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Cryopreservation of primary porcine liver cells in an organotypical sandwich model in a clinically relevant flat membrane bioreactor

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Abstract To overcome the logistical difficulties of continuously supplying freshly-isolated, primary porcine liver cells to bioartificial liver support bioreactors, we developed a cryopreservation method using an organotypical sandwich model in a flat membrane bioreactor (FMB). We measured albumin secretion rate, urea synthesis rate and 7-ethoxy coumarin (ECOD) in long-term cultures of cryopreserved cells (up to 14 days). The albumin secretion rate was 62% that of non-cryopreserved cells at days 11 and 14. The ECOD activity was 54% that of fresh, control cells initially and increased up to 79% by the 14th day. The urea synthesis rate was stable at 60% that of the control. This study showed that cryopreserved cells can recover liver-specific functions. This result has the potential to dramatically expand the clinical application of bioartificial liver supports.

Keywords Albumin · Bioartificial liver · Cryopreservation · Flat membrane bioreactor · Organotypical sandwich model · Porcine hepatocytes

Introduction

Due to the irregular availability of primary human liver cells, primary porcine liver cells have been widely used for bioartificial liver support, pharmacological research, and as a nonhuman model for pig-human clinical liver transplantation without risk of porcine endogenous retroviruses (PERV) cross transmission (Paradis et al. 1999; Levy et al. 2007). Daily isolation of primary liver cells is also a logistic difficulty. Conventional cryopreservation methods are not preferable for BAL because the cells have less metabolic capacity and their attachment capacity is impaired. To overcome these difficulties, we cultured the liver cells in an organotypical sandwich model and then cryopreserved them for a few weeks. After thawing these cells, we cultured them for up to 14 days to evaluate the liver specific potential of cryopreserved cells relative to freshly isolated liver cells. The sandwich model of cryopreservation in a tissue flask has been reported (Koebe et al. 1990), but little information is available about cryopreservation in an organotypical cellular model of a clinically relevant bioreactor.

Generally, when primary liver cells are removed from the complex architecture of the liver, hepatocytes

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rapidly lose liver specific gene expression and became phenotypically unstable in conventional culture (Strain and Neuberger 2002). Cells need an in vivo environment during in vitro culture. In vivo, each hepatocyte has direct contact with blood plasma and adjacent hepatocytes. Previously, we described an organotypical sandwich model in a flat membrane bioreactor (FMB) (De Bartolo and Bader 2000) and Petri dish (Acikgöz et al. 2009). This in vitro cellular model mimics the in vivo environment. In this FMB mode, each hepatocyte has direct contact with the perfusion medium. Our FMB is clinically relevant in long-term culture with a high density culture that enhances direct oxygenation.

Organotypical sandwich models are always superior to conventional collagen-coated cultures with respect to liver-specific functions, drug metabolizing enzymes (Bader et al. 1995, Kern et al. 1997) and other liver features such as cuboidal morphology, bile canaliculi, tight junctions, and gap junctions (Sidhu et al. 2004). In this study, primary porcine hepatocytes were cryopreserved in an organotypical sandwich model in an FMB and then evaluated for liver-specific functions with the goal of meeting the critical demand for BAL materials.

Materials and methods

Porcine hepatocyte isolation and organotypical culture in an FMB

Liver cells were isolated from female pigs weighing from 20 to 25 kg by a two-step perfusion method as described previously (DeBartolo et al. 2000) and modified from the method described by Seglen (1976). Upon disruption of the liver capsule, cells were liberated in ice-cold buffer and resuspended twice after sedimentation, as described previously (DeBartolo et al. 2000). The viability of the hepatocytes ranged between 90 and 95% as assessed by trypan blue exclusion.

The isolated hepatocytes were incubated in William's E medium (Biochrom, Berlin, Germany) supplemented with 5% (v/v) fetal bovine serum, 2.1 mM prednisolone, 0.038 mM glucagon (Novo, Mainz, Germany), insulin 0.16 U/ml (Hoechst,

Frankfurt, Germany), penicillin 200 U/ml, and streptomycin 137 mM (Biochrom, Berlin, Germany).

Technical design of the FMB

The technical design of FMB followed that in our recent report Maringka et al. (2010). Briefly, the bioreactor consists of a multitude of stackable flat membrane modules, each having an oxygenating surface area of 1150 cm². Up to 50 modules can presently be run in parallel mode, each module with 2×10^8 cells seeded, which results in a total seeded number of 1×10^{10} cells. We used up to 20 modules (4×10^9 cells). The hepatocytes were located at a distance of 10–20 μm in extracellular matrix in the FMB. Details of the FMB configuration are described in our previous reports (DeBartolo et al. 2000). The modules are separated from each other and are connected to a medium reservoir with tubing by a multichannel peristaltic pump, which controls the flow rate in the inlet and outlet streams. In these experiments the FMB was perfused with serum-free medium at 9 ml/h. The bioreactor was prepared for cell culture by coating of the oxygen-permeable membrane with rat tail type I collagen. After seeding and attachment of the hepatocytes, a second layer of matrix was placed on top to form an organotypical model (DeBartolo et al. 2000). The cells and controls were incubated at 37°C in a 5% CO₂/20% O₂ atmosphere with 95% relative humidity for the first 24 h and thereafter in a 5% CO₂/10% O₂ atmosphere for the whole culture time. The perfusion of the FMB was initiated at 9 ml/h for 24 h after cell loading. Media samples were collected daily from the inlet and outlet stream to determine product formation and substrate clearance. After 48 h, cultures were overlaid with a second layer of collagen gel. The method of bioreactor preparation and cell seeding is not clear here although there is more detail in the next section. This should be combined in the same methods section. Thereafter, the culture was continued under serum-free conditions. The albumin secretion rate and urea synthesis were measured using a method described previously (DeBartolo et al. 2000). The ECOD assay was based on a previous method (Schmitmeier et al. 2006). The position of liver cells in conventional cryovials and organotypical sandwich model is illustrated in Fig. 1.

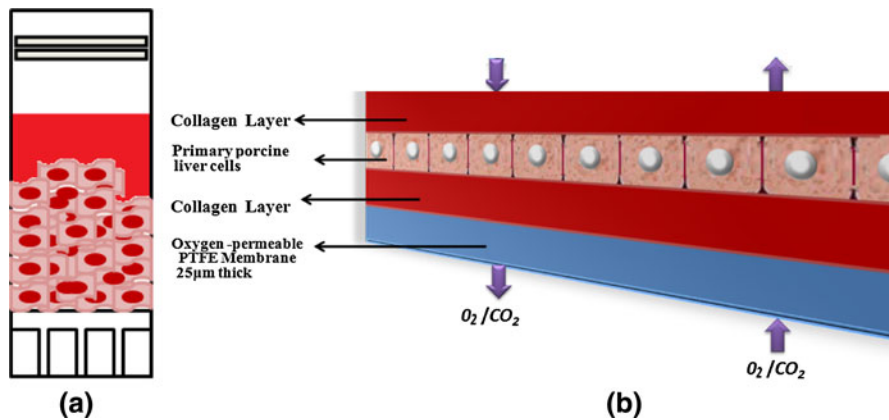


Fig. 1 **a** Conventional method of cryopreservation of liver cells in cryovial where cells are roughly distributed and lost adherence capacity and may not perform liver functions after thawing. **b** Cryopreservation of organotypical model culture model (reflect the vivo position) in clinically flat membrane bioreactor design and cryopreserved cells can recovery liver specific functions. The FMB permits a high-density hepatocyte culture and simultaneously offers cultured cells sufficient

oxygenation in an in vivo-like environment. It is believed that hepatocyte consumes ten times more oxygen than other cells. In hybrid liver support devices where oxygen is only transported by diffusion, the bioreactor has to provide an oxygen path length shorter than about 100–150 mm to meet the cell’s metabolic oxygen needs. The mean diffusion distance from the oxygen-permeable membrane to cells is about 20 µm in our FMB

Cryopreservation of the organotypical culture model and thawing

The bioreactor was frozen in a computer-controlled freezing chamber (IceCube 1810, version 2.02, SYSTEM LAB). The cooling chamber had the dimensions (L:B:H): 670:555:550 mm. Two Pt-100 thermocouples were situated in the chamber. One thermocouple was inserted into the bioreactor through the Teflon foil to measure the medium temperature during the freezing process. The other thermocouple measured the chamber temperature at short intervals. The regulation of the nitrogen supply took place by means of a PDI controller, which opened a magnetic vent when a difference between the chamber temperature and the nominal temperature was detected. Liquid N₂ was then distributed into the chamber by a ventilator.

The bioreactor was cryopreserved at day three in culture. After microscopic evaluation of the cells, medium was removed from bioreactor. Then 100 ml freezing medium (FCS + 10% DMSO) was filled into the bioreactor bottle and cooled in an ice bath. After 5 min, the freezing medium was transferred into the bioreactor and the bottle was disconnected. Then, the bioreactor was positioned immediately into the freezing chamber and the freezing program was immediately started.

The cryrogram started at a chamber temperature of 0°C (Fig. 2). With a cooling rate of -1°C/min the sample temperature was adjusted down to 0°C. The seeding of crystallization was executed and after an

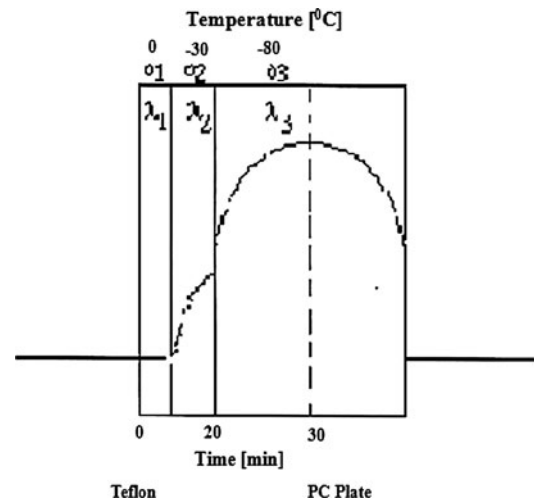


Fig. 2 Special thermal profile of the bioreactor while cooling down. PC plate (polycarbonate plate). The cryrogram started at a chamber temperature of 0°C. With a cooling rate of -1°C/min the sample temperature was adjusted down to 0°C. The seeding of crystallization was executed and after an adjustment time of 20 min the medium temperature was reduced with a cooling rate of -1°C/min down to -43°C. Afterwards a fast cooling (-30°C/min) down to -80°C followed

adjustment time of 20 min the medium temperature was reduced with a cooling rate of $-1^{\circ}\text{C}/\text{min}$ down to -43°C . Afterwards a fast cooling ($-30^{\circ}\text{C}/\text{min}$) down to -80°C followed. After a second adjustment time of 10 min, the bioreactor was thawed immediately to investigate the influence of cryopreservation on the viability of the cells. For thawing, the bioreactor was connected to the filling bottle again, which contained 250 ml Williams Medium E at 4°C with 5% (v/v) FCS. The bioreactor was thawed in a water bath at 40°C until the ice crystals disappear. Then medium was filled into the bioreactor so that the DMSO concentration was lowered slowly and stepwise (10, 6.6, 5, 2.85, 0%). Afterwards the bioreactors were incubated for 1 h at 37°C before biochemical assays were started.

For the albumin and urea assay, the supernatant was collected daily and stored at -20°C . For assaying the cytochrome P 450 activity of the cells, 7-hydroxyl coumarin synthesis (ECOD) was determined at day 3 in culture before and after cryopreservation, as well as on the 7th, 11th and 14th culture day according to methods previously published (Schmitmeier et al. 2006).

Results and discussion

Clinical treatment of hepatic failure requires a high cell concentration inside the bioreactor. It has been reported that 10% of liver mass is compatible with life support based on data from human studies. For a man weighing 70 kg, this value corresponds to approximately to 1.5×10^{10} hepatocytes (Kamlot et al. 1996; Kelly and Sussaman 1994). High density culture of primary hepatocytes in our FMB is possible (Bader et al. 2000). In summary, it can be stated that the biochemical performance of the organotypical sandwich model after cryopreservation and thawing were retained very well in the long-term culture. In many reports, cryopreservation of primary hepatocyte has been attempted using various cryoprotectants, programmable cell freezers and alginate encapsulation, but none of the major reports has attained satisfactory results. We used the organotypical model instead of uncoated or normal conventional collagen-coated plates since both monolayer and collagen-coated primary hepatocyte cultures are unstable with liver function being lost in 24–27 h. Primary pig liver

cells are used which are immobilized on a polycarbonate carrier structure as a cell monolayer between two collagen layers, and this is termed an organotypical sandwich model. It was proven that the typical polyhydric structure of the liver cell is retained for up to three weeks by this double-gel configuration, while cells that are cultivated without a second collagen layer dedifferentiate after approximately one week. We use primary porcine hepatocyte because of the increasing interest in extracorporeal liver support. Furthermore, isolation of primary hepatocytes from a single pig can provide sufficient cells for many bioreactors. For example, 10% of the human liver cell mass is needed for the survival of patients and this is equivalent to 2×10^{14} cells. Therefore, many organotypical culture bioreactors can be cryopreserved for future use.

Cultivating liver cells in a organotypical sandwich model an albumin synthesis of $1.23 \mu\text{g}$ albumin ($\text{h}/10^6$) at the 7th day in culture can be achieved (Fig. 3). This is six times higher than the synthesis rates achieved in a hollow fiber liver bioreactor. The albumin secretion rate was 61.6% compared to the rate of non-cryopreserved control cells at day eleven. Compared to other data, this synthesis is 13% higher, which stresses the good protein secretion of both cryopreserved and fresh cells when they are cultured more than seven days. The present results show that an FMB settled with pig liver cells can successfully be cryopreserved. The cells show DNA-specific urea synthesis of 78.7% and 7-hydroxycoumarin synthesis of 79.2% compared to fresh cells cultured at day 14 (Figs. 4, 5). From these results an average vitality of 79% was calculated. The secretion rate of 7-hydroxycoumarin in culture flasks was 89.4% at culture day seven. In contrast, a secretion rate of 54.3% compared to fresh control cells was found in our investigation. When cultured up to the 14th day, the secretion rate increased to 79.2%. This means that a recovery of the cytochrome P-450 enzyme occurred.

Only urea synthesis did not recover after cryopreservation. The cryopreserved cells showed 60% of the biochemical activity of the control cells. The urea synthesis of 33.3% found in alginate beads was about half of the amount assayed in the flat membrane bioreactor system (62.2%) on culture day five. This large difference can be explained both by the better cryopreservation of the bioreactor and the better immobilization procedure used. The present study

Fig. 3 Albumin secretion of cryopreserved porcine liver cells in comparison to fresh hepatocytes

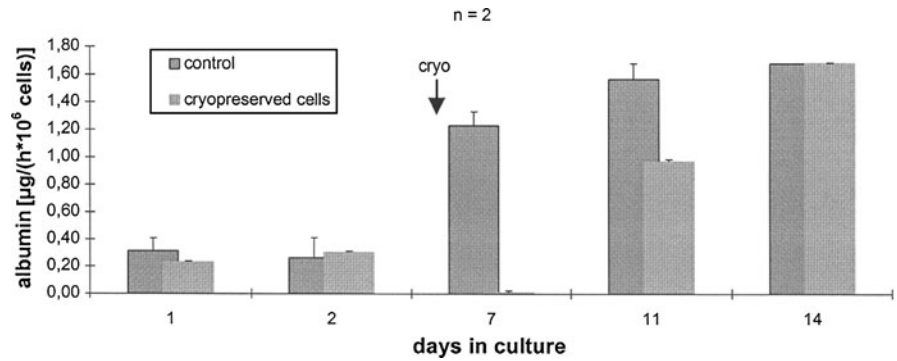


Fig. 4 Urea secretion of cryopreserved porcine liver cells in comparison to fresh hepatocytes

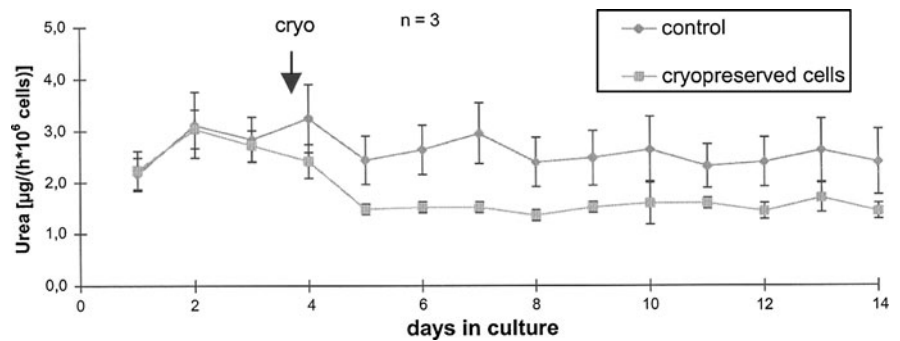
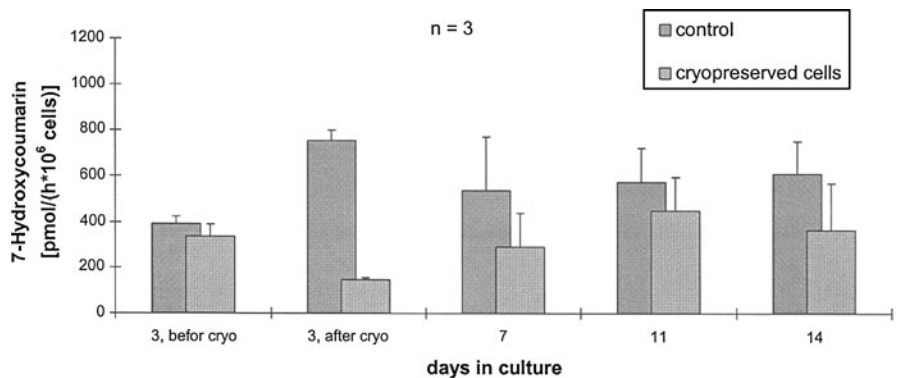


Fig. 5 ECOD assay: 7-hydroxycoumarin production of cryopreserved porcine liver cells in comparison to fresh hepatocytes



shows cells can recover their function after thawing. The albumin secretion and the 7-hydroxycoumarin synthesis reach their maximum about seven days after cryopreservation, which is almost same as with fresh isolation. The urea formation showed a constant final value two days after thawing. This means that to use an FMB to treat acute liver failure the bioreactor can be implemented immediately after thawing to eliminate nitrogenous substances. The full biochemical potential is reached approximately seven days after thawing.

Many protocols were developed for the cryopreservation of liver cells in suspension with many disadvantages and fewer advantages. However, after

thawing, liver cells of all species show reduced adherence. Thus, freshly isolated rat hepatocytes adhere with a rate of 68.12% ($n = 8$), whereas cryopreserved and thawed hepatocytes adhere only to $39 \pm 8.6\%$. This means that cryopreserved cells cannot be used for liver bioreactors because 2×10^{14} liver cells with an average vitality of 90% are necessary to build up the bioreactor, which requires even higher cell amounts to compensate for cell loss caused by adherence problems. Much less information is available on cryopreservation in well accepted organotypical cellular models in a clinically relevant FMB. To overcome this problem, cryopreservation of the

cells should be carried out when they are already immobilized in the bioreactor.

Calculation of the temperature at the cell monolayer.

By calculating the temperature at the cell monolayer the following results were found.

1. The polycarbonate plate determines the temperature at the cell monolayer when cooling down the bioreactor.
2. The medium is cooled primarily through the Teflon foil. The polycarbonate plate is responsible for a one-sided thermal insulation of the bioreactor.

Thus, a special spatial temperature profile is generated with time dependence when cooling down the bioreactor, which is shown in the following Fig. 2.

In this figure (Fig. 2), a temperature gradient between the medium and the polycarbonate plate can be observed. The temperature distribution gives warmth to the medium and warms up the cell monolayer, while in the medium crystallization occurs only near the Teflon foil. Thus, this temperature distribution has effects on the viability of the cells during the shock freezing. The polycarbonate plate prevents freezing of the cells when shock freezing is carried out and inhibits crystallization in the cells.

The FMB used in this investigation has a flat structure and cells are immobilized as a monolayer between two collagen layers. Thus, the settled bioreactor can theoretically be cryopreserved as a whole because the heat transport that occurs when cooling down or warming up the bioreactor is constant over the entire surface. Therefore, effects such as crystal formation and crystal growth as well as the osmotic reaction of the cells are equal everywhere. Because of this, the settled bioreactor could possibly be cryopreserved and hence an on-demand bioreactor could be available.

Each organ has its own pattern of cell organization as the tissue architecture and parenchymal morphology reflect the functional differentiation of an individual organ. For example, heart cells are arranged to form muscle fiber bundles, which are a form of tissue organization with direct contractile strength. Kidney cells are arranged in glomerular or tubular structures for excretory functions. Thus, every organ has its own form of cellular structure that determines its function. In the liver, hepatocytes are arranged as monolayer

plates, and the liver is efficiently provided with soluble nutrients and dissolved oxygen by a high rate of blood flow that reaches hepatocytes in the sinusoid space. Our FMB has the same pattern, with the hepatocyte culture receiving direct enhanced oxygenation from both sides. Existing conventional batch bioreactors (e.g., Petri dishes or T-flasks) for 2D cell or tissue culture have major limitations in supplying oxygen and nutrients to the cell surface across a stagnant layer of medium. Herein, we showed that cryopreservation and recovery of cell functions may be possible in an *in vivo*-based organotypical culture. This approach mimics the *in vivo* liver where the problem of oxygen and nutrient supply to the cells is solved by arranging them in cell plates with sinusoidal structures located on both sides.

Furthermore, hepatocytes consume ten times more O_2 than other cells (Cho et al. 2007). In hybrid liver support devices where O_2 is only transported by diffusion, the bioreactor has to provide an O_2 path length shorter than about 100–150 μm to meet the cell's metabolic O_2 needs. The mean diffusion distance from the O_2 -permeable membrane to cells is about 20 μm in our FMB.

According to the FDA, the minimum period is 14 days and the maximum period is 90 days for general toxicity assays. Therefore, we used 14 days of culture to meet the minimum period and we analyzed the ECOD activity along with albumin secretion and urea synthesis. Using this sandwich concept, cells can be considered stable at -80°C for at least one month since this already was examined for porcine liver cells in t-flasks (Koebe et al. 1990). We tested up to three weeks (28 days): longer periods of cryopreservation may be possible using this concept in an extracorporeal bioartificial-based bioreactor. Therefore, the hepatocytes cryopreserved using the organotypical model can be an effective source of hepatocytes and may greatly solve the logistic problem of fresh hepatocytes. Further research is needed at the molecular level during cryopreservation as well as on cell recovery.

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