

Postthaw Viability of Precultured Hepatocytes

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Hepatocytes are being studied for a wide variety of applications, including drug metabolism studies, gene therapy, and use in liver-assist devices for temporary liver support. The ability to cryopreserve isolated hepatocytes would permit the pooling of cells to reach the required therapeutic coordination of the cell supply with patient care regimes and the completion of safety and quality-control testing. The objective of this investigation was to develop a method of cryopreserving isolated hepatocytes that will retain high levels of function and facilitate the use of the cells in different applications. Freshly isolated hepatocytes were cultured in a spinner flask for different periods of time, up to 48 h. The cells were cryopreserved by use of a range of solution concentrations and cooling rates. For fresh, nonfrozen hepatocytes precultured for 24 h prior to being plated on collagen, the albumin secretion rate was 0.88 ± 0.62 mg/ml/h. When the cells were precultured for 24 h, frozen in a solution containing 10% Me₂SO with a cooling rate of 1°C/min, thawed, plated on collagen, and cultured, the albumin secretion rate was 0.21 ± 0.24 μg/ml/h. In contrast, freshly isolated hepatocytes cryopreserved without preculture and cultured on collagen had an albumin secretion rate of 0.07 ± 0.08 mg/ml/h. The influences of different solution compositions and cooling rates on postthaw function of precultured hepatocytes were also determined. These results indicate that the use of a preliminary culture step prior to cryopreservation can enhance the postthaw function of hepatocytes. © 2001 Academic Press

Key Words: cryopreservation; hepatocyte; *in vitro* culture.

Hepatocytes are being studied for a wide variety of applications. Xenogenic hepatocytes are being used in liver-assist devices for temporary liver support (8, 23). Genetically modified hepatocytes are being studied for correction of metabolic diseases (6). Hepatocytes are used extensively in pharmacology and toxicology research (1).

The clinical and commercial application of these technologies relies on the ability to cryopreserve isolated hepatocytes. The ability to cryopreserve isolated hepatocytes would permit the pooling of cells to reach the required therapeutic dose and facilitate the human therapy coordination of the cell supply with patient care regimes. Cryopreservation of hepatocytes would also permit completion of safety and quality-control testing before use of the cells with humans. Concerns over the transmission

of disease, in particular with xenogenic cells, make the cryopreservation of hepatocytes an essential element in the therapeutic application of these cells.

Various investigators have studied the freezing characteristics of isolated hepatocytes from rats (2, 5, 11, 31), humans (7, 13, 21, 27), dogs (15), and pigs (7, 16, 17). In general, cryopreserved hepatocytes from different species are viable postthaw (using trypan blue) and can attach to treated surfaces. There has been a wide range of results when the cryopreserved hepatocytes are tested for metabolic function (drug metabolism or albumin synthesis). One of the complexities in the determination of the postthaw viability of hepatocytes is the development of appropriate methods of *ex vivo* culture to assess functionality. Only recently have methods of *in vitro* culture been developed that retain differentiated function for hepatocytes and permit determination of metabolic function. One method of *ex vivo* culture used the culture of hepatocytes between two layers of collagen (9). Hepatocytes cultured using this

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method exhibit albumin synthesis and urea production and can maintain that function for weeks.

The freezing characteristics of hepatocytes cultured in a collagen gel have been studied in the past. These studies have shown that the cryobiophysical characteristics of the hepatocytes are influenced by *ex vivo* culture (14, 36). Hepatocytes cultured in a collagen gel could be frozen to -80°C and stored for periods up to 30 days with the majority of cells surviving (3, 4, 17, 28). This period of storage may be suitable for a variety of applications. The principal difficulty is that culture of the cells in the sandwich configuration is low density, resulting in the storage of large volumes (with the corresponding increase in cost). In addition, specific applications may require isolated cells, which may be difficult to recover from the collagen gel. Other approaches to cryopreservation of cultured hepatocytes have included use of hepatocytes cultured briefly on tissue-cultured plates, removed from the surface by use of trypsin, and then cryopreserved (2). Based on membrane integrity, high postthaw viability could be obtained (90%), but a loss of cells was observed if the cells were not frozen immediately after trypsinization. These results indicate that the additional step of trypsinization is stressful to the cells and can result in additional cell loss. Hypothermic storage of hepatocyte spheroids has also been studied (29). This method of storage is too limited in duration for most clinical applications.

The purpose of this investigation was to develop a method of cryopreservation of isolated hepatocytes for storage at liquid nitrogen temperatures. This method does not require extensive manipulation of the cells and permits long-term storage, the recovery of differentiated function, and the flexibility to incorporate these cells into different applications, such as encapsulation, monolayer culture, or culture in a bioreactor. These attributes facilitate incorporation of the cryopreservation protocol into the diverse applications for hepatocytes presently under investigation.

METHODS

Cell Isolation

All studies were performed with the approval of the Institutional Animal Care and Use Committee at the University of Minnesota. Pig hepatocytes were harvested from 8- to 10-kg male pigs using a two-step *in situ* collagenase perfusion technique that was modified from the original method developed by Seglen (30). The pig was initially anesthetized with ketamine and rompun to allow for intubation and mechanical ventilation, subsequently anesthetized with isoflurane, and paralyzed with succinylcholine. The liver was first perfused *in vivo* with oxygenated perfusion solution I (Per I) at 300 ml/min for 20 to 40 min. Per I is a calcium-free solution with 143 mM sodium chloride, 6.7 mM potassium chloride, 10 mM Hepes (Gibco, Grand Island, NY, U.S.A.) and 1 g/L EDTA (Sigma Chemical Co., St. Louis, MO, U.S.A.) at pH 7.4. The liver was then perfused *ex vivo* at 300 ml/min with oxygenated perfusion solution II (Per II). Per II consists of 100 mM Hepes, 67 mM sodium chloride, 6.7 mM potassium chloride, 4.8 mM calcium chloride, 1% (v/v) bovine albumin, and 1 g/L collagenase-D (Sigma), pH 7.6. Once the liver was visually dissolved (after 20–30 min), it was broken up and irrigated with cold William's E medium (Gibco) supplemented with 15 mM Hepes, 0.2 U/ml insulin (Lilly), 2 mM L-glutamine (Gibco), 100 U/ml penicillin (Celox, Hopkins, MN, U.S.A.), and 100 mg/ml streptomycin (Celox). The released cells were filtered through nylon mesh with 100- μm openings and washed via three centrifugations (50g) and resuspensions in the William's E medium. Viability for the harvests, as determined using trypan blue exclusion, ranged from 89 to 98%.

Preculture of Isolated Hepatocytes

In preliminary studies, we observed that hepatocytes cultured briefly before cryopreservation exhibited higher viabilities than those frozen directly postisolation. To establish the appropriate duration of culture, one phase of the

investigation involved evaluation of the influence of culture duration on postthaw viability. The preculture protocol consisted of the culture of freshly isolated hepatocytes for up to 24 h in a spinner vessel culture, by use of a technique described in more detail in (20, 25). Briefly, isolated hepatocytes were inoculated into a spinner flask containing 100 ml of media to a final concentration of $3 \times (10)^5$ viable cells per milliliter. The media consisted of William's E media (Gibco) containing 0.292 mg/ml L-glutamine (Gibco), 0.2 U/ml porcine insulin (Lilly Research Laboratories, Indianapolis, IN, U.S.A.), 25 ng/ml epidermal growth factor, 50 mg/ml linoleic acid, 500 mg/ml bovine serum albumin, 1 nM dexamethasone, 4 ng/ml glucagon, 6.25 mg/ml transferrin, 20 ng/ml liver growth factor, 6.25 ng/ml selenium, 0.1 mM $\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$, 3 nM H_2SeO_3 , 50 pM $\text{ZnSO}_4 \cdot 7\text{H}_2\text{O}$, 15 mM Hepes, 100 U/ml penicillin (Celox), and 100 mg/ml streptomycin (Celox). All components were from Sigma, unless otherwise indicated. The flask was cultured in a 37°C incubator with 5% CO_2 while being stirred using a magnetic stirring plate set at a speed of 90 rpm.

Cryopreservation Protocol

Cryopreservation solutions. To determine the role of solution composition on postthaw viability, several cryopreservation solutions were tested. Conventional cryopreservation solutions for hepatocytes have contained tissue culture solution supplemented (for this investigation, William's E media) with 10 v/v% of Me_2SO (Sigma). The other cryopreservation solutions evaluated contained Normosol-R (Abbott Laboratories, North Chicago, IL, U.S.A.) supplemented with 0–5% (v/v) Me_2SO and 0–15% (w/w) arabinogalactan (Larex, St. Paul, MN, U.S.A.). Arabinogalactin (AG), a polysaccharide with an approximate molecular weight of 20,000, was added to the solution. AG has recently been shown to have cryoprotective benefit (24). AG was used in this investigation for a variety of reasons. AG exhibits a low viscosity in solution up to approximately 30 w/w%. Preliminary calorimetry studies in-

dicating that AG also promotes glass formation. Previous studies have shown that AG interacts specifically with the asialoglycoprotein receptor on the surface of hepatocytes (12). Thus, the potential exists for AG to become internalized and act as an intracellular cryoprotectant. All solution compositions studied in this investigation were introduced and removed in a single step. For freshly isolated cells, the cryoprotective agent (CPA) solution of interest was introduced on ice. For cells that had been precultured, the cells were centrifuged and the CPA solution was introduced at room temperature. Once resuspended in the CPA solution of interest, the cells were placed into the controlled-rate freezer and the freezing protocol was initiated as quickly as possible. We did not over the course of the investigation see significant losses due to osmotic stresses or biochemical toxicity resulting from exposure to the CPA solution.

Freezing protocol. Hepatocytes were centrifuged at 100g for 10 min, resuspended at concentrations ranging from 6 to 25×10^6 cells/ml in the cryopreservation solution of interest, and transferred into Cryocyte freezing containers (50-ml bags, No. 4R-99-51; Baxter, Deerfield, IL, U.S.A.). As per manufacturer instructions, total cell volume was approximately 10 ml. The samples were placed in aluminum bag presses. The final thickness of the solution in the bag after placing in the press was approximately 3–4 mm. The cell suspensions were frozen using a Kryo 10 Series III programmable freezer (Planer, Sunbury on Thames, England). The freezing protocol involved taking the samples from room temperature to 0°C at a cooling rate of 10°C/min. The sample was then held for 15 min at 0°C and cooled using the cooling rate 1°C/min to –8°C. To induce nucleation of the extracellular solution, the sample was then cooled at 50°C/min to –45°C and warmed at 15°C/min to –12°C. The sample was then cooled at cooling rate 1°C/min to a temperature of –60°C and then at a cooling rate of 3°C/min to –100°C. At that temperature, the sample was removed from the controlled-rate freezer and

placed in liquid nitrogen storage. The frozen cells were stored in liquid nitrogen storage tanks in the vapor phase for a minimum of 24 h.

Thawing protocol. After removal from liquid nitrogen storage, the samples were taken and thawed in a 37°C water bath until all visible ice crystals were melted. The actual thawing rate was not measured directly. Based on the time required for the visible ice to disappear, the approximate warming rate for the samples was 250°C/min. Upon completion of the thawing process, the cells were centrifuged at 100g for 10 min and resuspended in William's E media at room temperature.

Collagen Plate Culture

Post freeze/thaw hepatocytes were cultured on specially prepared collagen plates for 24 and 48 h to observe their attachment and to measure albumin production. Three and one-half million cells suspended in 3 ml of William's E were seeded onto a 60 × 15-mm tissue culture dish (Becton-Dickinson, Franklin Lakes, NJ, U.S.A.) which had been coated with 1 ml of collagen mixture and incubated at 37°C overnight. The collagen mixture consisted of a 3:1 ratio of Vitrogen 100 (Cohesion, Palo Alto, CA, U.S.A.) and 4X William's E brought to a pH of 7.4 using NaOH. Four dishes were seeded for each trial and incubated at 37°C with 5% CO₂. After 24 and 48 h, two dishes were removed from incubation and observed under a microscope. The medium was then aspirated from the dish and centrifuged, and 1 ml of supernatant was removed and stored at -20°C. Albumin production was measured on the supernatant samples.

Size Distribution

The size of the hepatocyte aggregates was determined using a Coulter Counter (Model Z2, Coulter, Hialeah, HI, U.S.A.). Briefly, approximately 2 ml of hepatocyte culture was removed from the spinner flask and resuspended in isotonic saline solution. Due to limitations in the range of particle sizes that could be tested, the samples tested were analyzed over three different size ranges (15-45, 30-70, and 60-90 μm). In the regions of size overlap, the raw counts

obtained from two different measurements were averaged. The aperture size used was 200 μm, which allowed for measurement of aggregates up to 180 μm in diameter.

Albumin Concentration Measurement

The albumin production of the hepatocytes was determined using an enzyme-linked immunoassay (ELISA), as described in detail elsewhere (20). Briefly, 96-well plates (Nunc, Napierville, IL, U.S.A.) were coated with rabbit antiserum to porcine albumin (Cappell, Durham, NC, U.S.A.) in a ratio of 1:1000. The plates were incubated overnight at 4°C, washed with 0.05% Tween 20 (Bio-Rad, Hercules, CA, U.S.A.) in phosphate-buffered saline (PBS), blocked for 2 h at 37°C with 0.5% gelatin in PBS, and then re-washed with 0.05% Tween 20. Serial dilutions (1:4) of sample and porcine albumin standard (10 μg/ml) were added (100 μl/well). The plates were incubated for 1 h at 37°C and washed. Peroxidase-conjugated goat anti-swine albumin was diluted 1:16,000 in Tween/PBS, 100 ml of solution was added to each well, and the plates were incubated for 1 h at 37°C and washed. The plates were developed with 2,2' azino-di (3-ethylbenzthiazoline-6-sulfonate) (Boehringer Mannheim, Indianapolis, IN, U.S.A.) for 1 h at room temperature. The optical density of the wells was determined using an ELISA reader (Bio-Tek, Woon-ski, VT, U.S.A.). The concentration of albumin in the sample of culture medium was determined. For the studies described in this investigation, the culture period (24 h) and the volume of media (3 ml) were kept constant for all the cultures tested.

Data Analysis

Statistical analysis of the data obtained was done with StatView software (SAS Institute, Cary, NC, U.S.A.). Viability and cell recovery obtained from these experiments were analyzed by use of an unpaired *t* test.

RESULTS

Influence of Preculture

Non-frozen cells. Isolated hepatocytes were precultured between 0 and 48 h using the meth-

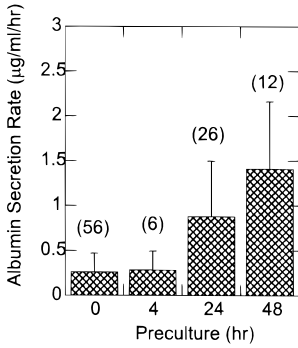


FIG. 1. Albumin secretion rate as a function of time of preculture for freshly isolated pig hepatocytes. Error bars indicate standard deviation. The numbers in parentheses indicate the number of samples tested.

ods described previously, seeded onto collagen-coated plates, and then cultured to determine albumin secretion as a function of time in preculture (Fig. 1). For freshly isolated cells and cells precultured for 4 h, the albumin secretion measured was statistically less than that observed after 24 or 48 h of preculture ($P < 0.03$). The albumin secretion for cells precultured for 48 h was greater than that observed at 24 h ($P = 0.014$). Thus, for the range of times tested, increasing the period of preculture increases the albumin secretion for fresh, nonfrozen cells cultured on a collagen matrix.

Postthaw viability. The next phase of the investigation involved determining the influence of preculture time on the postthaw viability of the hepatocytes. Pig hepatocytes were cultured for four different periods of time (0, 4, 24, and 48 h), cryopreserved using a solution containing 10 v/v% Me₂SO with a cooling rate of 1°C/min, and thawed and tested for viability. Albumin secretion was used as a marker for functional viability of the cells postthaw. As indicated previously, the preculture of the cells resulted in the formation of small aggregates. As such, it was difficult to quantify the total number of cells prefreeze and postthaw. An estimate of the overall viability was determined indirectly through measurement of albumin secretion. For all experiments, 200×10^6 cells were seeded into a bioreactor for preculture and the entire contents

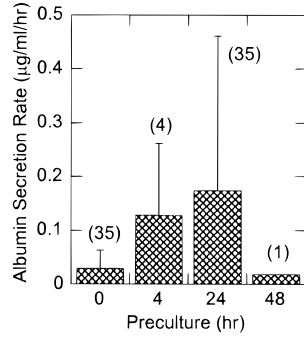


FIG. 2. Albumin secretion rate as a function of time in preculture for pig hepatocytes cryopreserved in 10% v/v Me₂SO solution (cooling rate: 1°C/min). Error bars indicate standard deviation. The numbers in parentheses indicate the number of samples tested.

cryopreserved at the end of the preculture period. Upon thawing, the samples were well mixed and aliquots taken for viability assessment. The same volume of cell suspension was removed from each bag and the albumin secretion determined for the same period of time postthaw. Thus, differences in albumin secretion reflect differences in postthaw viability.

For the range of preculture times tested, the highest postthaw albumin secretion was observed for 24 h of preculture (Fig. 2). The postthaw albumin secretion for cells precultured for 24 h prior to cryopreservation was statistically greater than that observed for no preculture ($P < 0.001$) and for 48 h preculture ($P = 0.007$). For a limited number of samples, the frozen and thawed hepatocytes were seeded into a spinner bioreactor and cultured using the same culture protocol as that used prefreeze. The frozen and thawed cells formed fully developed spheroids within 48 h. Thus, the frozen and thawed cells demonstrated the abilities to attach to collagen-coated surfaces and secrete albumin or to attach to other hepatocytes, become compacted, and form fully developed spheroids.

The preculture of the hepatocytes in spinner bioreactors promotes the formation of aggregates. After 48 h in culture, the hepatocytes form spheroids, which are compacted aggregates with spherical shapes (19). For shorter periods of culture, the size of the aggregates

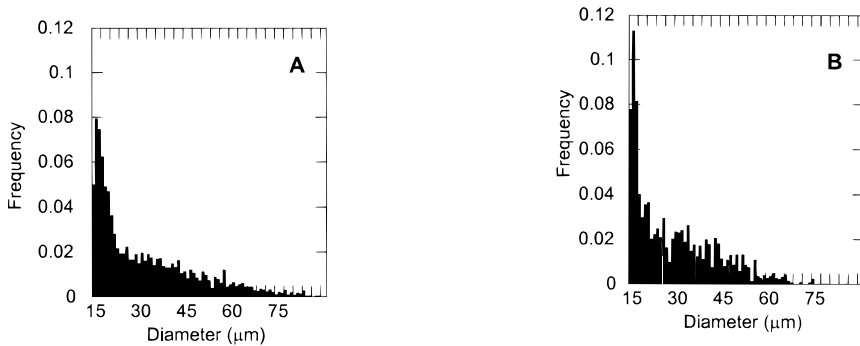


FIG. 3. Frequency as a function of diameter for pig hepatocytes (A) precultured for 24 h (B) precultured for 24 h, cryopreserved in 10% Me₂SO (cooling rate: 1°C/min), thawed, and counted.

varies and the physical structure is not compacted and spherical. The size and distribution of cells precultured for 24 h were determined before and after cryopreservation (Fig. 3). For hepatocytes precultured for 24 h, the average diameter of the cell/cell clusters was $30 \pm 16 \mu\text{m}$. Assuming that the average diameter of a pig hepatocyte is approximately $18 \mu\text{m}$ (7), this size of cluster corresponds to two or three cells. After cryopreservation and thawing, the average diameter of the cell/cell aggregates was $28 \pm 13 \mu\text{m}$. In Fig. 3, the distribution of aggregate diameters is given for fresh, nonfrozen hepatocytes precultured for 24 h and for precultured hepatocytes after freezing and thawing. Large-diameter aggregates ($>68 \mu\text{m}$ in diameter) were not measured in the samples postthaw. The measurements did not allow us to determine whether the large aggregates were disrupted by the freezing process and became smaller aggregates or whether the cells in larger aggregates lysed during the freezing and thawing processes. The differences in appearance between fresh, nonfrozen cells and frozen and thawed cells are seen qualitatively in Fig. 4.

Solution Composition and Cooling Rate

The previous studies indicate that culture prior to cryopreservation benefits the postthaw viability of isolated hepatocytes. The next phase of the investigation involved the determination of the optimum condition for cryopreservation of the precultured hepatocytes. The specification of a cryopreservation protocol requires the

specification of both the cooling rate and the solution composition. Typically, a cryopreservation solution contains (1) a balanced salt solution, (2) cryoprotective agents (glycerol, Me₂SO, polymers), and (3) a serum or protein source. For this investigation, we also examined the influence of an added buffering agent, histidine, on the postthaw viability of hepatocytes.

Based on the results obtained above, the next phase of the investigation involved the freezing of hepatocytes precultured for 24 h prior to cryopreservation by use of a variety of solution compositions and cooling rates. Changing of the solution composition may influence the cooling rate at which optimal postthaw survival is observed; so, both parameters cannot be varied independently. Two different CPAs that penetrate the cell membrane were tested: Me₂SO and glycerol. Glycerol was studied to enhance the compatibility of the cryopreservation solution for human therapeutic applications. For the studies using Me₂SO, two different concentrations were tested: 5 and 10 v/v%. Solutions containing 5% Me₂SO were also supplemented with AG. Cryopreservation solutions containing 15 w/v% AG supplemented with 3 or 6 v/v% glycerol were also tested (Table 1). These solution levels were selected based on preliminary studies examining the toxicity of different solution compositions using single-step introduction and removal protocols. The postthaw albumin secretion rate for the protocols tested was greatest for cells that were precultured for 24 h and cryopreserved by use of 10 v/v% Me₂SO with a

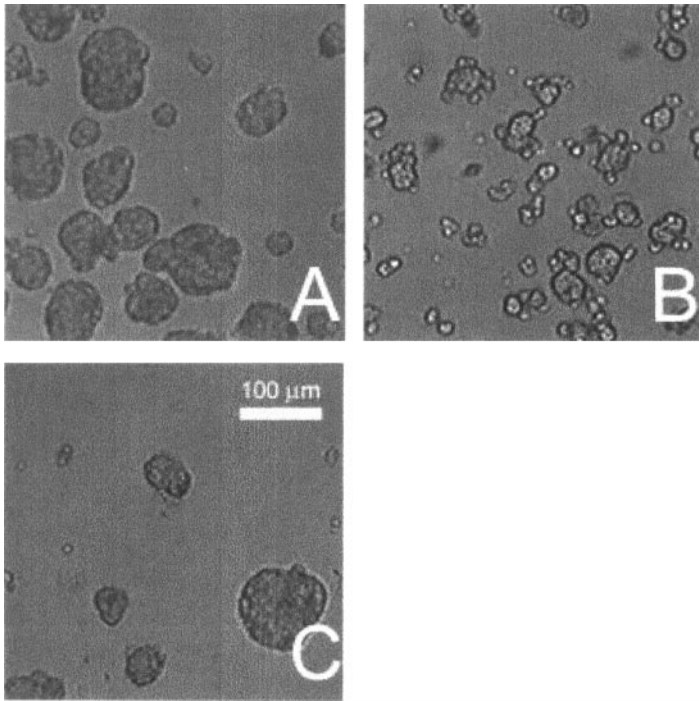


FIG. 4. Photomicrographs of hepatocyte aggregates: (A) Hepatocytes precultured for 24 h; (B) postthaw appearance of hepatocytes cryopreserved immediately postisolation; (C) postthaw appearance of hepatocytes cryopreserved after a 24 h preculture period. All cryopreserved samples were frozen in a solution containing 10% Me₂SO with a cooling rate of 1°C/min.

cooling rate of 1°C/min ($0.29 \pm 0.35 \mu\text{g/ml/h}$). The level of albumin secretion for this cryopreservation protocol was significantly less than that measured using nonfrozen, precultured cells ($0.88 \pm 0.63 \mu\text{g/ml/h}$) but was comparable to the albumin secretion rate of freshly isolated cells cultured on collagen without preculture ($0.26 \pm 0.21 \mu\text{g/ml/h}$).

Additional studies were performed to refine the final composition of the cryopreservation solution (10% Me₂SO solution with a cooling rate of 1°C/min). Specifically, we were interested in determining whether human serum albumin (HSA) or buffering of the solution with histidine (50 mM) resulted in an enhancement of the postthaw viability. For hepatocytes pre-

TABLE 1
Postthaw Albumin Secretion for Hepatocytes PreCultured for 24 h Prior to Cryopreservation

Cryopreservation solution	Cooling rate (°C/min)	Albumin concentration ($\mu\text{g/ml/h}$)
5% v/v Me ₂ SO	3	0.028 ± 0.018 ($n = 12$)
5% v/v Me ₂ SO + 15% w/v AG	3	0.14 ± 0.19 ($n = 34$)
10% v/v Me ₂ SO	1	0.29 ± 0.35 ($n = 46$)
3% v/v glycerol + 15% w/v AG	3	0.026 ± 0.030 ($n = 10$)
6% v/v glycerol + 15% w/v AG	3	0.020 ± 0.018 ($n = 12$)

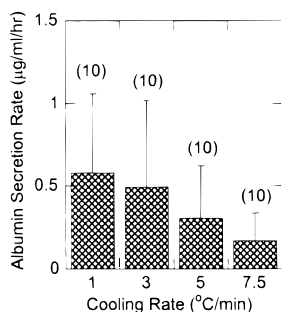


FIG. 5. Postthaw albumin secretion rate as a function of cooling rate for pig hepatocytes precultured for 24 h and cryopreserved using 10% Me₂SO solution. Error bars indicate standard deviation. The numbers in parentheses indicate the number of samples tested.

cultured for 24 h and cryopreserved in a solution containing 10 v/v% Me₂SO with a cooling rate of 1°C/min, the postthaw albumin secretion was $0.35 \pm 0.33 \mu\text{g/ml/h}$. For the same cryopreservation solution supplemented with histidine, the postthaw albumin secretion was $0.56 \pm 0.48 \mu\text{g/ml/hr}$. The difference in the albumin secretion with and without histidine in the cryopreservation solution was not statistically significant ($P = 0.18$). For a cryopreservation solution supplemented with HSA, the postthaw albumin secretion measured was $0.64 \pm 0.47 \mu\text{g/ml/h}$. The difference in the albumin secretion with and without HSA in the cryopreservation solution was marginally not statistically significant ($P = 0.06$).

The final phase of the investigation involved determining whether the cooling rate (1°C/min) used for the cryopreservation of the precultured hepatocytes using 10 v/v% Me₂SO was optimal. Hepatocytes were precultured for 24 h and cryopreserved using a 10% Me₂SO solution with a range of cooling rates between 1 and 7.5°C/min (Fig. 5). On average, there was a decrease in viability with increasing cooling rate for the range of cooling rates tested. Only the postthaw albumin secretion for cells cooled at 7.5°C/min was statistically less than that observed at 1°C/min ($P = 0.02$).

DISCUSSION

This study illustrates the role of prefreeze culture on the ability of hepatocytes to survive the stresses of freezing and thawing. As indicated under Results, culturing of the freshly isolated pig hepatocytes in spinner bioreactors and then plating of those cells on collagen-coated plates resulted in a statistically significant increase in the albumin secretion with time in preliminary culture. Whereas this study does not provide specific evidence concerning the mechanism for this increase in metabolic activity, previous investigators have shown an increase in albumin secretion with time in culture (9). Additional studies from the same group determined that, specifically, the isolation process resulted in damage to polyribosomes, and with time in culture, polyribosome size increased and protein translation was restored (10). Another factor in the increase in albumin secretion is the formation of aggregates. Several studies have found that biotransformation functions of hepatocytes cultured in spheroids are much higher than those of isolated cells (18, 19, 22, 32, 34, 35). Either or both of these factors may account for the increase in albumin secretion rate with time in preculture for nonfrozen cells observed in this investigation.

As discussed in the introduction, previous studies have looked at the influence of different culture conditions on the postthaw viability of cultured hepatocytes. Several investigators have found that hepatocytes cultured in a collagen gel sandwich configuration can survive when frozen to -80°C (4, 16, 17) with albumin secretion rates of between 70 and 100% of control values. Other methods of preculture for hepatocytes have involved the use of brief culture on tissue-cultured plates followed by trypsinization to remove the cells prior to cryopreservation (2). Based on membrane integrity, high postthaw viability could be obtained (90%); a loss of cells was observed if the cells were not frozen immediately after trypsinization. The method used in this investigation provides for the preservation of cells in suspension, which in turn can be used for the formation of spheroids, culture in collagen or other extracellular matrix proteins, or monolayer culture.

As indicated previously, there is a significant increase in albumin secretion from 24 to 48 h of preculture for nonfrozen cells. However, the postthaw albumin secretion rate for cells precultured for 48 h prior to cryopreservation is statistically less than that observed for cells precultured for 24 h. The loss of larger aggregates during freezing is the most likely reason for the drop in postthaw viability observed. As shown in this investigation, spheroids larger than approximately 68 μm in diameter do not survive the freezing process. Thus, the decrease in albumin secretion measured may reflect the higher cell losses associated with larger aggregates.

A variety of different cryopreservation solutions were tested in this investigation. In general, solutions containing Me_2SO performed better than those containing glycerol. All the solutions described in Table 1 were also tested over a range of cooling rates, and no statistically significant improvement in albumin secretion rates was observed. The postthaw viability of hepatocytes cryopreserved using solutions containing glycerol was uniformly poor. This observation was consistent with previous studies (for review, see 11) in which higher postthaw viabilities were for cryopreservation solution containing Me_2SO rather than glycerol.

Previous studies have shown that cryobiophysical characteristics of hepatocytes can vary with species (7, 33). We have also been interested in applying this method of cryopreservation to hepatocytes isolated from other species. The differences in isolation protocol (specifically, duration of the isolation process) and the difference in cryobiophysical characteristics may imply that hepatocytes from different species require different culture and freezing conditions. Further studies are needed to elucidate the role of preculture for hepatocytes obtained from other species.

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