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Preservation and Transportation of Hepatocyte Monolayers in A No Spill Format at Non Freezing Temperatures

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Abstract

Hepatocyte monolayers were preserved under a gelatin overlay at 10 °C. Morphological changes were observed after 4 days, as structure less areas at the cell periphery. The changes could be prevented by adding sucrose (0.05M) to the gel. Cell viability remained high for 7 days. Cell spreading occurred rapidly when the cells were reactivated. Basal enzyme levels were maintained during the preservation period. The hormonal induction of tyrosine aminotransferase was stable for 4 days but declined subsequently. Sucrose addition extended the period of preservation where induction was evident. The method provides a robust system that allows preservation in the absence of potentially deleterious chemicals. Time is allowed to send cells anywhere in the World in a no spill format whist arriving at their destination as if freshly produced.

Keywords: hepatocyte monolayer preservation, gelatin overlay, no spill format, transportation

1. Introduction

The developing areas of cell based therapies, artificial organs and in vitro toxicology all require a method that allows cells to be transported from their site of production to where they are needed. Ideally the cells should arrive with the same characteristics as their freshly prepared counterparts. Cryopreservation is useful for many purposes but invariably the viability is decreased and metabolic function is sub-optimal. Of particular concern is the use of chemicals that affect subsequent cell behaviour (Acker, 2006), changes in the membrane as a consequence of freezing and the requirement for a recovery period (de Loecker et al., 1993). The use of hypothermic, non freezing, temperatures does not damage membranes directly but its use often causes apoptosis (Rauen et al., 2003) and other types of injury (Dai & Meng, 2011). This has led to a range of potential protective agents being added to preservation solutions. The use of "protecting" chemicals may compromise other cellular systems or produce unexpected non-enzymic complications (Evans, 1983). A system where cell change could be stopped, without adding potentially damaging chemicals during the transportation process, but quickly reactivated when required would be of considerable value. Such a method is described in this report for hepatocytes.

Traditionally, hypothermic preservation has been carried out at 4 °C. The rationale is to reduce enzymic activity to a minimum. However, little attention has been paid to the continued requirement for compartmentalisation of the cell during preservation. In particular the cytoskeleton is known to be disrupted at 4 °C (Feldmann, 1989). Energy must be produced continually to maintain cellular integrity. Evidence has been presented that that the optimal preservation temperature is above 4 °C (Evans, 1995; Kruuv et al., 1995).

In the present investigation hepatocyte monolayers were preserved at 10 °C. This is a temperature above where a detrimental negative Arrhenius plot was observed (Kruuv et al., 1995) but below the temperature of 15 °C where autophagy is inhibited (Seglen, 1987). Autophagy is the major mechanism of protein degradation in the liver (Mortimore & Ward, 1981). The cells were stored under a semi-solid matrix of gelatin, containing various additions. Hepatocytes attach to gelatin. However, the cells cannot spread on this protein. With a gelatin overlay, the cells were held in position. Moreover, this method allows the cells to be transported in a "no spill" format and there is no liquid shear force on the cells.

The preservation of hepatocytes monolayers under gelatin overlays was monitored. The morphology of the attached cells was determined by microscopy. The viability of the monolayers, with time of preservation, was

investigated by trypan blue staining and retention of the cytosolic marker enzyme, lactate dehydrogenase. Retained cell function was measured by cell spreading to form a confluent layer and the hormonal induction of the enzyme tyrosine aminotransferase (E.C. 2.6.1.5). Both the latter phenomena are completed in 7.5 h.

2. Method

2.1 Preparation and Preservation of Hepatocytes

Rat hepatocytes were prepared as described previously (Evans, 1981). The cells were plated on collagen coated dishes (60 mm) or Primaria plates (Falcon). Hepatocytes spread rapidly on collagen but more slowly on Primaria dishes. The latter support is useful in observing the behaviour of attached, single cells. 2.5×10^6 Hepatocytes were added to each plate in 3ml Leibovitz L-15 medium (pH 7.4) containing hepes (25 mM), glucose (8.3 mM), insulin (0.8 μg/ml), gentamycin (50 μg/ml) and 10% v/v heat inactivated newborn calf serum. The dishes were placed in a humidified 37 °C incubator for 1.5h to allow cell attachment. The medium was changed and supplemented with solubilised gelatin (1.5%) ± other additions (for details see figure legends). The medium was solidified and the cells preserved by putting the plates in a humidified 10 °C incubator.

2.2 Cell Morphology

The morphology of the cells was examined throughout the preservation period by light microscopy. Trypan blue staining was used in some cases (Evans, 2012). Preliminary experiments, using "killed monolayers", established that 30min were required for trypan blue, added to the surface of the solidified gelatin, to reach the hepatocyte monolayer underneath it.

2.3 Cell Harvesting

Hepatocyte viability was determined by the retention of the cytosolic enzyme, lactate dehydrogenase in the monolayer. At the times indicated plates were reactivated by adding 3 ml of plating medium and placing the dishes in a humidified 37 °C incubator for 20 min. The medium containing the aqueous gelatin was aspirated away. The exposed monolayer was washed with Leibovitz L-15 medium. The cells were harvested by scraping in 2 × 0.5 ml potassium phosphate buffer (0.1 M pH 7.6) and stored at -20 °C prior to assay.

2.4 Cell Behaviour

Hepatocyte functionality was monitored by observing the spreading of the cells to form a confluent monolayer and the hormonal induction of tyrosine aminotransferase. The latter enzyme was induced by reactivating the hepatocyte in the same way as for the lactate dehydrogenase determination but rather than scraping the cells immediately, fresh plating medium supplemented with dexamethasone (10^{-6} M) and dibutyryl cyclic AMP (1.5×10^{-5} M) was added. The plates were returned to the 37 °C incubator. The cells were harvested, at the times indicated in the figure legends. The medium was removed and the monolayer washed with Leibovitz L-15 medium. The cells were scraped into 2 × 0.5 ml phosphate buffer (0.1 M pH 7.6 containing 0.2 mM pyridoxal 5' phosphate) and stored at -20 °C.

2.5 Enzyme Assays

Lactate dehydrogenase and tyrosine aminotransferase were assayed as described previously (Evans, 1981). The continuous keto-enol tautomerism method was used for the aminotransferase.

3. Results and Discussion

The attached hepatocytes were not damaged by local forces when the gelatin solidified above them. The morphology of the cells was unchanged for the first 3 days of preservation under gelatin (Figure 1a). On the fourth day hypothermic haloing was observed in the attached hepatocytes (Figure 1c). A similar phenomenon has been observed in cells preserved on top of gelatin (Evans, 2012). The hepatocytes exclude trypan blue so that hypothermia has not killed the cells at this stage. The haloing can be prevented by the addition of 0.05 M sucrose to the preservation medium (Figure 1e).

The halos disappeared when the cells were reactivated by increasing the temperature. The change in morphology was reversible for the first nine days but thereafter halos persisted in the cells.

The viability of the cells with preservation time is shown in Figure 2. Lactate dehydrogenase was maintained at its initial level in both the presence and absence of sucrose. This is explained by the mild reactivation procedure. The reactivation medium was not being added directly to the cells but to the gelatin layer above them. The haloing hepatocytes (in the absence of sucrose) were not dislodged by medium addition. The halos disappeared before the protective gelatin was removed.

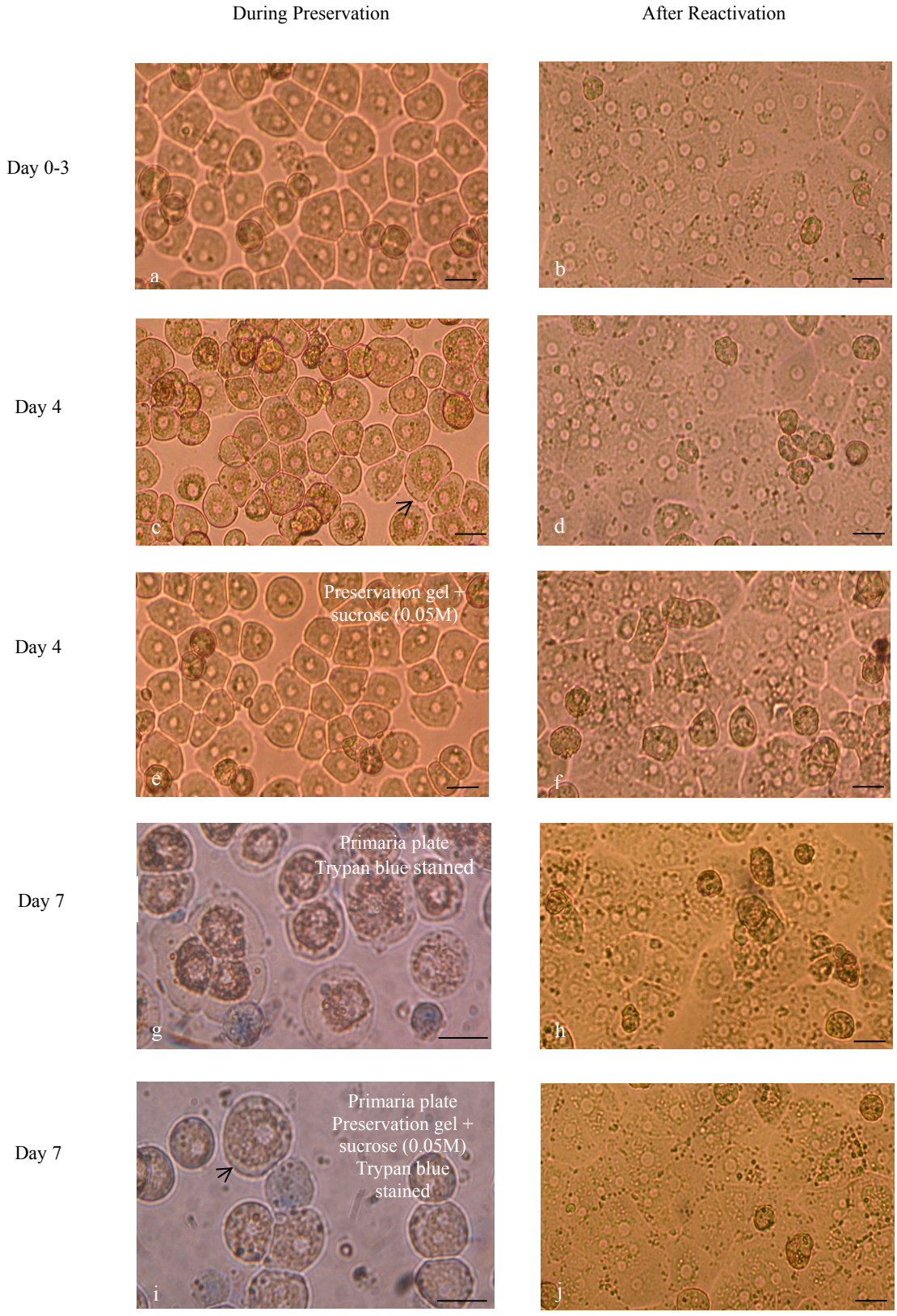

Figure 1. Light microscopy of hepatocytes preserved under gelatin gels at 10 °C

The photographs were taken either during the preservation process or 7.5 h at 37 °C after cell reactivation. Except where indicated, the cells were on collagen coated plates and unstained. Similarly unless specified, standard preservation gel was used. The arrows in (c) and (i) show the start of haloing. Bar, 20 μm.

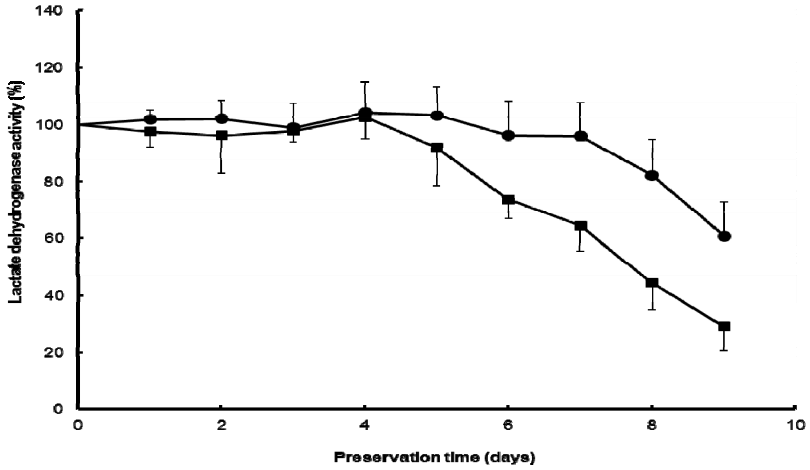

Figure 2. Viability of hepatocytes after preservation under gelatin gels at 10 °C

Cells attached to collagen coated plates.

■ Standard preservation gel; • Preservation gel supplemented with 0.05 M sucrose.

The values are the means ± S.D. (n = 6).

The monolayer retention of lactate dehydrogenase showed that there was no rewarming damage immediately. However, to be a useful preservation method the cells should behave normally following reactivation. This was demonstrated by the plated cells forming a confluent monolayer within 7.5 hours (Figure 1b, d, f, h, j). There was no lag in the motility of the cells. The cytoskeleton had not been compromised by the preservation. General cell function is maintained.

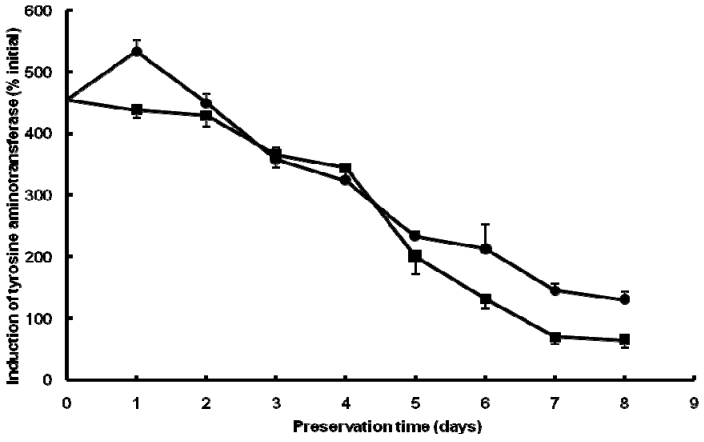

Figure 3. Hormonal induction of tyrosine aminotransferase in hepatocytes after preservation under gelatin gels at 10 °C

The peak of induction was 7.5 h after reactivation.

■ Standard preservation gel. • Preservation gel supplemented with 0.05 M sucrose.

The values are the means ± S.D. (n = 3).

Tissue specific properties of the preserved hepatocytes were investigated by the hormonal induction of tyrosine aminotransferase. The results are shown in Figure 3. Hormonal induction was shown for 6 days in the absence of sucrose and extended to 8 days when sucrose (0.05 M) was included in the preservation gel. The decrease in

inducibility of tyrosine aminotransferase was temporally related to the appearance of hypothermic halos (4 days and 7 days in sucrose free and 0.05 M sucrose supplemented media respectively, Figure 1c and 1i). The time course for enzyme induction remained the same at all preservation times. It involves hormone receptor binding, occupied receptor translocation, transcription and translation (Evans, 1981). A rapid, cell type specific process has been maintained in this preservation system. The cells can be used after the 20 min reactivation procedure. This contrasts with the 24-48 h recovery period required for cryopreserved cells (de Loecker et al., 1993).

A useful property of this preservation is that enzyme levels are kept at the freshly isolated levels for several days. This includes cytochrome P-450s (Evans, 1995). Since reactivated, attached cells form a monolayer in 7.5 h (Figure 1), the toxicity of potential pharmaceutical candidate drugs can be screened by seeing if they interfere with this process.

Gelatin preservation of cells is a versatile technique. Under a gelatin gel, the attached cells are protected. Transportation to distant sites is facilitated in a no spill format. The demonstration that hepatocyte viability is not compromised by the cells being present during the gelation process means that cells required for suspension cultures can be carried in a similar manner. In the latter case the cells are allowed to attach to a preformed gelatin gel (Evans, 2012). The plating medium is removed and medium supplemented with liquefied gelatin is added on top of the cells. The gelatin is allowed to gel at 20 °C. The hepatocytes are encased in a semi-solid gelatin sandwich. The plates are stored at 10 °C until required. Reactivation, at 37 °C, solubilises the gelatin and the cells are released as single spherical entities. Trypan blue (molar mass 872.9) reached hepatocytes under gelatin in 30 min. Medium can be added above the gel to change the composition of the preservation medium. Sucrose (molar mass 342.3) can be introduced to extend the duration of conservation.

Transportation of cells in the no spill arrangements does not require a special courier. Commercial suppliers sell boxes which can maintain a temperature of 10 °C for several days. This is sufficient for distribution to distant sites. On receipt the plates can be maintained in a 10 °C incubator.

In conclusion, embedding hepatocytes in a gelatin gel at 10 °C with supplements provides mechanical and osmotic protection to the cells. A no spill transportation and preservation method is provided without adding, potentially harmful, “protective chemicals”.

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