Development of a Protocol for Cryopreservation of Hepatocytes for Use in Bioartificial Liver Systems

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Abstract. Porcine hepatocytes are considered the cell type of choice for bioartificial liver and cell transplantation techniques in support of patients with liver failure. A protocol for cryopreservation of hepatocytes that function adequately after thawing would allow on-demand usage and long-term storage. We conducted experiments to evaluate the freeze rate, concentration of hepatocytes during storage, and the effect of a pre-incubation step prior to freezing on cryopreserved hepatocytes isolated from 15 porcine livers. Cell return, attachment, LDH leakage, bilirubin conjugation, and lignocaine metabolism were tested to assess the effects of the interventions. No significant differences were found between a computer-controlled freeze rate, the Nalgene propan-2-ol device, or simply using -20°C and -80°C freezers. Trials at a range of hepatocyte concentrations did not produce significant differences. Pre-incubation did not confer any advantage to the thawed hepatocytes. In conclusion, porcine hepatocytes can be cryopreserved using a simple method of temperature reduction at a concentration of 5x10^6 hepatocytes/ml. Such hepatocytes can potentially be used for bioartificial livers; however, further investigation is required to explain the large cell losses that occur during cryopreservation. (received 1 November 2003; accepted 21 November 2003)

Keywords: Bioartificial liver, hepatocytes, cryopreservation, hepatocyte culture

Introduction

Isolated porcine hepatocytes are currently being used extensively for bioartificial liver development and cell transplantation studies [1-9]. Although human hepatocytes are considered the ideal cell source for clinical use, their supply has been reported to be erratic and it is difficult to obtain sufficient numbers [10]. The ability to isolate and bank porcine hepatocytes would allow on-demand clinical usage of characterised cells. Cryopreservation is currently considered the only practical method for this long-term storage [11].

An ideal protocol for hepatocyte cryopreservation has been sought for many years without any one protocol gaining universal acceptance [11,12]. Many studies have been performed on various species, with different methods and endpoints, making comparisons difficult [13-20]. Despite this uncertainty, it appears that a slow rate of temperature reduction, use of dimethylsulphoxide (DMSO) as the cryoprotective agent, and a rapid rate of thawing are prerequisites to a successful protocol.

As part of our project to develop a porcine bioartificial liver, we assessed the suitability of cryopreserved porcine hepatocytes to provide hepatic support in vitro. A simple, easily applicable protocol was developed to facilitate mass production and enhance consistency. Three facets of the porcine hepatocyte cryopreservation process were selected for this study. The first was to establish the optimal freeze rate protocol, the second was to determine...
the optimal concentration of porcine hepatocytes in the cryopreservation solution, and the third was to test the influence of a pre-incubation step prior to cryopreservation and subsequent culture.

**Materials and Methods**

**Animals.** Porcine livers were obtained from an abattoir. All standard abattoir procedures for animal treatment were followed. Fifteen pigs (body weight 15 to 20 kg) were used for the study.

**Hepatocyte isolation.** The whole liver was removed and the inferior vena cava was cannulated with a tube (1 cm diameter) sutured into position. The liver was perfused with pre-cooled (4°C) Soltran kidney perfusion solution (Baxter Healthcare, Ltd., UK), containing 1 µl/ml of gentamicin (Gibco, Scotland). A clip was placed on the portal vein to prevent uncontrolled leakage and to maintain adequate tissue perfusion. Each liver was immediately placed on ice and transported in this solution to the laboratory.

Liver digestion and hepatocyte isolation were performed as described by Koebe et al [21]. Three oxygenated porcine perfusion buffers were sequentially perfused through the liver circuit prior to liver digestion and were then discarded. The stock buffer (5 L) contained NaCl, 154 mM; KCl, 5.6 mM; glucose, 5 mM; NaHCO3, 25 mM; and HEPES, 20 mM. Buffer 1 contained 500 ml of the stock buffer with dexamethasone, 40 mg/L. Buffer 2 contained 500 ml of the stock buffer with EGTA, 1 mM. Buffer 3 was 100 ml of the stock buffer. Liver digestion was achieved by addition of pre-warmed oxygenated porcine digestion medium (Buffer 4, 800 ml of stock buffer containing 0.5 mg of collagenase (Sigma-Aldrich, UK)) and perfused through the liver. This was circulated at a rate of 180 ml/min for 15-25 min. When the liver became soft to the touch, it was emulsified manually and the cell suspension was filtered (250 µm, 100 µm, and 75 µm pore meshes), washed, and viable cells were counted using trypan blue exclusion.

**Hepatocyte culture.** Immediately following isolation, the porcine hepatocytes were cultured on collagen-coated 12-well plates (Becton-Dickinson, France) at a seeding density of 7x10^5 hepatocytes/well. Porcine hepatocytes were cultured in an adapted tissue medium described by Koebe et al [21], which consisted of Dulbecco’s Modified Eagle’s Medium (DMEM) supplemented with glucose, 4.5 g/L; insulin, 125 mU/ml; hydrocortisone, 60 ng/ml; glucagon, 10 ng/ml; foetal calf serum, 10% v/v; and fungizone (Gibco, Scotland), 1.6 ml/500 ml.

**Cryopreservation and thawing.** Porcine hepatocytes were suspended in ice-cold DMEM at a concentration of 5x10^6 hepatocytes/ml (except during the concentration experiment, when other hepatocyte densities were investigated) and 20% (by final volume) foetal calf serum (Sigma) was added. A 10% (by final volume) solution of DMSO (Sigma) was added over 3-4 min, just prior to pipetting the hepatocytes into 2 ml cryovials (Nalgene). The cryovials were subjected to the appropriate freezing protocols and stored in liquid nitrogen for 7-10 days before thawing. Thawing was performed rapidly by submerging the vials in a 37°C water bath prior to evaluation.

**Determination of optimal freezing rate.** Three methods were evaluated. The first was the method described by Diener et al [22], utilising a step-wise program with a computer-controlled Planer Cryo-10-16 series machine (Planer, Sunbury on Thames, UK). The second method used the Nalgene propan-2-ol device, which allows a reduction in temperature of approximately 1°C/ min when placed in a -80°C freezer. The device was left in the freezer for 4-6 hr before the cryovials were removed for storage in a liquid nitrogen container. The third method was to put the cryovials in a container and place the container in a -20°C freezer for 1 hr before transfer to a -80°C freezer for 3 hr. The vials were then stored in liquid nitrogen.

**Determination of optimal hepatocyte concentration.** Four cell concentrations were evaluated (2.5x10^6, 5x10^6, 1x10^7, and 2x10^7 cells/ml of solution). The constituents of the cryopreservation solution were used as outlined above. The cryopreservation was performed using the Nalgene propan-2-ol device.
Investigation of pre-incubation step. Following hepatocyte isolation, $1.5 \times 10^8$ viable hepatocytes were suspended in 80 ml of pre-warmed culture medium within two spinner flasks in a 37°C incubator (5% CO$_2$). A further 20 ml of culture media was used to wash the tube to ensure suspension of all cells, resulting in a cell density of $1.5 \times 10^6$ hepatocytes/ml. The spinner flasks (Cellspin, Integra Biosciences) were set to rotate at 30 rpm and left for 1 hr and 16 hr, respectively. After incubation, the cells were transferred to a 150 ml tube and centrifuged (100 x g, 4°C, 5 min) to pellet the cells. Following trypan blue exclusion cell count, $2.5 \times 10^7$ viable hepatocytes were cultured as above. The remaining viable cells were cryopreserved in solution at a concentration of $5.0 \times 10^6$ cells /ml in the Nalgene device.

Of the remaining isolated hepatocytes, $1.0 \times 10^8$ were cryopreserved with the Nalgene device and $3 \times 10^7$ hepatocytes were cultured directly on collagen-coated 12-well plates to serve as a control.

Determination of cell viability and attachment. Total cell number and percentage cell viability were determined using trypan blue exclusion. Cell return refers to the percentage of viable hepatocytes returned following cryopreservation, compared to the number of viable hepatocytes originally frozen. The number of attached hepatocytes was estimated by measuring the protein content of the cells using the Bio-Rad protein assay kit (Bio-Rad, Hemel Hempstead, UK), based on the intensity of the coomasie blue colour formed with protein. The absorbance was measured at 405 nm and compared to a standard curve.

Biochemical parameters. Release of lactate dehydrogenase (LDH) was measured using the Sigma kit. Briefly, lactate dehydrogenase catalyses the reversible conversion of lactic acid to pyruvic acid. Pyruvic acid reacts with 2,4-dinitrophenylhydrazine to form hydrazine, which is measured by spectrophotometry.

Phase II metabolic activity was evaluated by measuring the ability of the hepatocytes to conjugate bilirubin. Bilirubin mixed isomers (Sigma-Aldrich, Gillingham, UK) were added (29 µl of a solution containing 2.9 mg of bilirubin mixed isomers plus

1.25 ml of 50 mM NaOH and 3.75 ml of 100 mM tris-HCl) to hepatocytes in culture and the supernatant was collected after 4 hr. Bilirubin was assayed by a reagent kit (Sigma-Aldrich, Gillingham, UK), based on reaction of conjugated and unconjugated bilirubin with diazotized sulfanilic acid in the presence of dimethylsulphoxide (DMSO) to generate azobilirubin, which was measured by spectrophotometry.

Cytochrome P450 3A4 activity was measured by the de-ethylation of lignocaine to produce monoethylglycinexylidide (MEGX), which was isolated by HPLC and monitored at 214 nm. The assay was developed in our department, based on published methods [23-25].

Statistics. Statistical analyses were performed by the SPSS computer program, using the ANOVA general linear model. Results were expressed as mean ± SD; p values <0.05 were considered significant.

Results

Cell return post-cryopreservation

Freeze rate. Of the methods investigated, the Nalgene device returned the highest percentage of viable cells ($54 \pm 25\%$) of those frozen ($n = 16$). The simple freezer procedure returned more cells ($47 \pm 23\%$) than the complex Planer computer-controlled rate apparatus ($37 \pm 25\%$). As shown in
Fig. 1. no significant differences were observed in recovery of viable porcine cells post-cryopreservation when the 3 methods for freezing hepatocytes were compared (Nalgene device vs Planer apparatus, p = 0.22; Nalgene device vs freezer, p = 0.95; Planer apparatus vs freezer, p = 0.39).

**Hepatocyte concentration.** The hepatocyte return was consistent across all 4 hepatocyte concentrations. Although a concentration of 2.5x10^6 hepatocytes/ml yielded the lowest cell return (41.9±6.6%), there were no significant differences among the hepatocyte densities investigated (Fig. 2).

**Effect of pre-incubation.** After cryopreservation, a pre-incubation step for 1 hr did not significantly improve the hepatocyte return. The return appeared to be improved by incubation for 16 hr (81.3±28% at 16 hr vs 50.5±22.1% at 1 hr), but this did not reach statistical significance (p = 0.08). Comparable cell return was evident when these data were compared to hepatocytes that were cryopreserved immediately post-isolation (Fig. 3).

**Hepatocyte attachment**

**Freeze rate.** Attachment was higher when porcine hepatocytes were cultured immediately post-isolation than when they were cultured after cryopreservation. At day 2, no significant differences were observed between the attachment of the post-cryopreservation cultures (fresh vs Nalgene device, p = 0.83; fresh vs Planer, p = 0.73; fresh vs freezer, p = 0.84). At day 5, there was marked reduction in the attachment of the post-cryopreserved cells compared to values observed on day 2 and those cultured immediately post-isolation. The mean protein content of the attached hepatocytes averaged 0.59±0.09 mg for fresh hepatocytes, which was significantly higher than for cryopreserved hepatocytes prepared with the Nalgene device (0.40±0.09 mg, p <0.05), the Planer apparatus (0.32±0.07 mg, p <0.05), or the freezer method (0.33±0.05 mg, p <0.05).

**Hepatocyte concentration.** When hepatocyte attachment at day 2 was compared, there was little variation among the 4 hepatocyte concentrations. Fresh hepatocytes maintained their attachment up to day 5 of culture. Cryopreserved hepatocytes showed significantly reduced attachment at day 5, compared to fresh hepatocytes (p <0.05), but no significant differences were noted among the cryopreserved hepatocytes prepared by the three methods (Nalgene device vs Planer apparatus vs freezer method).

**Effect of pre-incubation.** The data suggested that pre-incubation for 1 hr prior to cryopreservation may improve the attachment of cryopreserved hepatocytes in culture, but this finding did not reach statistical significance. As described above, there was a general reduction of hepatocyte attachment at day
Table 1. Biochemical parameters (mean ± SD) of cultured fresh hepatocytes and cryopreserved hepatocytes, comparing the effects of different methods of freezing (Device = Nalgene apparatus, Planer = Planer apparatus, Freezer = placing cryovials in a -20°C freezer for 1 hr and in a -80°C freezer for 3 hr). The assays were made after the hepatocytes had been cultured for 2 or 5 days.

<table>
<thead>
<tr>
<th>Hepatocyte preservation</th>
<th>LDH activity (units/mg)</th>
<th>Bilirubin concentration (mg/dl)</th>
<th>MEGX concentration (µg/dl)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Fresh hepatocytes</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>day 2</td>
<td>777±461</td>
<td>1.8±0.9</td>
<td>7.5±9.7</td>
</tr>
<tr>
<td>day 5</td>
<td>767±276</td>
<td>1.8±1.1</td>
<td>13.9±11.9</td>
</tr>
<tr>
<td>Nalgene device</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>day 2</td>
<td>1712±1187</td>
<td>2.6±2.4</td>
<td>12.4±8.9</td>
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<td>day 5</td>
<td>874±454</td>
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<td>Planer apparatus</td>
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</tr>
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<td>8.7±4.8</td>
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<td>day 5</td>
<td>1263±772</td>
<td>4.4±2.8</td>
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<td>Freezer procedure</td>
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<td>day 5</td>
<td>1098±590</td>
<td>5.5±1.8</td>
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</table>

Table 2. Biochemical parameters (mean ± SD) of cultured fresh hepatocytes and cryopreserved hepatocytes, comparing the effects of different hepatocyte concentrations during storage. The assays were made after hepatocytes were cultured for 2 or 5 days.

<table>
<thead>
<tr>
<th>Hepatocyte cryopreservation</th>
<th>LDH activity (units/mg)</th>
<th>Bilirubin concentration (mg/dl)</th>
<th>MEGX concentration (µg/dl)</th>
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<tr>
<td>Fresh hepatocytes</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>day 2</td>
<td>856±441</td>
<td>1.9±0.9</td>
<td>7.5±9.6</td>
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<tr>
<td>day 5</td>
<td>760±269</td>
<td>2.4±1.0</td>
<td>13.9±11.9</td>
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<tr>
<td>Hepatocyte conc. = 2.5x10⁶</td>
<td></td>
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</tr>
<tr>
<td>day 2</td>
<td>1701±977</td>
<td>2.0±1.0</td>
<td>21.4±17.0</td>
</tr>
<tr>
<td>day 5</td>
<td>637±129</td>
<td>3.2±1.1</td>
<td>9.4±8.1</td>
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<tr>
<td>Hepatocyte conc. = 5x10⁶</td>
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<td></td>
<td></td>
</tr>
<tr>
<td>day 2</td>
<td>2028±1036</td>
<td>2.7±2.4</td>
<td>16.1±12.9</td>
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<td>day 6</td>
<td>952±443</td>
<td>4.2±1.0</td>
<td>8.2±3.7</td>
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<td>Hepatocyte conc = 1x10⁷</td>
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<td></td>
<td></td>
</tr>
<tr>
<td>day 2</td>
<td>1201±673</td>
<td>2.6±2.3</td>
<td>12.8±8.0</td>
</tr>
<tr>
<td>day 5</td>
<td>953±451</td>
<td>3.1±1.8</td>
<td>9.8±4.3</td>
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<tr>
<td>Hepatocyte conc. = 2x10⁷</td>
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<td></td>
<td></td>
</tr>
<tr>
<td>day 2</td>
<td>1077±767</td>
<td>2.8±2.4</td>
<td>18.7±13.0</td>
</tr>
<tr>
<td>day 5</td>
<td>1847±2868</td>
<td>3.6±1.2</td>
<td>10.0±6.9</td>
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</table>

2 and day 5 of culture following cryopreservation. Pre-incubation did not have a significant positive impact on this decline.

**Biochemical assays**

**Freeze rate.** Cryopreserved hepatocyte cultures (2 and 5 days) showed higher LDH leakage than cultured fresh hepatocytes, reflecting the expected damage caused by cryopreservation (Table 1). No significant differences were noted between the mean LDH leakage values following hepatocyte culture at day 2 or day 5.

**Bilirubin conjugation.** The ability to conjugate bilirubin increased as the hepatocyte cultures were maintained from day 2 to 5 post-cryopreservation. Bilirubin conjugation stayed at about the same level in the cultured fresh hepatocytes. Increased bilirubin conjugation did not reach significance in any of the cryopreserved hepatocytes, but tended towards
significance in hepatocytes cryopreserved using the Nalgene device (day 2, 2.6±2.4 mg/dl; day 5, 4.2±1.0 mg/dl; p = 0.14). No significant differences were observed on day 5 among the hepatocytes cultured fresh (1.8±1.1 mg/dl) and those cryopreserved using the Nalgene device (4.2±1.0 mg/dl) (p = 0.28) or the Planer apparatus (4.4±2.8 mg/dl) (p = 0.27). The bilirubin conjugation of hepatocytes cryopreserved by the freezer method (5.5±1.8 mg/dl) approached significance (p = 0.09) compared to those cultured fresh.

**Cytochrome P450 3A4 activity.** The mean MEGX value for hepatocytes cultured immediately following isolation was lower than those cryopreserved with the Nalgene device (4.2±1.0 mg/dl) (p = 0.28) or the Planer apparatus (4.4±2.8 mg/dl) (p = 0.27). The bilirubin conjugation of hepatocytes cryopreserved by the freezer method (5.5±1.8 mg/dl) approached significance (p = 0.09) compared to those cultured fresh.

<table>
<thead>
<tr>
<th>Parameter</th>
<th>LDH activity (units/mg)</th>
<th>Bilirubin concentration (mg/dl)</th>
<th>MEGX concentration (µg/dl)</th>
</tr>
</thead>
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<tr>
<td>Fresh hepatocytes</td>
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<tr>
<td>day 2</td>
<td>786±471</td>
<td>1.4±0.3</td>
<td>3.5±2.2</td>
</tr>
<tr>
<td>day 5</td>
<td>767±277</td>
<td>1.9±1.0</td>
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<td>Cryopreserved hepatocytes with no pre-incubation (0 hr)</td>
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<tr>
<td>day 2</td>
<td>1696±694</td>
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<td>17.0±11.8</td>
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<tr>
<td>day 5</td>
<td>702±271</td>
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<td>Cryopreserved hepatocytes with pre-incubation for 1 hr</td>
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<td></td>
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<tr>
<td>day 2</td>
<td>1235±837</td>
<td>2.0±1.0</td>
<td>10.4±10.2</td>
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<td>day 5</td>
<td>649±530</td>
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<td>Cryopreserved hepatocytes with pre-incubation for 16 hr</td>
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<td>day 2</td>
<td>1370±1207</td>
<td>2.0±1.2</td>
<td>10.1±11.8</td>
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</table>

**Hepatocyte concentration.** As shown in Table 2, on day 2 of culture less LDH leakage was observed for the fresh cells than for cryopreserved hepatocytes. A concentration of 5x10^6 hepatocytes/ml was associated with the highest LDH leakage. By day 5, LDH leakage from hepatocytes cultured fresh was not greatly changed, but cryopreserved cells showed a marked reduction. Significant reduction in leakage from day 2 to day 5 occurred at the 5x10^6 hepatocyte concentration (p <0.05). Comparing the other values on day 2 and 5 revealed no significant differences in leakage. At day 2 of culture, LDH leakage for 5x10^6 hepatocytes/ml vs fresh hepatocytes approached significance (p = 0.10). At day 5 of culture, no significant differences were observed.

**Effects of pre-incubation.** As shown in Table 3, pre-incubation of the hepatocytes following isolation did not significantly alter the LDH leakage at day 2 or day 5 of culture.

There was a tendency for bilirubin conjugation to increase as the hepatocytes were maintained in culture from day 2 to day 5. The increased ability to conjugate bilirubin of the non-pre-incubated
cryopreserved hepatocytes from day 2 to day 5 of culture was significant (1.5±1.1 mg/ml vs 4.1±1.4 mg/ml, respectively, p = 0.01). The increase in bilirubin conjugation ability of hepatocytes preincubated for 1 hr however was not significant (2.0±1.0 mg/ml at day 2 vs 3.4±2.0 mg/ml at day 5, p = 0.23). There was no significant improvement in bilirubin conjugation by pre-incubating the hepatocytes for 16 hr.

There were no significant differences among the MEGX results obtained in the pre-incubation experiments, indicating that cryopreserved cells are as good as non-cryopreserved cells and that pre-incubation has a negligible effect on hepatocyte function, based on cytochrome P450 3A4 activity.

Discussion

Cryopreservation of hepatocytes has been widely studied in recent years. Many species have been studied with a variety of protocols, but porcine hepatocytes have received particular attention due to their potential therapeutic use in bioartificial livers (BALs) and cell transplantation. Despite the numerous studies using porcine hepatocytes, a universally acceptable method of cryopreservation has not been found. Some have claimed success and are using the hepatocytes clinically [26].

Effect of freeze rate on post-cryopreservation hepatocytes. The freezing rate is considered a major factor that affects post-thaw viability of hepatocytes. Many studies have compared simple rates of freezing (for example placing hepatocytes directly into a freezer [27]) and complex controlled rates (where the temperature is reduced in a controlled fashion using sophisticated freezing apparatuses). The controlled rate methods have been advocated following experiments using rat hepatocytes [28]. The whole freezing process hinges on the avoidance of intracellular ice formation, especially for cells in suspension. Although DMSO provides some protection by allowing more controlled cellular dehydration, the actual rate of freezing is also important. If the rate is too fast, water cannot exit and the formation of intracellular ice damages the cells. If the rate is too slow, hepatocytes are exposed to excessive cellular dehydration and mechanical effects of external ice are evident [29,30].

By controlling and altering the freeze process, it is hoped to optimize the time and temperature at critical stages of this process. Some processes involve a shock-cooling phase [31]. Here the temperature is suddenly reduced to compensate for the latent heat of fusion, which increases the temperature as ice forms and may be harmful to cells. By quickly reducing the temperature, the extra energy is absorbed and smoother cooling progression occurs. The ideal freezing protocol is far from clear and discrepancies exist, which may reflect differences in hepatocyte preparation and experimental variations [32,33]. Guillouzo et al [12] reviewed the situation in 1999 and concluded that there was no convincing benefit from complex controlled cooling, compared to simply placing the hepatocytes first in a -20°C freezer and then in a -80°C freezer.

In experiments using porcine and rat hepatocytes, various step-wise cooling programs have been advocated [1,2,7,22]. In this study, we found that a complex computer-controlled cooling system (Planer apparatus) was not significantly superior to other techniques. The simple freezer method, in agreement with Guillouzo et al [12], proved as effective. Alexandre et al [13] found with human hepatocytes that the Nalgene device was as effective as computer-controlled rate reduction. Although we did not find any method to be superior in terms of biochemical parameters, the Nalgene device produced the highest return of viable hepatocytes and was therefore chosen for future experiments.

Deterioration of hepatocyte attachment was consistently seen as the post-cryopreservation hepatocyte cultures progressed from day 2 to 5, in comparison to fresh cultures. This appears to be an inevitable consequence of cryopreservation, although the hepatocytes that remain show good cellular repair, with comparable LDH leakage and functional ability [1].

Effect of hepatocyte concentration on post-cryopreserved hepatocytes. Storage at high cell density would save space, which is an important factor for large-scale tissue banking. Published studies have investigated variations in cell densities
during cryopreservation, but few studies have established an optimum cell density. Madan et al [34] noted that in published studies the concentration of stored hepatocytes has ranged from $10^6$-$10^7$ cells/ml of solution. De Loecker et al [35] found with rat hepatocytes that as the density of cells decreased, cell viability as measured by trypan blue exclusion increased. As cell density increased, the attachment measured by radioactive uptake diminished, following 1 hr of culture. In canine hepatocytes, cell viability measured by trypan blue exclusion was better at concentrations $<10^7$cells/ml than at higher density [22,36]. These findings support the hypothesis that, although there are many reasons for cellular injury during freezing, membrane to membrane contact between hepatocytes leads to more serious damage; hence lower cell densities should yield improved viability [35].

The hepatocyte concentrations evaluated in this investigation had no significant influence on the measured parameters. The $5 \times 10^6$ hepatocyte/ml concentration appeared to return more hepatocytes and to sustain bilirubin conjugation better than the others concentrations tested. The least dense and the most dense concentrations showed the widest variations. One problem was cell clumping following isolation, which may have impaired the accuracy of trypan blue exclusion and associated haemocytometer cell counts.

**Effect of pre-incubation on hepatocytes post-cryopreservation.** Culturing hepatocytes in suspension following isolation, but prior to cryopreservation, is thought to allow the cells time to recover from the isolation process before the rigors of the freezing process. Darr and Hubel [37] cultured porcine hepatocytes in Williams E solution (with additives) in a spinner flask for 4 to 48 hr at 90 rpm following isolation, before the cells were seeded for culture. On fresh culture, the albumin secretion increased after a long pre-incubation period (0.25 $\mu$g/ml/hr at time 0, increasing to 1.4 $\mu$g/ml/hr at 48 hr). Further experiments examined pre-incubation and subsequent cryopreservation and thawing. Pre-incubation up to 24 hr conveyed significant improvement of albumin production upon thawing. Unlike fresh cells, preincubation for more than 24 hr showed marked reduction in albumin secretion. They concluded that aggregates of cells form during the culture in suspension, which do not survive the freezing process as efficiently and thus do not last as long in culture [37].

Koebe et al [38,39] performed experiments on cryopreservation of hepatocytes in monolayer culture, using rat and porcine cells. Prior to cryopreservation, they cultured the hepatocytes on a monolayer in DMEM with additives. Culturing for 3 days before cryopreservation gave the best results. Moreover, culturing for 7 days prior to freezing in a sandwich configuration conferred survival benefits after thawing [40].

The effect of pre-incubation on porcine hepatocytes was disappointing in our studies. Pre-incubation for 16 hr did increase the percentage of viable hepatocytes after cryopreservation, but the process itself reduced the viable cell population by 80%. Possible explanations include too high a spin rate and hence detrimental shear forces. In respect to functional ability, pre-incubated hepatocytes did not perform any better than fresh cells before or after cryopreservation. Further investigations on this facet of cryopreservation are underway in our laboratory.

**Conclusions**

This investigation supports the following observations:

- Porcine hepatocytes can be cryopreserved with return to nearly normal function.
- There is an inevitable decrease in attachment over time with cryopreserved hepatocytes.
- Further work is needed to optimise the pre-incubation method, since others have reported success.
- A complex computer-controlled program for freezing hepatocytes was not superior to simpler methods.
- The effect of hepatocyte concentration on the test parameters was variable and no significant superiority of any concentration was found.
- Cryopreserved porcine hepatocytes function adequately to be considered for therapeutic or research applications.
Acknowledgements

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References


