

Dear Editor:

Many studies have reported on aspects of liver metabolism using isolated hepatocytes from a variety of species including mouse (1), rat (2), rabbit (3), dog (4) and human (5). The collagenase perfusion methods, originally described by Berry & Friend (6) and Seglen (7) have been improved and standardized to yield consistently high numbers of hepatocytes. One problem that this can create, particularly when hepatocytes are isolated from valuable or scarce sources, such as large animals or human tissue, is that a large number of cells are produced at a single time and there is likelihood of wastage of cells which cannot all be used at once. The most efficient use of resources would necessitate storage of cells until they can be used, and freeze - preservation would seem the method of choice. However, cryopreservation of isolated hepatocytes has consistently been found to yield only poor recoveries of viable cells, particularly if those cells are required for culturing experiments after freezing (8). Another option might be refrigerated storage of the cells in the liquid state at 0° C and the present study was undertaken to investigate this possibility. Since long-term culture of isolated hepatocytes is, in our experience, a more demanding test of cell function than dye exclusion or enzyme leakage (8), the stored hepatocytes were cultured for 24 hours at 37° C before metabolic assessment was made using gluconeogenesis (9). Results were compared with the response of freshly-isolated cells in culture.

Hepatocytes were isolated from the livers of adult Sprague Dawley rats by a two-step collagenase perfusion method as described previously (8). Cell numbers were counted using trypan blue dye (0.2% wt/v) and yields were commonly between 200-300 $\times$ 10^6 cells with a dye exclusion of 85-90%. Details of the culture conditions have been published previously (8,10). For fresh cells attachment was achieved in 3 hours. In those experiments where hepatocytes were stored, the cells were sedimented by centrifugation at 50 $\times g$ for 2 minutes, and the cell pellet resuspended either in cold L15 or UW solutions (11). The UW solution is a synthetic solution formulated to allow improved storage of organs for transplantation (11). The cells were resuspended at a concentration of 10 $\times$ 10^6/ml and packed in ice for 24 hours. They were then washed twice in an equal volume of fresh L15 before culturing. For stored cells, it became apparent that a longer attachment period was necessary (*see* later) and these cells were left for 24 hours.

After 24 hours in culture, the flasks were washed twice with fresh medium and new L15 medium was added. A multiple time-point analysis of glucose production over 2 hours was made in these flasks. The L15 medium contains pyruvate (5 mmol) which is a stimulatory concentration of pyruvate for gluconeogenesis. Glucose production was measured using a commercial reagent kit (Boehringer Corporation Ltd.). The cells were harvested from the flasks using a rubber policeman in 2 washes (1 and 0.3 ml) and the resultant suspension was used for cell protein measurement by the method of Lowry et al. (12).

The production of glucose by cultures of freshly-isolated cells can be seen in Figure 1. There was a progressive, linear increase in glucose concentration over the 2 hour period, and this was shown to be an active metabolic process since inhibition by cyanide plus azide, or derangement of function by freezing, resulted in abolition of gluconeogenesis. In Table 1 are shown the values for protein content of attached cells, and glucose synthesis, for fresh cells and cells stored either in L15 or UW before culture. When the stored hepatocytes were placed in culture, it was noted that the cells only weakly attached during the first 3 hours. However, if the washing procedure was delayed until the cells had been in culture overnight, firm attachment was achieved and the gross morphology of the cultures was similar to that of fresh cultures. This procedure was adopted in all experiments on stored hepatocytes.

The values for attached protein and glucose synthesis (Table 1) show significant differences between the two groups of stored cells. Approximately twice as many cells (as judged by attached protein) were capable of adhering and surviving in culture when previously stored in UW when compared with those stored in L15 ($P < 0.01$). In fact the protein content was not significantly different in UW-stored cells than in fresh hepatocyte cultures. The difference in attachment between the stored groups was reflected by a noticeable difference in cell covering of the flasks as judged by visual inspection.

When glucose synthesis per unit of cell protein in the two stored groups was compared, again, the UW-stored cells gave higher values ($P < 0.05$) than those stored in L15, although the difference in this aspect was less marked than in cell attachment. Similarly, the UW-stored cells were not significantly different in glucose synthesis than were cultures of freshly-isolated cells. However, cultures of L15-stored cells showed statistically less gluconeogenesis than those of fresh cells ($P < 0.05$).

The present studies indicate that hepatocytes can be stored in the isolated state as a suspension for 24 hours at 0° C and still retain sufficient function to allow subsequent culture experiments to be performed. The overall capacity for glucose synthesis in stored cells following culture for 24 hours was not significantly different from fresh cells provided the hepatocytes had been maintained in a good storage medium (UW). Even those cells maintained at 0° C in normal culture

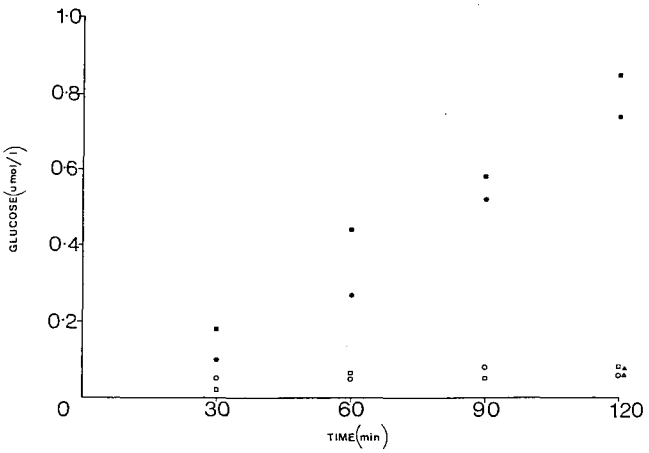

FIG. 1. Glucose synthesis by cultured hepatocytes over time at 37° C. Hepatocytes were plated onto 25 cm flasks (Primaria, Falcon Ltd.) in L15 medium containing 10% foetal bovine serum (GIBCO, UK Ltd.), 80 U/ml gentamycin, 100 U/l insulin (Novo, Denmark) and attachment was achieved for fresh cells during 3 hours at 37° C in an humidified atmosphere containing 5% v/v CO_2 in air. Points (■) & (●) are for two separate fresh cell cultures (means of triplicate measurements at each time). Other flasks from each cell preparation (□) and (○) were subjected to metabolic inhibition with chemicals (cyanide and azide) or were lethally frozen (△) and (▲). Glucose synthesis was abolished by metabolic inhibition.

medium showed considerable gluconeogenesis (on a unit protein basis), which was only just outside the range statistically for fresh cells. The major difference between fresh and stored cells was the speed of attachment in culture, where fresh cells attached within 3 hours as previously noted (10), whilst the stored cells required a longer period (somewhere between 3 and 24 hours) to attach. This delayed attachment may reflect recovery of some particular essential function in the stored hepatocytes since attachment has been shown to occur only in metabolically-active, viable cells. Again, the cells stored in the specifically-designed storage solution showed better recovery in terms of attachment than did those maintained in standard L15 medium.

Our results suggest that, provided care is taken in the design of experiments and appropriate controls are included, stored suspensions of hepatocytes can be used for subsequent culture assays up to a day after isolation.

TABLE 1

PROTEIN CONTENT OF ATTACHED CELLS AND GLUCONEOGENESIS AFTER 24 HOURS IN CULTURE FOR FRESHLY-ISOLATED HEPATOCYTES AND IN HEPATOCYTES STORED ON ICE[a]

	Protein Content mg/plate	Glucose Produced μmol/h/mg protein
Freshly-isolated cells	1.35 ± 0.40	3.22 ± 0.62
Stored in L5 for 24 h	0.88 ± 0.29[b]	2.26 ± 0.30[b]
Stored in UW for 24 h	1.56 ± 0.25	2.89 ± 0.38

[a]Values are means ± SD for triplicate flasks from 5 separate cell isolates.

[b]Denotes statistical difference from freshly-isolated cells.

This may be important where studies on hepatocyte metabolism are performed on cells isolated from human or large animal livers, when large numbers of cells may be produced which cannot all be used at the time of isolation. The one-day storage method may usefully bridge the gap between the need for good viability and a delay to allow more efficient use of the cells which have been produced. Another, and different, aspect of the work suggests that suspensions of hepatocytes might be a useful tool to investigate the constituents of preservation solutions for organs in clinical transplantation.

REFERENCES

1. Renton, K. W.; Deloria, L. B.; Mannering, G. Effects of polyriboinosine acid - polyribocytidylic acid and a mouse interferon preparation on cytochrome P450-dependent monooxygenase systems in cultures of primary mouse hepatocytes. Mol. Pharmacol. 14:672-681; 1978.
2. Bengtsson, G.; Kiessling, K.; Smith-Kiell, A. Partial separation and biochemical characteristics of periportal and perivenous hepatocytes from rat liver. Eur. J. Biochem. 118:591-597; 1987.
3. Corona, G. L.; Santagostino, G.; Facino, R. M., et al. Cell membrane modifications in rabbit isolated hepatocytes following a chronic amitryptilline treatment. Biochem. Pharmacol. 22:849-856; 1973.
4. Powis, G.; Santone, K. S.; Melder, D., et al. Cryopreservation of rat and dog hepatocytes for studies of xenobiotic metabolism and activation. Drug Metab. Disposition 15:826-832; 1987.
5. Houssin, D.; Capron, M.; Celier, C., et al. Evaluation of isolated human hepatocytes. Life Sci. 331:1805-1809; 1983.
6. Berry, M. N.; Friend, D. S. High-yield preparation of isolated rat liver parenchymal cells. A biochemical and fine structural study. J. Cell Biol. 43:506-520; 1969.
7. Seglen, P. O. Preparation of isolated rat liver cells. Methods Cell. Biol. 131:29-83; 1975.
8. Innes, G.; Fuller, B. J.; Hobbs, K. E. F. Functional testing of hepatocytes following their recovery from cryopreservation. Cryobiology 25:23-30; 1988.
9. Hue, L.; Feliu, J.; Hers, G. Control of gluconeogenesis and enzymes of glycogen metabolism in isolated rat hepatocytes. Biochem. J. 218:165-170; 1984.
10. Innes, G.; Fuller, B. J.; Hobbs, K. E. F. Lipid peroxidation in hepatocyte cultures: modulation by free radical scavengers and iron. In Vitro Cell. Dev. Biol. 24:126-132; 1988.
11. Belzer, F. O.; Southard, J. Principles of solid-organ preservation by cold storage. Transplantation 45:673-676; 1988.
12. Lowry, O.; Rosebrough, N.; Farr, A., et al. Protein measurement with the Folin phenol reagent. J. Biol. Chem. 193:265-275; 1951.

B. J. Fuller[1] K. Cheetham

Academic Department of Surgery
Royal Free Hospital School of Medicine
Rowland Hill Street
London NW3 2QG
ENGLAND

(Received 29 January 1990)

[1] To whom correspondence should be addressed at Academic Department of Surgery, Royal Free Hospital School of Medicine, Pond Street, London NW3 2QG, England.

We wish to thank Dr. J. Southard, Organ Preservation Laboratory, Department of Surgery, University of Wisconsin, Madison, for the kind donation of UW preservation solution.