Cryopreservation of Suckling Pig Hepatocytes

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Abstract. To determine the best and simplest method for cryopreservation of pig hepatocytes, we compared immediate cryopreservation with cryopreservation after short-term culture. Suckling pig hepatocytes were isolated by a modified 2-step in situ collagenase perfusion method, suspended in serum-free medium, and preserved for 10 da by two cryopreservation methods. Serial measurements were made of cell viability, LDH release, synthesis of protein, urea and glucose, glucose-6-phosphatase (G-6-Pase) activity, and diazepam transformation after thawing. These measurements were performed on both groups of cultured hepatocytes, and on freshly isolated hepatocytes, which served as a control. High viability (>95%) of thawed hepatocytes was obtained and maintained in both cryopreservation groups. There were no significant differences in cell viability, protein synthesis, glucose synthesis, G-6-Pase activity, or diazepam transformation between the two cryopreservation groups. In the immediate cryopreservation group, urea synthesis was less than in the group with cryopreservation after short-term culture. Protein synthesis, glucose synthesis, and diazepam transformation were lower in both cryopreserved groups than in the controls. The results showed that a protocol of immediate cryopreservation of hepatocytes in RPMI-1640 medium containing 10% DMSO, hormones, growth factors, and 10% newborn bovine serum, together with rate-controlled freezing and rapid thawing, provides indices of cell viability and function during subsequent serum-free culture that are comparable to hepatocytes cryopreserved after short-term culture, except for lower urea production. This simple procedure can be used in studies of bioartificial liver and hepatocyte transplantation. (received 23 April 2001; accepted 24 July 2001)

Keywords: hepatocyte culture, cryopreservation, bioartificial liver, liver transplantation

Introduction

Preliminary clinical trials reveal that bioartificial liver (BAL) is a promising therapy for fulminant hepatic failure (FHF) and may serve as a bridge to liver transplantation [1]. However, the development of BAL has been impeded by the difficulty of acquiring high-density cultures, maintaining hepatocyte function, and uncertainty about the optimal preservation method. There is need for long-term preservation of isolated hepatocytes, so that they can be available upon demand. Banking of cryopreserved hepatocytes would fulfill the requirement, but until now there has been no ideal cryopreservation technique.

Zaleski et al [2] reported that cryopreserved rat hepatocytes retain metabolic capacity similar to fresh hepatocytes when the cells are preincubated in Krebs-Ringer bicarbonate buffer prior to freezing. Silva et al [3] found that hepatocytes preincubated prior to freezing show excellent plating efficiency and respond to classical inducers in a manner indistinguishable from fresh hepatocytes. They found that preincubation of hepatocytes at 37°C before cryopreservation, in order to allow their recovery from cellular trauma relating to isolation, enhances post-thaw viability and the efficiency of attachment in serum-containing media. It remains
uncertain, however, whether cryopreservation after short-term culture is superior to immediate cryopreservation of hepatocytes in regard to hepatocyte functions during subsequent serum-free culture [4].

Although primary cultures of human hepatocytes are most suitable for BAL, their availability is limited; consequently, porcine hepatocytes are generally employed. In the present study, using a rate-controlled freezing program for cryopreservation of suckling pig hepatocytes, we compared immediate cryopreservation versus cryopreservation after short-term culture, examining the functions of thawed hepatocytes when subsequently cultured in serum-free medium.

Materials and Methods

Animals. Healthy Chinese experimental miniature suckling pigs of either sex (n = 10; body wt 2.7 to 4.0 kg) were provided by the Experimental Animal Center of Drum Tower Hospital. The research protocol was in compliance with Chinese guidelines for the humane care of experimental animals.

Hepatocyte isolation. Hepatocytes were isolated by a modified two-step in situ collagenase perfusion method [5]. Briefly, suckling pigs were anesthetized with ketamine (20 mg/kg body wt, iv). After the portal vein was cannulated, the liver was perfused in situ at 37 °C for 15 min with 1.5-2.0 L of Hanks solution. The perfusion was followed by recirculation of Hanks solution supplemented with 0.05% (w/v) collagenase (Gibco BRL Life Technologies, USA). The liver was resected and hepatocytes were immediately isolated and washed by centrifugation (1000 rpm, 5 min) 3 times at room temperature with resuspension of the sedimented cells in RPMI-1640 medium (Gibco BRL Life Technologies, USA). Cell viability was assayed by trypan blue exclusion. Hepatocytes were then resuspended in serum-free RPMI-1640 medium supplemented with 200 µg/L hydrocortisone, 1 mg/L HGF, 10 µg/L EGF, 20 µg/L NGE, 100 µg/L insulin, 4 µg/L glucagon, 6.25 mg/L transferrin, 10 mg/L linoleic acid, 2 mM glutamine, 0.5 g/L bovine serum albumin, 3 nmol Na2SeO3, 0.1 µM CuSO4·5H2O, 50 pM ZnSO4·7H2O, 15 mM HEPES, 200 µg/L cefoperazone sodium, 100,000 U/L penicillin, and 100 mg/L streptomycin) [6,7].

Short-term culture prior to cryopreservation. Hepatocytes were incubated in the supplemented serum-free RPMI-1640 medium at 1 x 10⁷/ml in 5% (v/v) CO₂ at 37°C for 2 da.

Cryopreservation, thawing, and culture. After addition of 10% (v/v) DMSO (Merck Co., USA) and 10% newborn bovine serum (NBS, Sigma Chemical Co., USA), suspensions of freshly isolated hepatocytes and of hepatocytes cultured in the supplemented culture medium were transferred into 2 ml cryogenic tubes at 1 x 10⁷ cells/ml and placed in the freezing chamber of a programmable freezing unit (Kryo 10 series III, Planer, UK) for cryopreservation by a rate-controlled freezing program [8]. The sample temperature was reduced at 1°C/min from 4 to 0°C, then reduced at 0.5°C/min from 0 to -11°C, kept at -11°C for 15 min, and then reduced at 3°C/min from -11 to -80°C. Finally, the cryogenic tubes were stored in liquid nitrogen for 10 da. The cells were rapidly thawed by immersing the cryogenic tubes in a 37°C waterbath (<1 min), and the cells were washed by centrifugation (1000 rpm, 5 min) 3 times with Hanks solution. The cells were resuspended (5 x 10⁵/ml) in supplemented serum-free RPMI-1640 culture medium, as specified above, and incubated at 37°C in 5% (v/v) CO₂ for 7 da. The culture flasks were precoated with rat tail collagen.

Experimental groups. Hepatocytes isolated from each pig were divided into the three groups, as follows:

Group IC (immediate cryopreservation, n=10). Freshly isolated cells were immediately cryopreserved for 10 da, then thawed and cultured in serum-free medium at 5 x 10⁵/ml for 7 da, as specified above;

Group SC (cryopreservation after short-term culture, n=10). Cells were cryopreserved for 10 da following short-term culture for 2 da, then thawed and cultured in serum-free medium at 5 x 10⁵/ml for 7 da, as specified above; and

Group C (control group, n=10). Freshly isolated cells were cultured in serum-free culture medium at
5 x 10^5/ml for 7 days as specified above. The culture flasks were all precoated with rat tail collagen.

**Cell viability assay.** Viability of hepatocytes was determined by trypan blue exclusion.

**Release of LDH.** Release of LDH from hepatocytes into the culture supernatant was measured using an automated chemical analyzer (MEGA Toshiba) [9].

**Protein synthesis.** The protein neosynthesis of hepatocytes was analyzed after incorporation of 1 µCi/ml [\(^3\)H]-leucine in 5% (v/v) CO\(_2\) at 37°C for 24 hr. The medium was separated by centrifugation and hepatocytes were washed twice with Hanks solution. To determine [\(^3\)H]-leucine incorporation during 24 hr into total newly synthetized proteins, each sample was precipitated with 10% (v/v) trichloroacetic acid (TCA) and 100% (v/v) ethanol on cellulose filter paper and counted in scintillation solution using a model LS-9800 liquid scintillation system (Beckman Instruments, USA) [10].

**Urea production.** After addition of 10 mM (final concentration) of NH\(_4\)Cl to a suspension of porcine hepatocytes cultured in vitro for 24 hr, the urea concentration in the supernatant was determined using a urea assay kit (Randox Laboratories, Ltd., Antrim, UK) [3,11].

**Glucose synthesis.** The culture medium was replaced with Hanks-HEPES solution containing 160 g/L (w/w) fructose, and the hepatocytes were incubated again. Glucose concentration in the supernatant after 1, 4, and 8 hr of incubation was measured using an automated chemical analyzer (MEGA Toshiba) [12].

**Diazepam transformation.** After addition of 20 mg/L (w/v) diazepam, the hepatocytes were incubated in 5% (v/v) CO\(_2\) at 37°C for 24 hr, and the diazepam concentration in the supernatant was determined using a fluorescence polarization immunoassay (TDx analyzer, Abbott Labs, USA).

**Glucose-6-phosphatase (G-6-Pase) activity.** Hepatocytes were harvested and crushed in distilled water. G-6-Pase activity was determined by quantitation of phosphoric acid formed after addition of glucose-6-phosphate; the activity was expressed as nmol phosphoric acid/10^10 cells [13].

**Statistical analysis.** Quantitative results were expressed as mean ± SD; differences among the test groups were evaluated by ANOVA. The threshold for statistical significance was considered p <0.05.

<table>
<thead>
<tr>
<th>Day of culture</th>
<th>Group C (controls)</th>
<th>Group IC (immediately cryopreserved)</th>
<th>Group SC (cryopreserved after short-term culture)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>98.9±10.3</td>
<td>96.1±19.4*</td>
<td>95.8±20.0*</td>
</tr>
<tr>
<td>1</td>
<td>96.1±19.3</td>
<td>96.2±19.1</td>
<td>95.7±20.4</td>
</tr>
<tr>
<td>2</td>
<td>93.9±23.9</td>
<td>96.1±19.4</td>
<td>94.8±22.2</td>
</tr>
<tr>
<td>3</td>
<td>90.6±29.2</td>
<td>96.6±18.1*</td>
<td>94.2±23.4*</td>
</tr>
<tr>
<td>4</td>
<td>92.3±26.7</td>
<td>94.3±23.2*</td>
<td>93.6±24.4</td>
</tr>
<tr>
<td>5</td>
<td>88.9±31.4</td>
<td>94.9±21.9*</td>
<td>91.8±27.4</td>
</tr>
<tr>
<td>6</td>
<td>90.5±29.3</td>
<td>93.9±24.0*</td>
<td>93.3±25.1*</td>
</tr>
<tr>
<td>7</td>
<td>88.1±32.3</td>
<td>90.9±28.7</td>
<td>92.1±26.9</td>
</tr>
</tbody>
</table>

*p <0.05 versus group C

Table 1. Viability of hepatocytes (%) during culture (mean ± SD; n = 10 cultures for all groups).

**Fig. 1.** Time-course of LDH release by hepatocytes during culture; group C (controls): circle; group IC (immediate cryopreservation): triangle; group SC (cryopreservation after short-term culture): diamond. Values are means ± SD.
Results

Cell viability. The yield of isolated hepatocytes averaged $4.32 \times 10^9 \pm 1.48 \times 10^9$ cells/total liver ($4.25 \times 10^7 \pm 1.98 \times 10^9$ cells/g liver). Viability data for the groups of hepatocytes from da 0 to 7 of culture are listed in Table 1. Viability gradually declined in all groups. No significant differences in viability were noted between groups IC and SC. Groups IC and SC had lower viability than group C on da 0 (IC = 96.1±19.4%; SC = 95.8±20.0%; C = 98.9±10.3%), but higher than C after da 2.

LDH release. As shown in Fig. 1, there were no significant differences in the rates of LDH release in the three groups of cultured hepatocytes. Release of LDH was higher on da 1, 2 and 3 than on da 0, 4, 5, 6, and 7 in the two cryopreserved groups.

Protein synthesis. Hepatocytes in groups IC and SC showed significantly less incorporation of [3H]-leucine, compared to group C (Fig. 2). No significant differences of [3H]-leucine incorporation were noted between groups IC and SC. In all groups, [3H]-incorporation increased gradually from da 1 to 7.

Urea production. Urea production by hepatocytes in group IC was substantially less than in groups SC and C throughout the culture period (Fig. 3). The small differences of urea production in groups SC and C were statistically significant (p <0.05).

Glucose synthesis. Glucose production in the 2 groups of cryopreserved hepatocytes was less than in the control cells from da 1 to 5 (Fig. 4). Glucose synthesis declined with time in all groups. There were no significant differences in glucose synthesis between groups IC and SC.

Diazepam transformation. Concentrations of diazepam in the supernatants throughout the period of culture are shown in Fig. 5. The diazepam transformation capacity of groups IC and SC was slightly reduced, compared to group C on da 0, 1 and 7. Diazepan concentrations in the supernatants of groups IC and SC were not significantly different.

Glucose-6-phosphatase activity. The time courses of G-6-Pase activity in the cultured hepatocytes are plotted in Fig. 6. In all groups, the G-6-Pase activity was higher on day 0 than on da 1 to 7. No significant differences of G-6-Pase activity were found between groups IC and SC. G-6-Pase activity of hepatocytes...
in group SC slightly exceeded that of group C on da 4, 5, and 6.

**Discussion**

Cryopreservation of hepatocytes has been widely applied in studies of BAL, hepatocyte transplantation, drug metabolism, and toxicology [6,14-17]. Even the most refined cryopreservation procedures are attended by reduced viability and deterioration of metabolic functions [6,18-22]. Hepatocytes are easily influenced by physical and chemical factors that result in destruction of cell membranes, loss of organelles, intracellular edema, reduced function, and even death during the freeze-thaw process. Until now, there has been no optimal freeze-thaw protocol.

Kardssis et al [23] found that enzyme release reached stable levels after 4 to 5 da of hepatocyte culture. Flendrig et al [9] found that LDH release by hepatocytes was only significant on da 1, GOT release was significant and tended to fall during a 3-da period, and GPT release was low but significant on da 1 and 2. These results suggest that the hepatocyte membrane needs 2 to 3 da to recover integrity. Koebe et al [8] studied cryopreservation with cultured hepatocytes for 48 hr prior to storage. The present study compared immediate cryopreservation after short-term culture; 48 hr was used as the duration of the short-term preincubation of hepatocytes in this study.

Pulain et al [24] found that Leibovitz medium is superior to the University of Wisconsin solution as cryopreservant for hepatocytes. Addition of polyethylene glycol (PEG) to Leibovitz medium resulted in slightly higher viability of hepatocytes after cold storage. DMSO has also been used with various media and shown to be a potent cryoprotectant at 10% [25]. DMSO may delay and reduce ice formation during the freezing process, resulting in decreased cellular damage. DMSO interacts electrostatically with phospholipid membranes, which may increase membrane stability during the freeze-thaw process. Hormones (ie, hydrocortisone, insulin, glucagon) and growth factors (ie, HGF, EGF, NGF) also have beneficial effects on cryoprotection. Accordingly, in the present study, we used RPMI-1640 medium containing 10% DMSO, HGF, EGF, NGF, hydrocortisone, insulin, glucagon, with 10% neonatal bovine serum (NBS) as a cryoprotectant, and several other ingredients.

As to the freezing program, many investigators recommend slow cooling and rapid thawing rates [4]. Viability is enhanced by (a) adjusting the cooling rate for the heat of crystallization during
freezing and (b) thawing rapidly to minimize cellular damage from intracellular ice-crystal formation. Guillouzo et al [26] noted that optimal freeze-thaw conditions are not yet well defined; the most critical parameters appear to be the choice of cryoprotectant, composition of the freezing medium, and cooling and thawing rates. Little work has been done to assess the continuing viability and function of porcine hepatocytes during culture after cryopreservation. In the present study, a program reported by Koebe et al [8] for rate-controlled freezing was used, together with rapid thawing, and the functions and integrity of hepatocytes were assayed during subsequent culture in serum-free medium for 7 da.

Guillouzo et al [26] found that viability of thawed hepatocytes was decreased by 10-25% depending on the species and conditions. Our results showed that the viability was >95% in each group. This may reflect our method for hepatoceyte isolation and choice of cryopreservant. We used a modified in situ collagenase perfusion method described by Seglen [5]; the period of warm ischemia was 0 min. The pigs were still alive when the perfusion stopped.

Urea synthesis is a sensitive marker for cell viability since it requires high energy demand from cells, as well as coupled transport in different organelles. In this study, the capacity for urea synthesis of cells in group IC was inferior to group SC. The higher urea synthesis in Group SC was possibly related to preincubation, which allowed recovery from cellular trauma during hepatocyte isolation and subsequent repair of cell membranes. This result is consistent with reports of Watts and Grant [18] and Siva et al [3].

Production of proteins and glucose are two main synthetic functions of hepatocytes. In this study, these functions were impaired in the two cryopreservation groups, compared to the control group, but no significant differences were found between the two cryopreservation groups. The diazepam transformation assay reflects cytochrome P450 activity, which is an important biotransformation function of hepatocytes. Although thawed hepatocytes showed slightly lower diazepam transformation than controls, there were no consistent differences between groups IC and SC. There were no significant differences among the groups in the activity of glucose-6-phosphatase, a key enzyme in the homeostatic regulation of blood glucose levels. Insofar as the authors are aware, such studies of protein synthesis, glucose synthesis, diazepam transformation, and G-6-Pase activity of thawed hepatocytes have not previously been reported.

LDH release from hepatocytes was similar in groups C, IC, and SC, indicating that severe damage in cell membrane did not occur owing to the freeze-thaw process. This may reflect the composition of the cryopreservant, which contained a mixture of factors and components. The release of LDH activity into the culture supernatant was greatest on da 2 and diminished thereafter, consistent with repair of cell membrane damage [9,23].

In summary, this study shows that a protocol for immediate cryopreservation of hepatocytes in RPMI-1640 medium containing 10% DMSO, hormones, growth factors, and 10% NBS, in conjunction with a rate-controlled freezing program and rapid thawing, provides high cell viability and maintains various metabolic functions of hepatocytes during subsequent culture. In these respects, immediate cryopreservation was comparable to cryopreservation after short-term culture, except for lower urea production. This simple protocol can
reduce contamination of cells in subsequent culture
and can be used in research on bioartificial liver and
hepatocyte transplantation.

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