Drug transport across the hepatocyte plasma membranes is a key factor in hepatic clearance. Hepatobiliary transport of endogenous and exogenous compounds is mediated by the co-ordinated action of multiple transport systems present at the sinusoidal (basolateral) and canalicular (apical) membrane domains of hepatocytes. The lipophilic properties of drugs enable them to cross the sinusoidal membrane by passive diffusion. However, for others, entry into the hepatocyte can be facilitated via a variety of sinusoidal transporters that augment passive diffusion. Such drugs with molecular weights ≤400 tend to be hydrophilic since they usually contain polar groups and are ionized (either anionic or cationic) (Meijer et al., 1990; Aytton and Morgan, 2001). These drugs enter the liver by sinusoidal drug transporters, including the sodium-dependent taurocholate cotransporting polypeptide (NTCP<sup>1</sup>) (Hagenbuch and Meier, 1994), members of the organic anion-transporting polypeptide (OATP) (Bossuyt et al., 1996; Cattori et al., 2001), and the organic cationic transporter (Gründemann et al., 1994). Biliary elimination of drugs is mediated by different ATP-binding cassette-transporters as exemplified by the multidrug-resistance P-glycoprotein (MDR1, MDR2) for neutral and cationic compounds (Meijer et al., 1997), and the canalicular multi-drug resistance-associated transporter (MRP2) (Payen et al., 2000) for anionic and conjugated drugs.

In our laboratory, rat and human hepatocytes have been routinely used for studies related to hepatic metabolism and transport of xenobiotics. For transport studies, freshly isolated hepatocytes have been used to investigate drug uptake to determine the role of transporters in the overall drug disposition by the liver (Tan et al., 1999; Abu-Zahra et al., 2000; Kusuhara and Sugiyama, 2002; Meng et al., 2002). To this effect, freshly isolated rat hepatocytes have been commonly used since several reports have demonstrated good correlation between results obtained with rat hepatocytes with those from perfused liver and whole animal studies (Tan et al., 1999; Abu-Zahra et al., 2000; Kusuhara and Sugiyama, 2002). One of the limitations of this technique is the requirement for isolation of cells from fresh tissue every time an experiment is to be conducted. Another major hurdle is the scarcity of liver tissue available for some species, especially human.

It would be highly desirable if daily preparation could be avoided. This may be achieved by cryopreservation of surplus hepatocytes after isolation so that they can be stored for use when needed. We and others have already demonstrated that cryopreserved hepatocytes re-

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**ABSTRACT:**

The success of cryopreservation of isolated hepatocytes with existing methodologies is assessed with respect to the retentivity of cell integrity/viability (defined by trypan blue) and metabolic activities upon thawing in comparison to those of freshly prepared cells. But the ability of the cryopreserved cells to transport xenobiotics relative to that of freshly prepared cells has not been investigated. In this study, we optimized our previous methodology for cryopreservation and evaluated the metabolism and transport of thawed hepatocytes. Half of the freshly isolated rat hepatocytes prepared by collagenase perfusion were immediately used for studies of transport of [<sup>14</sup>C]taurocholate, [<sup>3</sup>H]estrone sulfate and [<sup>3</sup>H]estradiol 17β-glucuronide (1 μM) and metabolism of 7-hydroxy-4-(trifluoromethyl)-coumarin (100 μM), (3,4-difluorobenzoyloxy)-5,5-dimethyl-4-(4-methylsulfonylphenyl)-(5H)-furan-2-one (250 μM), bufuralol (100 μM), and tolbutamide (100 μM), probes for UDP-glucuronol transferase (UGT) and CYP3A, CYP2D, and CYP2C, respectively. The remaining half was cryopreserved using an optimized, programmed-freezing protocol, which was developed to minimize the prolonged release of latent heat during freezing. With the exception of the UGT probe, no significant difference (P > 0.05) was found in both metabolism and transport with freshly isolated versus cryopreserved hepatocytes upon thawing. In conclusion, we have demonstrated for the first time that thawed rat hepatocytes cryopreserved by a programmed-freezing protocol retain drug transport activities.
tain metabolic capacities that are comparable with freshly prepared cells (Zaleski et al., 1993; Steinberg et al., 1999; Silva et al., 1999). Although cryopreserved hepatocytes may be used and are suitable for drug metabolism studies, the transport of drugs within cryopreserved hepatocytes has not been reported (Kusuhara and Sugiyama, 2002). In this study, we further improved and optimized the cryopreservation methodology of rat hepatocytes to attain cells with drug transport capacities comparable with fresh cells. We furthered assessed whether a controlled freezing procedure would improve cell yield over a two-stage freezing protocol. Hengstler et al. (2000) have recently reported that a controlled slow-freezing protocol with a supercooling step minimized the release of latent heat and resulted in significant increases in viable cells after thawing. They reasoned that, as the temperature decreases and cell mixture began to freeze, crystallization would start, and the latent heat of fusion would be released resulting in the warming of the cells mixture. Since freezing and thawing are damaging to cells, these processes are major hindrances for successful cryopreservation. One way to minimize this phenomena is to supercool the freezing chamber at the moment when the cells are beginning to freeze to minimize the warming of the cell mixture as latent heat is being released. This process can be recorded by measuring the temperature of the cells in the cryovial as well as the temperature in the freezing chamber. Results from cryopreserved cells by this method clearly demonstrated that rat hepatocytes thus prepared were useful for the study of drug transport and metabolism.

Materials and Methods

Materials. 14C-Dextran carboxy (6.5 mCi/ml), DMSO, polyvinyl-pyrolidone. BSA (35%), trypsin inhibitor, L15 medium Leibovitz, and Krebs-Henseleit buffer were obtained from Sigma-Aldrich (St. Louis, MO). Tauro-[carboxy-14C]-cholic acid (sodium salt) 54.0 mCi/mmol and Percoll were purchased from Amersham Pharmacia Biotech Inc. (Baie d’Urfe, QC). Collagenase type 2 was procured from Worthington Biochemical Corp. (Freehold, NJ). [3H]Estrone sulfate (specific activity, 40 Ci/mmol) and [3H]estradiol-17β-glucuronide (specific activity 40.5 Ci/mmol) were obtained from PerkinElmer Life Science Inc. (Boston, MA). n-Butyl phthalate was obtained from Fisher Scientific (Nepean, ON). Perchloric acid (70%) was purchased at A&C Inc. (Montreal, QC). All Falcon sterile cell culture labwares were purchased from Becton Dickinson (Franklin Lakes, NJ).

Isolation of Rat Hepatocytes. All animal studies were approved by the institution’s animal care and use committee and were conducted in accordance with all applicable regulation. Rat hepatocytes were isolated from male Sprague-Dawley rats purchased from Charles River (200 g, 250 g) by two-step collagenase (70 U/ml) perfusion as described by Moldeus et al. (1978). Viability of freshly isolated hepatocytes was assessed by trypan blue uptake (0.2%). Hepatocyte preparations with a cell viability <85% were rejected.

Cryopreservation and Thawing. Cryopreservation of rat hepatocytes was conducted by two methods. The first was one described previously (Silva et al., 1999) and involved a two-step freezing protocol. The second method employed a controlled freezing protocol with a programmable freezer (Cryomed system from Thermo Forma, Marietta, OH). In these methods, freshly isolated hepatocytes were incubated for 30 min in Krebs-Henseleit buffer containing 15 mM glucose and 12 mM HEPES, pH 7.4, at 5 × 10^6 cells/ml in a shaker-water bath at 37°C under an atmosphere of 95% O2/5% CO2. The cells were centrifuged and resuspended in cold L15-media containing 40% fetal bovine serum and 5% BSA, pH 7.4. Then a mixture containing an equal volume of cold L15-media containing 40% fetal bovine serum and 5% BSA, pH 7.4 was removed into a scintillation vial. This mixture contained an equal volume of cold L15-media containing 26% DMSO and 4% polyvinyl-pyrolidone was added slowly (1 ml/min). Cells were transferred into 5-ml cryogenic tubes (Corning, Palo Alto, CA) and kept on ice for 10 min. For the freezing method of Method 1, the cells were placed at −4°C for 1 h and at −20°C for another hour before being stored in liquid nitrogen (Silva et al., 1999). For Method 2, we furthered assessed whether a controlled freezing procedure would improve over the two-stage freezing protocol of Method 1. A freezing procedure controlled by a Cryomed Programmable freezer (Thermo Forma) was used. That included a slow freezing at −1°C/min until the temperature reached −7°C in the sample, which is 2°C before the expected freezing point. That was followed by a supercooling step at −60°C/min to −80°C in the chamber to rapidly adsorb the release of latent heat. Then the chamber was reheated at +40°C/min to −20°C, and the freezing process was continued at −1°C/min to −40°C in the chamber. A rapid freezing step at −10°C/min to −90°C in the chamber was done to complete the freezing Method 2. Cryotubes were then rapidly placed into a liquid nitrogen storage reservoir and stored for at least 1 week prior to thawing.

When needed, hepatocytes were rapidly thawed by immersing the cryogenic tubes in a 45°C water bath just long enough to melt the solution. Iced-cold L15-media containing 0.2% BSA was added slowly to 2 ml/min to dilute the DMSO, followed with washing of the cells by gentle centrifugation at 50g. The cell pellet was then resuspended in Krebs-Henseleit buffer containing 12 mM Heps, pH 7.4, and cell viability was assessed by the Trypan blue exclusion test. A 30% isotonic Percoll centrifugation step was performed to remove dead cell when viability was lower than 85%.

Metabolism Studies. Freshly prepared or thawed, isolated rat hepatocytes (2 × 10^6 cells/ml) were preincubated at 37°C under a 95% O2/5% CO2 atmosphere for 15 min before the addition of substrate. 7-Hydroxy-4-(trifluoromethyl)-coumarin (7-HFC, 100 μM) and (3,4-difluorobenzyl)-5,5-dimethyl-4-(4-methylsulfonylphenyl)-4H-furan-2-one (DFB, 250 μM), probes for UGT and CYP3A activity (Chauret et al., 1999), respectively, were incubated with the cells for 10 min. Bufuralol and tolbutamide (100 μM), probes for CYP2D and C2 (Kronbach et al., 1987; Miners et al., 1988), respectively, were incubated with the cells for 2 h. Each incubation was stopped by addition of an equal volume of acetonitrile for protein precipitation. Parent compounds remaining in the incubation mixtures and metabolites were analyzed with a high-performance liquid chromatography/UVP system equipped with a Waters 717 plus auto-sampler, a Waters 6005 controller, and a Waters 996 photodiode array detector. The data were collected and processed by Millennium version 3.20 software (Waters, Milford, MA).

The analysis of all samples was performed using a Zorbax RX-C18 column, 4.6 × 150 mm, and the following detection wavelengths were used: 355 nm (7-HFC), 305 nm (DFB), 250 nm (bufuralol) and 235 nm (tolbutamide). A gradient mobile phase was used, consisting initially of 85:15, 20 μM ammonium acetate in water and acetonitrile, and was brought to 50:50 in 15 min and then to 90:10 in 3 min at a flow rate of 1.0 ml/min. Under these conditions, parent compounds (7-HFC, DFB, bufuralol, and tolbutamide) eluted at 12.7, 17.4, 12.0, and 7.5 min, respectively. The main metabolites formed were glucuronide of 7-hydroxy-4-(trifluoromethyl)-coumarin, (hydroxy)-4-(4-methyl-sulfonylphenyl)-5,5-dimethyl-(5H)-1′-OH-bufuralol, and 3-OH-tolbutamide, and eluted at 9.2, 10.8, 4.9, and 9.5 min for 7-HFC, DFB, bufuralol, and tolbutamide, respectively.

Transport Studies. Hepatocytes suspended in Krebs-Henseleit buffer at 2 × 10^6 cells/ml were preincubated in a shaker-water bath at 37°C for 30 min under an atmosphere of 95% O2/5% CO2. The uptake studies were initiated by the addition of radiolabeled substrate (1 μM final concentration) to the incubation mixture (1.4 ml final volume); sampling (125 μl) was performed in duplicates at specified times. The cells were rapidly separated from the incubation medium by a centrifugation method described by Fariss et al., 1985. Briefly, each cell aliquot was added into an Eppendorf microtube consisting of incubation medium by a centrifugation method described by Fariss et al., 1985. duplicates at specified times. The cells were rapidly separated from the incubation medium by a centrifugation method described by Fariss et al., 1985. Briefly, each cell aliquot was added into an Eppendorf microtube consisting of incubation medium by a centrifugation method described by Fariss et al., 1985. Briefly, each cell aliquot was added into an Eppendorf microtube consisting of incubation medium by a centrifugation method described by Fariss et al., 1985.
Schematic of freezing records of cryopreservation by Method 1 (two steps, 
$-20^\circ$C and $-70^\circ$C) and Method 2 (Thermo Forma automated freezer) as described under Materials and Methods showing temperatures in the freezing chamber and in the cryovial as a function of time. The critical zones correspond to freezing point ($-9^\circ$C), latent heat released and removed period.

Method 1

Method 2

FIG. 1. Recording of cryopreservation.

Schematics of the cryopreservation process for Method 1 and Method 2. The graphs show the temperature change during the freezing process.

Results

Comparison of Methods 1 and 2. When the hepatocytes were precooled to $4^\circ$C and placed at $-20^\circ$C for 1 h (Method 1), the release of latent heat occurred when the temperature in the cell mixture reached approximately $-9^\circ$C with an increase of temperature of approximately $2^\circ$C, as shown in Fig. 1. It took 5 min for the freezing chamber to absorb the released heat. In contrast, under conditions in which the rate of freezing was controlled and the cells were supercooled prior to the freezing point (Method 2), the release of the latent heat was dramatically minimized (Fig. 1). These conditions were found to be the ones that resulted in the least increase in temperature during the freezing process.

Both methods for cryopreservation resulted in an initial high yield of cells with good viability as measured by trypan blue exclusion (Table 1). Upon further separation of viable cells from damaged ones by Percoll centrifugation, viabilities of the resulting cells increased albeit the yields decreased. This is to be expected since the Percoll centrifugation step will remove damaged cells (less dense than intact cells), but some viable cells will also be lost. Cells cryopreserved by Method 2 consistently gave a higher yield of viable cells after Percoll centrifugation compared with cells cryopreserved by Method 1 (Table 1).

Furthermore, viability of the thawed hepatocytes after a 2-h incubation was not significantly different from that of freshly isolated cells incubated over the same time periods (Table 2). In contrast, thawed cells from cryopreservation with Method 1 had significantly lower viability at the end of the 2-h incubation period (Table 2). This has also been our experience with hepatocytes obtained from other species, including dog and human (results not shown). Since we were trying to obtain the highest yield of cells after thawing, we decided at this stage to favor the Method 2 over the Method 1 to characterize cryopreserved hepatocytes in transport studies.

Effect of cryopreservation on viability and recovery of viable rat hepatocytes*

<table>
<thead>
<tr>
<th>Method</th>
<th>Thawing</th>
<th>Percoll</th>
<th>Thawing</th>
<th>Percoll</th>
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</thead>
<tbody>
<tr>
<td>Method 1</td>
<td>78 ± 4</td>
<td>80 ± 4</td>
<td>82 ± 2</td>
<td>87 ± 6</td>
</tr>
<tr>
<td>Method 2</td>
<td>72.0 ± 3.4</td>
<td>39.5 ± 4.8</td>
<td>71.1 ± 3.6</td>
<td>47.2 ± 5.1</td>
</tr>
</tbody>
</table>

*Viability was measured by trypan blue exclusion assay immediately after thawing the cells and after a Percoll gradient to remove the dead or damaged cells, as described under Materials and Methods. Recovery was defined as the percentage of cells left relative to the number of cells initially cryopreserved. Data are expressed as mean ± S.E.M. There was no significant difference in cell survival immediately after thawing, but there was a significant difference according to the t test ($P < 0.05$; $n = 6$) between the two cryopreservation methods after the Percoll step.

Metabolic Studies. To insure that cryopreserved hepatocytes retained their metabolic capacity, substrates known to be metabolized by several cytochromes P450 (bufuralol, tolbutamide and DFB and UGT (7-HFC) were incubated with hepatocytes before and after cryopreservation. The parent remaining after a 2-h incubation period with each probe was determined in fresh hepatocytes and compared with that observed in cryopreserved cells. Although not shown, the metabolite profiles of the probes showed similar patterns in fresh and cryopreserved cells were identical. As shown in Fig. 2, there was no significant difference in the relative metabolism of most of these probes when incubated with fresh versus thawed cryopreserved hepatocytes. Metabolism of 7-HFC was significantly decreased ($p < 0.05$) in cryopreserved hepatocyte compare to freshly isolated cells. About 50% of the UGT activity was lost following cryopreservation. Reports by other groups have also

Relative amount of metabolism of 7-HFC, DFB, bufuralol, and tolbutamide in freshly isolated hepatocytes (open) were compared with that observed in cryopreserved (gray) cells as described under Materials and Methods. Isolated rat hepatocytes were cryopreserved using Method 2 as described under Materials and Methods. Data are expressed as mean ± S.E.M. * significantly different from freshly prepared cells, $P < 0.05$ according to the t test, $n = 4$.  

FIG. 2. Metabolic activities.
TABLE 3
Lack of difference in uptake of various radiolabeled substrates

<table>
<thead>
<tr>
<th>Compounds</th>
<th>Uptake Rate (nmol/min/10^6 cells)</th>
</tr>
</thead>
<tbody>
<tr>
<td>[14C]Taurocholate</td>
<td>0.15 ± 0.02</td>
</tr>
<tr>
<td>[3H]Estrone Sulfate</td>
<td>0.13 ± 0.01</td>
</tr>
<tr>
<td>[3H]Estradiol 17β-D-glucuronide</td>
<td>0.14 ± 0.03</td>
</tr>
</tbody>
</table>

*a The rate of uptake was extrapolated from the slope of the linear portion of the curve (points of 0.15 to 1 min). No statistical difference, n = 3.

demonstrated that hepatocytes lose a part of conjugative pathways after cryopreservation (Diener et al., 1995; Madan et al., 1999; Steinberg et al., 1999).

Transport Studies. Upon the admixture of the substrate and cells to result in 1 μM of substrate (radionabeled and unlabeled) in 1.4 ml of 2 × 10^6 cells, aliquots (125 μl) were quickly removed, and the intracellular concentration determined by a centrifugation technique. As shown in Fig. 3A, uptake of [3H]taurocholate into freshly isolated hepatocytes was rapid and linear within the first minute. The rate of accumulation was indistinguishable from that obtained with the cryopreserved hepatocytes obtained upon thawing (Fig. 3A; Table 3). Similarly, the initial rates of uptake for [3H]estrone sulfate and [3H]estradiol 17β-D-glucuronide were also not significantly different in fresh cells versus the thawed cells (Fig. 3B and C; Table 3). This limited set of results clearly suggests, for the first time, that cryopreserved hepatocytes retain their capacity to transport compounds across the plasma membrane. Furthermore, the transport of these compounds was temperature dependent and dose-saturating (results not shown), consistent with the mechanism of an active-transport mechanism. As an example, the K_m and V_max of taurocholate uptake were 36 μM and 12.3 nmol/10^6 cells/min in freshly isolated cells, and 42 μM and 11.3 nmol/10^6 cells/min in thawed cryopreserved (Method 2) cells.

Discussion

The isolated hepatocyte system that contains intact membranes serves as an important, physiologically relevant experimental tool for uptake studies. Some work has been carried out with human hepatocytes (Olinga et al., 1998a,b) whereas rat hepatocytes are used more routinely. The preparation is facile (Moldeus et al., 1978) and the intact cells contain the entire complement of transporters, including passive permeation and transporter-assisted uptake. The incubation of drug with freshly isolated suspended hepatocytes is usually conducted over a short time-frame (usually 1 min) such that the net uptake rate is mostly influx with little efflux and is time-independent. Conventional cultured cells tend to rapidly lose hepatic transport activity (Liang et al., 1993). Recently, it has been reported that hepatocytes cultured in collagen-sandwich environment regain polarity and form bile canalicul networks (Liu et al., 1998). In this model the billiary efflux transporters such as MRP2 are maintained, however, uptake transporters such as NTCP are significantly down-regulated (Liu et al., 1999). In contrast, freshly isolated hepatocytes are reported to reflect the relative uptake rates found in vivo (Sandker et al., 1994; Zhou et al., 1994). Thus, cryopreservation poses as a viable alternate procedure for the storage of human and animal hepatocytes. Cryopreserved hepatocytes have been shown to retain most of the drug-metabolizing activities (Li et al., 1999) as well as inducibility of the drug-metabolizing enzymes (Silva et al., 1999; Hengstler et al., 2000). However, use of cryopreserved hepatocytes for uptake studies has not been attempted.

We have previously described a technique for cryopreserving rat and human hepatocytes (Silva et al., 1999). Our previous, two-step freezing method (Method 1) demonstrated the importance of allowing freshly isolated hepatocytes to recover their ATP levels by preincubation with ethanol and 3 mM isoprenaline before cryopreservation. In contrast, freshly isolated hepatocytes incubated with 2.5 μM of 3H-D-glucuronide, a substrate of the bile salt export pump (BSEP), demonstrated that uptake of 3H-D-glucuronide was temperature dependent and dose-saturating (results not shown). However, uptake of radiolabeled cholecystokinin-B was not significantly different in freshly isolated cells versus the thawed cells (Fig. 3A, Table 3). This limited set of results clearly suggests, for the first time, that cryopreserved hepatocytes retain their capacity to transport compounds across the plasma membrane. Furthermore, the transport of these compounds was temperature dependent and dose-saturating (results not shown), consistent with the mechanism of an active-transport mechanism. As an example, the K_m and V_max of taurocholate uptake were 36 μM and 12.3 nmol/10^6 cells/min in freshly isolated cells, and 42 μM and 11.3 nmol/10^6 cells/min in thawed cryopreserved (Method 2) cells.

![Figure 3](Image)

**FIG. 3.** Effect of cryopreserving rat hepatocytes on the uptake transport of tracer concentrations of model compounds. Cryopreserved or freshly isolated rat hepatocytes in Krebs-Henseleit buffer, pH 7.4, were incubated as described under **Materials and Methods.** [14C]taurocholate (A), [3H]estrone sulfate (B), and [3H]estradiol 17β-D-glucuronide (C) transport into hepatocytes was measured as described under **Materials and Methods.** Cells were incubated with 1 μM of compounds. At each time point an aliquot was spun through oil to pellet viable cells as described under **Materials and Methods.** Data are expressed as mean ± S.E.M. There is no significantly difference. n = 4 (A), n = 3 (B) and (C).
Cryopreservation on Hepatocytic Transport and Metabolism

by Nathan L. Payen, L. Céline Courtois, Alain Guillaud, and Alain Guillouzo

Cryopreservation of hepatocytes by rapid freezing is a widely used technique in the field of biotechnology and drug discovery. This method allows the preservation of cellular function and viability, making it a valuable tool in the evaluation of drug metabolism and transport across species. The technique was originally developed for the cryopreservation of mammalian tissues, particularly focusing on liver cells, due to their crucial role in drug detoxification and excretion.

A. Introduction

Cryopreservation by rapid freezing has been shown to preserve hepatocytic transport and metabolic activities, including the expression of organic anion transporters (OATPs), bile acid transporters (NTCP), and uptake transporters of organic anions (OATPs). The approach is based on the rapid cooling of the hepatocytes to temperatures below their freezing point to arrest cellular metabolism, followed by slow freezing to avoid ice crystal formation and damage. This process allows the maintenance of cellular function for cryopreserved hepatocytes.

B. Cryopreservation of Hepatocytes

1. Methods

- The method involves harvesting hepatocytes from liver biopsy or by perfusion of the liver with a dissociation media.
- The hepatocytes are then rapidly frozen to a temperature below -10°C to arrest metabolism.
- They are then cooled at a controlled rate to -196°C to be cryopreserved in liquid nitrogen.

2. Cryoprotective Agents

- Cryoprotective agents (CPAs) are commonly used to protect hepatocytes during freezing. They include sugars, glycerol, dimethyl sulfoxide (DMSO), and dimethylformamide (DMF).

3. Functional Preservation

- Cryopreserved hepatocytes retain drug transport activity after thawing. For example, OATPs and NTCP exhibit functional activity.

C. Metabolic Activity

1. UGT and Phase II Metabolism

- Phase II metabolic enzymes, such as UDP-glucuronosyltransferases (UGTs), are preserved in cryopreserved hepatocytes.

2. Phase I Metabolism

- Phase I enzymes, like cytochrome P450, are also preserved in cryopreserved hepatocytes.

D. Applications

1. Drug Discovery

- Cryopreserved hepatocytes are used in high-throughput screening (HTS) for drug metabolism and transport. They can provide valuable information on drug clearance and toxicity.

2. Clinical Applications

- Cryopreserved hepatocytes are used in drug development, particularly for assessing drug metabolism and transport across species.

E. Challenges

1. Cryopreservation at high resolution

- Cryopreservation at high resolution is necessary to ensure the preservation of cellular function and viability. This requires optimizing the cryopreservation protocol and cryoprotective agents.

2. Cryopreservation in 3D environments

- Cryopreservation in 3D environments is crucial for maintaining the integrity of hepatocytes and their functions.

References
