Wheat Extracts as an Efficient Cryoprotective Agent for Primary Cultures of Rat Hepatocytes

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Abstract: Hepatocytes are an important physiological model for evaluation of metabolic and biological effects of xenobiotics. They do not proliferate in culture and are extremely sensitive to damage during freezing and thawing, even after the addition of classical cryoprotectants. Thus improved cryopreservation techniques are needed to reduce cell injury and functional impairment. Here, we describe a new and efficient cryopreservation method, which permits long-term storage and recovery of large quantities of healthy cells that maintain high hepatospecific functions. In culture, the morphology of hepatocytes cryopreserved with wheat protein extracts (WPE) was similar to that of fresh cells. Furthermore, hepatospecific functions such as albumin secretion and biotransformation of ammonium to urea were well maintained during 4 days in culture. Inductions of CYP1A1 and CYP2B in hepatocytes cryopreserved with WPEs were similar to those in fresh hepatocytes. These findings clearly show that WPEs are an excellent cryopreservant for primary hepatocytes. The extract was also found to cryopreserve other human and animal cell types such as lung carcinoma, colorectal adenocarcinoma, Chinese hamster ovary transfected with TGF-b1 cDNA, cervical cancer taken from Henrietta Lacks, intestinal epithelium, and T cell leukemia. WPEs have potential as a universal cryopreservant agent of mammalian cells. It is an economic, efficient and non-toxic agent.

Keywords: liver; cryopreservation; wheat proteins; viability; metabolic activities

INTRODUCTION

Among the different cell types, hepatocytes are the most important for liver function, representing about 70% of the total cellular population and 80% of hepatic tissue volume (Meeks et al., 1991). They are responsible for the majority of hepatospecific functions (Clément and Guillouzo, 1992) such as synthesis and secretion of essential proteins (e.g., ceruloplasmin, clotting factors, albumin). Hepatocytes are also involved in biotransformation of endogenous and exogenous hydrophobic compounds such as xenobiotics (e.g., pharmaceuticals, environmental pollutants) into water-soluble products, which are excreted into the extracellular medium (e.g., urine, bile) (Glicklis et al., 2000).

Hepatocytes represent the most physiologically relevant model of the liver, especially as an in vitro experimental system for the evaluation of the metabolic fate and biological effects of xenobiotics. The use of other hepatic models such as cell-free microsomes would bias drug biotransformation towards phase I oxidation, whereas liver slices would have a major artefactual problem of limited drug penetration across multiple cell layers. Due to their intact cell properties, hepatocytes represent a self-contained system with complete, undisrupted enzymes and cofactors, which are at physiological levels. For studies of xenobiotic metabolism by the liver, the use of hepatocytes is more likely to yield results which are representative of those obtained in vivo, both in terms of metabolic profiles and rates of metabolic

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†In memory of Professor Francine Denizeau, her endless energy, and her dedication to the pursuit of scientific excellence (March 25, 2004).

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Abbreviations: AFPs, antifreeze proteins; CA, cold acclimated; CYP, cytochrome P450; DMSO, dimethyl sulfoxide; EROD, 7-ethoxyresorufin-*O*-deethylase; FBS, fetal bovine serum; FT, freezing tolerance; HRP, horse radish peroxidase; IRI, inhibition of ice recrystallization; L-15, Leibovitz medium; LDH, lactate dehydrogenase; NA, non-acclimated; PI, propidium iodide; PROD, 7-pentoxyresorufin-*O*-depentylase; WME, Williams' medium E; WPE, wheat protein extracts.

clearance (Billings et al., 1977; Houston, 1994; Kedderis, 1997).

Primary cultures of isolated hepatocytes do not replicate without addition of specific growth factors. Furthermore, major xenobiotic-metabolizing enzymes such as inducible isoforms of cytochrome P450 (CYP) decline rapidly in culture. Therefore, freshly isolated hepatocytes are required for most studies on xenobiotic metabolism and toxicity. The successful cryopreservation of freshly isolated hepatocytes, retaining high viability and adequate liver functions after thawing, would significantly decrease the need for freshly procured livers for the preparation of hepatocytes for experimentation. Cryopreserved hepatocytes of high quality would be of considerable value for investigations in the fields of hepatology, pharmacology, and toxicology (Powis et al., 1987; Santone et al., 1989; Zaleski et al., 1993). The use of cryopreserved hepatocytes from one source would allow investigations on well-defined cell batches. In addition, the same cells could be used in different laboratories, offering the advantage of experimental standardization. Cryopreserved hepatocytes could also be useful for the development of bioartificial liver devices that depend highly on readily available functional units (Gerlach et al., 1989, 1994; Takahashi et al., 1993; Uchino et al., 1991).

Hepatocytes are extremely sensitive to damage during freezing and thawing, even after addition of classical cryoprotectants. Thus improved cryopreservation techniques are needed to reduce cell injury and functional impairment of hepatocytes. Cryoprotective agents such as DMSO are currently used to protect cells and tissues from dehydration caused by the formation of intracellular ice during freezing. However, they are either toxic and need to be eliminated rapidly after freezing (Fahy, 1986) or cause osmotic stress that affects the metabolic competence of cells (Schneider and Mazur, 1984). Consequently, the cryopreserved cell does not represent the native metabolic state of cells or tissues and can lead to erroneous interpretation of results.

To find better alternatives to existing cryoprotectants, scientists have long considered using natural substances made by organisms that survive freezing conditions. To ensure survival, several plants such as winter wheat have evolved efficient strategies that help them to tolerate extreme winter conditions. These mechanisms are genetically controlled and induced upon exposure to low temperatures (Guy, 1990; Thomashow, 1990). This process, termed cold acclimation (CA), is associated with the development of freezing tolerance (FT). One strategy that hardy plants like wheat use to tolerate subzero temperatures is the accumulation of freezing associated proteins such as antifreeze proteins (AFPs) and dehydrins (Breton et al., 2000). AFPs exhibit two related activities in vitro. The first is the noncolligative depression of the freezing temperature of aqueous solutions relative to their melting temperature, known as thermal hysteresis. The second is inhibition of ice recrystallization (IRI), which is inhibition of the growth of larger ice crystals at the expense of smaller crystals (Knight et al., 1984). Larger ice crystals cause severe dehydration and increase the possibility of physical damage within frozen tissues. IRI occurs at low AFP concentrations (nM) and may represent the physiological function of AFPs in freezing tolerant organisms (Knight and Duman, 1986; Marshall et al., 2004). In addition, AFPs may protect cell membranes from cold-induced damage (Breton et al., 2000). The dehydrins are hydrophilic, resistant to denaturation by boiling and many are composed largely of repeated amino acid sequence motifs. They possess regions capable of forming an amphipatic α-helix. These properties may enable them to protect cells against freezing damage by stabilizing proteins and membranes during conditions of dehydration (Breton et al., 2000).

This study aims to explore the properties of these wheat proteins to develop an improved method of cryopreservation of isolated hepatocytes and other mammalian cells for long-term storage at liquid nitrogen temperature. Therefore, winter wheat protein extracts (WPE) were investigated as an alternative to the classical cryoprotectant DMSO. This new cryopreservation method permits long-term storage and recovery of large quantities of healthy cells, which maintain the differentiated functions of hepatocytes. This user-friendly technique is inexpensive, does not require extensive manipulation of cells and was successfully used to cryopreserve other cell types. Wheat extracts have potential as a universal cryopreservant agent of mammalian cells.

MATERIALS AND METHODS

Chemicals

Collagenase, insulin, Williams' medium E (WME), dimethyl sulfoxide (DMSO), resofurin, and other chemicals were from Sigma Chemical Company (St. Louis, MO). Leibovitz medium (L-15), gentamicin, and MEM vitamins were from Gibco/Life Technologies (Burlington, Ont.). Calcein, 7-ethoxyresorufin-*O*-deethylase (EROD) and 7-pentoxyresorufin-*O*-dependentylase (PROD) were from Molecular Probes (Eugene, OR). Propidium iodide (PI) was from Calbiochem (San Diego, CA). Antibodies for cytochrome P450 (CYP 1A1 (G-18) goat polyclonal IgG) and anti-goat IgG (horse radish peroxidase (HRP) conjugated mouse anti-goat IgG) were from Santa Cruz Biotechnology (Santa Cruz, CA). Fetal bovine serum (FBS) was from Medicorp (Montreal, Que).

Plant Materials and Growth Conditions

Winter wheat genotype (*Triticum aestivum* L. cv Clair, LT_{50} (lethal temperature that kills 50% of seedlings) $-19^{\circ}C$) was used in this study. Wheat plants were grown and treated as previously described (Danyluk et al., 1998). Briefly, control plants were grown for 10 days at 20°C and CA was performed at 4°C for a 7-day period.

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Total Protein Extraction

The aerial parts of seedlings were collected and blended until a homogeneous solution was obtained with cold ultrapure water. The homogenate was filtered through three layers of miracloth and centrifuged at 30,000*g* for 45 min at 4°C. The pH of the supernatant was adjusted to 7.4 and sterilized using a 0.22 μm filter. The extract was concentrated by freeze-drying and stored at −20°C, or dialyzed against ultrapure water, using 12–14,000 MWCO tubing (Spectra/Por, Spectrum Laboratories, Inc., Los Angeles, CA) before concentration. The extract was resuspended in ice-cold WME medium before being added to the hepatocyte suspension.

Hepatocyte Isolation and Culture

Hepatocytes were isolated from male Sprague–Dawley rats (120–180 g) (Charles River Canada, Saint-Constant, Que), in a two-step collagenase digestion technique (Guillemette et al., 1993; Seglen, 1976). Animals were maintained and handled in accordance with the Canadian Council on Animal Care guidelines for care and use of experimental animals (Canadian council on animal care, 1993). Cell viability was evaluated by flow cytometry (FACScan, Becton Dickinson, Oakville, ON) with 2 μM PI (Reader et al., 1993). Isolated cells were diluted to 3.5×10^5/mL and cultured in tissue culture plates (Corning, Acton, MA) in WME medium supplemented with 10% FBS, insulin (0.2 μg/mL), and gentamicin (50 μg/mL) in a humidified atmosphere of 5% CO_2 and 95% air at 37°C. After 3 h, medium was changed and cells were incubated overnight in L-15 medium (Reader et al., 1993) supplemented with insulin and gentamicin.

Cryopreservation of Hepatocytes and Cell Lines

Immediately after isolation, the hepatocyte suspension was added to ice-cold WME medium supplemented with 10% FBS and non-acclimated (NA) or CA WPEs in cold cryovials. The following cell lines were cryopreserved in their respective growth media supplemented with NA WPE: A549 (human lung carcinoma), Caco-2 (human colorectal adenocarcinoma), CHO-B1 (Chinese hamster ovary transfected with TGF-b1 cDNA), HeLa (cervical cancer taken from Henrietta Lacks), HIEC (human intestinal epithelium cells), and Jurkat (human T cell leukemia). Positive (15% DMSO + 50% FBS) and negative (WME) controls were also prepared. Tubes containing cells were frozen at a cooling rate of 1°C/min in a controlled freezing container (Nalgene, Rochester, NY) to −80°C for 1 day, and then transferred to liquid nitrogen.

Thawing of Cryopreserved Hepatocytes and Cell Lines

Frozen cells were thawed quickly by gentle agitation in a 37°C water bath and viability was determined. For adherence and metabolic assays, the hepatocyte suspension was diluted 10-fold with cold WME medium, immediately after thawing. When viability was lower than 80%, dead cells were removed by a 30% isotonic Percoll centrifugation step. After centrifugation (4°C, 50*g*, 2 min), hepatocytes were suspended in 10 mL of WME medium and washed twice. Hepatocytes (3.5×10^5/mL) were cultured in tissue culture plates in WME medium supplemented with insulin and gentamicin in a humidified atmosphere of 5% CO_2 and 95% air at 37°C. After 3 h, medium was changed and cells were incubated overnight in L-15 medium supplemented with insulin and gentamicin.

Viability Assays

Live-Dead Assay

After freeze/thaw cycles, cell suspensions were stained immediately with the fluorescent probes 4 μM calcein and 2 μM PI in WME medium for 5 min and analyzed by flow cytometry (excitation at 488 nm) using a Becton Dickinson FACScan. The numbers of live cells expressing green fluorescence of calcein and dead cells expressing red fluorescence of PI were determined with Cell Quest software (Becton Dickinson).

Lactate Dehydrogenase (LDH) Assay

LDH activity was determined in the medium of seeded, adherent hepatocytes as a measure of hepatocyte deterioration (Moldeus et al., 1978). The hepatocyte culture medium was removed daily and activity of LDH released into the medium from adherent cells was quantified. The live cells were then lysed with 10% Triton X-100 at the end of the experiment and activity of LDH was quantified to give the total cell number (Moffatt et al., 1996).

Plating Efficiency

Plating efficiency was determined by measuring LDH activity in cells prior to seeding and in 3 and 24 h-old cultures. Plating efficiency was defined as LDH activity in 24 h-old cultures divided by LDH activity in pre-culture cells.

Adherence and Cellular Morphology

Adherence and morphology of hepatocytes in tissue culture plates coated with collagen were evaluated by confocal microscopy. The confocal microscope MRC1024 (BioRad, Microscience, Cambridge, MA) was equipped with an argon laser (excitation at 488 nm) combined with an inverted microscope Eclipse Model TE 3000 (Nikon, Montreal, Que) with objectives of 40× Hoffman.

Albumin Secretion

Albumin secretion from hepatocytes was quantified every 24 h, until 96 h, in culture media by a ELISA assay (Uotila

et al., 1981), with minor modifications (Wan et al., 1993). Albumin concentrations were determined at 550 nm using a standard curve of rat albumin (0–250 ng/dL) using an ELISA reader (SPECTRAFluor Plus, Tecan, CA) and the LDH viability test, then expressed as microgram albumin/10^6 cells/24 h.

Urea Determination

To evaluate hepatocyte-mediated biotransformation of ammonia to urea, seeded hepatocytes were exposed to 10 mM NH_4Cl in L-15 culture medium. Samples of media were collected at the beginning and after 24 h intervals of exposure to ammonia, during 3 days. Urea concentration was measured colorimetrically using the urea nitrogen reagent set (BioTron Diagnostics, Hemet, CA) and an ELISA reader at 540 nm. Concentrations of urea were determined using a standard curve of urea (0–45 mg/dL) and the LDH viability test, then expressed as micrograms urea/10^6 cells.

Enzymatic Activity and Protein Expression of Cytochrome P450 Isoforms

CYP1A1 and 2B enzymatic activities were measured in hepatocyte cultures induced with benzo-a-pyrene (10 μM). Cells were washed twice with PBS and incubated for 1 h with the substrates EROD (8 μM) or PROD (17 μM) (λ_{exc}: 530 nm; λ_{em}: 585 nm). Enzymatic activity was determined using a standard curve of resorufin (0–200 μM).

CYP1A1 protein expression was determined after 24 h induction with benzo-a-pyrene (10 μM). Cells were washed with PBS, scraped off plates and suspended in 100 μL of lysis buffer (20 mM Tris-HCl, 2 mM EGTA, 2 mM EDTA, 6 mM β-mercaptoethanol) and homogenized by sonication. Proteins were separated by SDS–PAGE with 30 μg protein on 12% polyacrylamide gels (Laemmli, 1970) and then blotted onto polyvinylidene fluoride membrane. Membranes were blocked with 5% dry milk in TBS (2 mM Tris-HCl, 13.7 mM NaCl) supplemented with 0.1% Tween-20 and incubated with CYP1A1 antibody (1/1,000). Protein bands were detected by chemiluminescence (western lightning chemiluminescence reagent plus; PerkinElmer Life Sciences, Boston, MA) using a HRP-conjugated anti-goat IgG (1/1,000). Proteins were quantified by densitometry using a Molecular Dynamics scanner (Amersham, Baie d'Urfe, Que) and IP Lab gel software (Scanalytics, Inc., Fairfax, VA).

Statistical Analysis

Quantitative results were expressed as mean ± SD of at least three replicate dishes for each condition with a minimum of three experimental repeats using different cell preparations. Data were normalized to non-cryopreserved experimental controls at each time interval in the same experiment. Comparison between groups and analysis for differences between means of control and treated groups were performed using ANOVA followed by the post hoc test Newman–Keuls ($P < 0.05$). The threshold for statistical significance was: $P < 0.05$ (*), $P < 0.01$ (**), and $P < 0.001$ (***).

RESULTS

Cryopreservation of Rat Hepatocytes Using Classical Techniques

The optimal protocol for cryopreservation of freshly isolated hepatocytes was determined using DMSO. Hepatocyte concentrations ranging from 1.5 to 5×10^6 cells/mL were frozen to −80°C in Williams' medium supplemented with 10% FBS and 5–25% DMSO (data not shown). The rate of freezing was assessed using three different freezing methods: Styrofoam (4 h at −20°C, 18 h at −80°C), a programmable freezer (−6°C/h until −20°C, then 18 h at −80°C) or a Nalgene apparatus (−1°C/min until −80°C for 18 h). Optimal conditions for cryopreservation of hepatocytes were a concentration of 5×10^6 cells/mL and freezing in the Nalgene apparatus using 15% DMSO. These conditions served as a reference for classical cryopreservation in subsequent experiments.

Cryopreservation Potential of WPEs on Rat Hepatocytes and Cell Lines

The ability of WPEs to improve the viability of cryopreserved rat hepatocytes and other cell types, was compared to the classical standard, DMSO. Figure 1A presents the viability of suspensions of hepatocytes after 7 days of freezing in the presence of WPEs, proteins and DMSO, compared to fresh hepatocytes. The viability of hepatocytes that were cryopreserved with 15% DMSO + 50% FBS (positive control) was 62.5%, compared to 86.3% for freshly isolated hepatocytes. When 15% DMSO + 20 mg of BSA were used, the viability of cryopreserved hepatocytes was only 38.7%. On the other hand, very low viability was obtained with 20 mg of BSA, WME medium, 20 mg of FBS, or *E. coli* proteins (3.9, 1.6, 6.5, and 3.3%, respectively). However, significant results were obtained with 20 mg of NA WPE, giving viability of 68.4%, which was comparable to that obtained with the classical cryoprotectant, DMSO. In comparison, 20 mg of CA WPE gave a viability of 35.8% (Fig. 1A). Certain phenolic compounds accumulate during CA of wheat and may contribute to this decrease in the viability of hepatocytes. Furthermore, increases in the solute concentration of the CA WPE (927 mOsm) compared to the NA WPE (602 mOsm) could further dehydrate the hepatocyte cells and increase damage due to dehydration. This could also contribute to the reduction in the post-thaw viability of hepatocytes that were cryopreserved with CA WPE (35.8%, Fig. 1A) compared to the NA WPE (68.4%, Fig. 1A). Furthermore, dialysis of the CA and NA WPEs to remove contaminants such as phenolic compounds increased the post-thaw viability of hepatocytes to 66.1 ± 2.4 and 78.6 ± 1.7%, respectively. This was accompanied by a

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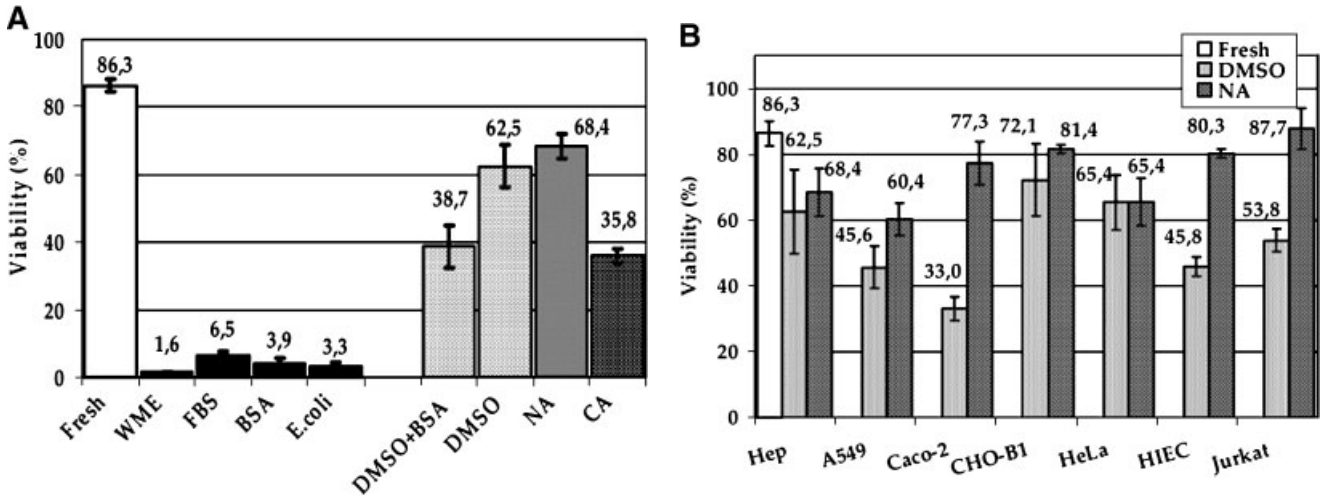

Figure 1. Cryopreservation potential of WPEs on isolated rat hepatocytes and other cell types. **A**: Viability of suspensions of hepatocytes (5×10^6 cells/mL) after 7 days of freezing was evaluated with calcein/PI by flow cytometry. Hepatocytes were frozen in WME 10% FBS supplemented with 50% FBS (FBS), 20 mg of BSA (BSA), or 20 mg of *E. coli* proteins (*E. coli*) or 15% DMSO, and 20 mg of BSA (DMSO + BSA) or 15% DMSO, and 50% FBS (DMSO) or 20 mg of NA or CA WPEs. **B**: Viability of several eukaryotic cell types after 7 days of freezing was evaluated by flow cytometry using calcein/PI. Viability of A549 (human lung carcinoma), Caco-2 (human colorectal adenocarcinoma), CHO-B1 (Chinese hamster ovary transfected with TGF-b1 cDNA), HeLa (cervical cancer cells taken from Henrietta Lacks), HIEC (human intestinal epithelium cells), and Jurkat (human T cell leukemia) cell lines (5×10^6 cells/mL) was evaluated in their respective growth media supplemented with DMSO or WPEs NA Clair (NA). Freshly isolated hepatocytes (Fresh) served as reference. Data (mean ± SEM) represent triplicate measurements of three different preparations of WPEs in at least six independent experiments with different cell preparations ($n = 54$).

reduction in their respective solute concentrations to 411 and 330 mOsm, which is 66% and 45% less than their initial osmolarity.

Cryoprotective activity of WPEs was also evaluated for the cryopreservation of several cell lines. Figure 1B presents viability of the different cell lines after 7 days of freezing in the presence of WPEs and DMSO, compared to fresh hepatocytes. Viability of A549, Caco-2, CHO-B1, HeLa, HIEC, and Jurkat cells cryopreserved with DMSO was 45.6%, 33%, 72.1%, 65.4%, 45.8%, and 53.8%, respectively, compared to 60.4%, 77.3%, 81.4%, 65.4%, 80.3%, and 87.7%, respectively, for cryopreservation with WPEs (Fig. 1B). These findings demonstrate that WPEs contain specific compounds with cryoprotective activity at least equivalent to the commonly used cryoprotectant, DMSO.

Hepatocyte viability was also assessed by the release of LDH, which measures loss of viability in culture by providing indirect measurement of cellular membrane integrity. Viability for adherent hepatocytes cryopreserved with WPEs was better than that obtained with DMSO (Fig. 2). After 24 h in culture, high viabilities of 76.4% and 89.3% were obtained for hepatocytes cryopreserved with NA and CA WPEs, respectively, compared to 60.2% for DMSO. WPEs improved viability, compared to DMSO, throughout the 96 h culture period. WPEs improved viability to similar levels as in fresh hepatocytes during 96 h.

Plating Efficiency, Adherence, and Morphology of Cryopreserved Rat Hepatocytes

The ability of thawed cells to survive in culture is one of the most important criteria for successful cryopreservation of hepatocytes. Plating efficiency of cells was assessed 3 and 24 h after seeding and culture. After 3 h in culture, plating efficiencies of thawed hepatocytes cryopreserved with NA and CA WPEs were very similar to the optimized DMSO standard (63.7 and 62.5%, respectively, compared to 64.9% for DMSO, Table I). After 24 h, plating efficiency was about 50% for thawed hepatocytes cryopreserved with DMSO, NA, and CA WPEs, relative to non-cryopreserved hepatocytes

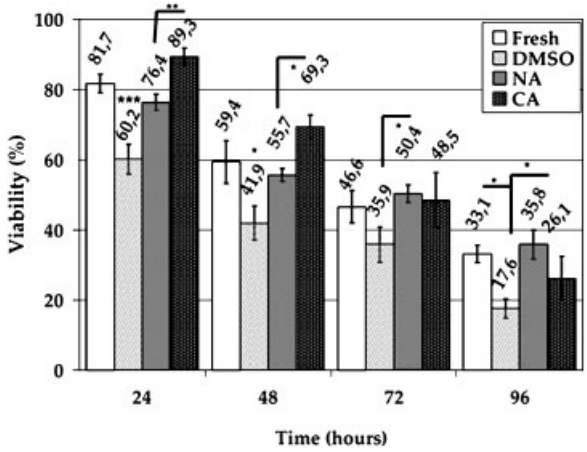

Figure 2. Viability of cryopreserved hepatocytes: effect of WPEs. Viability was determined over a 4-day period after seeding thawed rat hepatocytes that had been cryopreserved for 7 days in WME supplemented with 15% DMSO and 50% FBS (DMSO), NA and CA WPEs. Viability (%) was obtained by subtracting LDH released by damaged hepatocytes from total cellular LDH. Total LDH was evaluated by lysing cells with 10% Triton X-100. Freshly isolated hepatocytes served as reference. Controls were used to subtract intrinsic plant activity. Data (mean ± SEM) represent triplicate measurements of three different preparations of WPEs from four independent experiments with different cell preparations ($n = 36$). $^*P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$.

Table I. Plating efficiency of thawed rat hepatocytes following cryopreservation with WPEs compared to an optimized DMSO standard*.

Culture period (hours)	Fresh	Cryopreserved		
		DMSO[a]	NA	CA
3	77.3 ± 0.5	64.9 ± 1.7	63.7 ± 1.5	62.5 ± 0.4
24	100.0 ± 2.4	50.3 ± 4.1	50.9 ± 2.9	52.5 ± 3.4

Data are expressed as mean ± SEM from three different experiments.
*Plating efficiency was evaluated by LDH activity in freshly isolated and cryopreserved hepatocytes, as described in the Materials and Methods.
[a]Optimized DMSO concentration of 15% for cryopreservation of rat hepatocytes.

(100%) (Table I). These findings demonstrate that hepatocyte plating efficiency was comparable in the presence of WPEs and the optimized DMSO standard, after 3 h and 24 h in culture.

Morphological analysis by confocal microscopy is shown 24 h after seeding for thawed hepatocytes that were cryopreserved with WPEs (Fig. 3C and D) or DMSO (Fig. 3B), compared to freshly isolated hepatocytes (Fig. 3A). At the same cell concentration, fresh and WPE-cryopreserved hepatocytes showed similar rounded cellular morphology (Fig. 3A, C, and D), whereas those cryopreserved with DMSO had shrunk (Fig. 3B). Moreover, cell to cell contacts were observed for the fresh, NA and CA WPE-cryopreserved hepatocytes, but not for DMSO-cryopreserved hepatocytes in which we can observe aggregates of dead cells (uptake of PI) appearing as cells adhering to one another. Cells cryopreserved with WPEs appear to be slightly larger than the fresh cells, however, their measured size under the microscope was not significantly different. They appear larger because of their apparent opacity under confocal microscopy with the Hoffman objective. In fact, the NA and CA protein extracts were added to the hepatocyte cells at relatively high protein concentration. After cryopreservation, it is plausible that the WPE proteins have aggregated at the cell membrane of the hepatocytes, giving rise to their apparent microscopic opacity. Indeed, it is possible to see these protein aggregates attached to the Petri dishes between the cells.

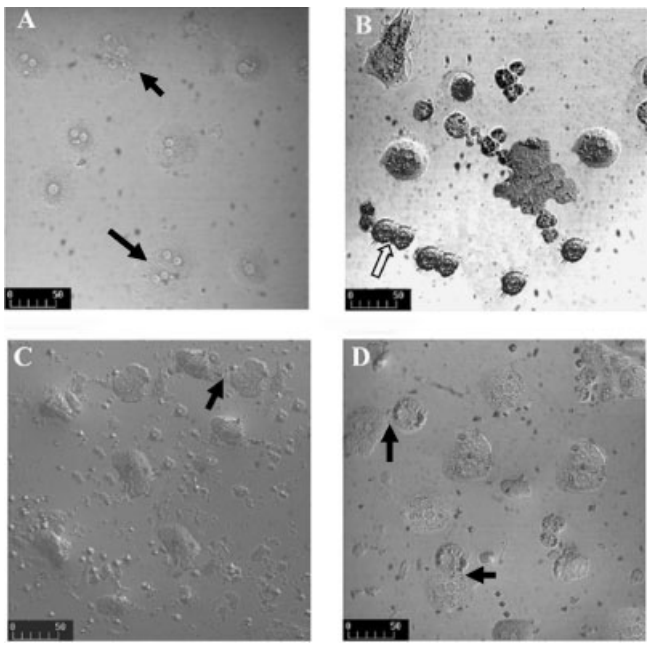

Figure 3. Analysis of adherence and cellular morphology of cryopreserved hepatocytes. Adherence was visualized 24 h after seeding thawed rat hepatocytes, following cryopreservation for 7 days in WME 10% FBS supplemented with 15% DMSO and 50% FBS (**B**), WPEs NA (**C**), or CA (**D**). Freshly isolated hepatocytes (**A**) served as reference. Solid arrows indicate cell to cell contacts; empty arrows indicate aggregates of dead cells. Hepatocytes (175×10^3) were visualized by confocal microscopy under 40× Hoffman (A–D). Photographs are from one representative experiment, which was repeated at least in triplicate.

Albumin Secretion by Cryopreserved Rat Hepatocytes

Albumin secretion is a specific marker for protein synthesis in hepatocytes because it requires liver-specific gene expression and intact translational and secretory pathways. The effects of WPEs on albumin production by cryopreserved hepatocytes were monitored during 4 days after seeding in culture (Fig. 4A). Albumin secretion by freshly isolated hepatocytes, supplemented or not with WPEs, decreased progressively with time from days 1 to 4, although the decrease was much more rapid in cells that had been cryopreserved with DMSO. In fresh hepatocytes, 85% of albumin secretory activity was maintained after 4 days in culture, compared to 48% in DMSO-cryopreserved cells. However, 83% of activity was maintained in hepatocytes that were cryopreserved with CA WPEs after 4 days, which is comparable to that of fresh hepatocytes. When hepatocytes were cryopreserved with NA WPEs, levels of albumin secretion were approximately 30% less than those cryopreserved with CA WPEs. These results demonstrate that the hepatospecific function of albumin secretion was well maintained throughout the 4-day culture period in WPE-cryopreserved hepatocytes and was considerably improved with CA WPEs, compared to DMSO (Fig. 4A).

Ammonium Detoxification by Cryopreserved Rat Hepatocytes

The effects of WPEs on ammonium detoxification by cryopreserved hepatocytes were measured at days 2, 3, and 4 after seeding in culture, compared to fresh cells (Fig. 4B). Urea production by freshly isolated cells decreased progressively with time and was much lower in DMSO-cryopreserved hepatocytes. Fresh hepatocytes, supplemented or not with WPEs, maintained 55% of initial detoxification activity, compared to 16% for DMSO-treated cells after 4 days in culture. However, after subtraction of plant arginase activity, ammonium detoxification in hepatocytes cryopreserved with CA and NA WPEs was similar to fresh cells from 48 to 96 h. These findings indicate that the hepatospecific function of ammonium detoxification was well maintained throughout the 4-day culture period with WPEs, relative to DMSO (Fig. 4B).

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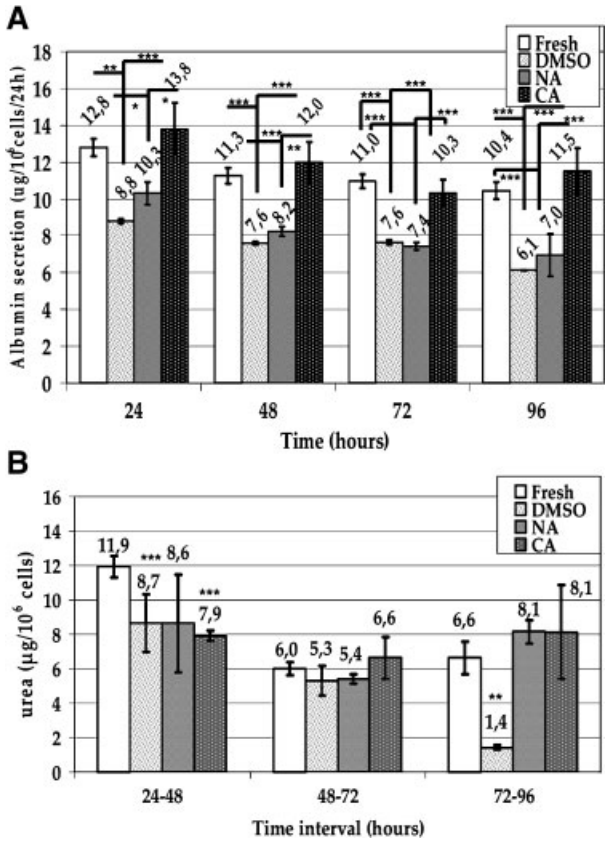

Figure 4. Albumin secretion and detoxification of ammonium to urea by cryopreserved hepatocytes: beneficial effect of WPEs. **A**: Albumin secretion (µg/10^6 cells/24 h) in culture medium over a 4-day period after seeding thawed rat hepatocytes and (**B**) production of urea (µg/10^6cells) during 24 h intervals after 1, 2, and 3 days in culture, for thawed rat hepatocytes following cryopreservation for 7 days in WME supplemented with 15% DMSO and 50% FBS (DMSO), WPEs NA, and CA. Total cell number was evaluated by the LDH activity assay. Freshly isolated hepatocytes supplemented with WPEs served as reference. Controls were used to subtract intrinsic plant activity. Data (mean ± SEM) represent triplicate measurements of three different preparations of WPEs from four experiments with different cell preparations ($n = 36$). $^*P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$.

Cytochrome P450 Enzyme Activities of Cryopreserved Rat Hepatocytes

The activity of xenobiotic-metabolizing CYP enzymes was evaluated as a third marker of hepatospecific functions. Metabolic activity of isoforms CYP1A1 and CYP2B was measured by the EROD (CYP1A1) and PROD (CYP2B) assays after a 24 h induction with benzo-a-pyrene (Fig. 5A). Compared to fresh hepatocytes, supplemented or not with WPEs, the relative activity of CYP1A1 and CYP2B enzymes decreased slightly in DMSO-cryopreserved hepatocytes, while it was maintained in hepatocytes cryopreserved with NA and CA WPEs. Western blot analysis of the CYP1A1 isoform demonstrated that increased benzo-a-pyrene inducible activity was associated with increased protein expression (Fig. 5B). This indicates that metabolic activity of the CYP1A1 and CYP2B isoforms was also improved in WPE-cryopreserved hepatocytes, compared to DMSO (Fig. 5A and B).

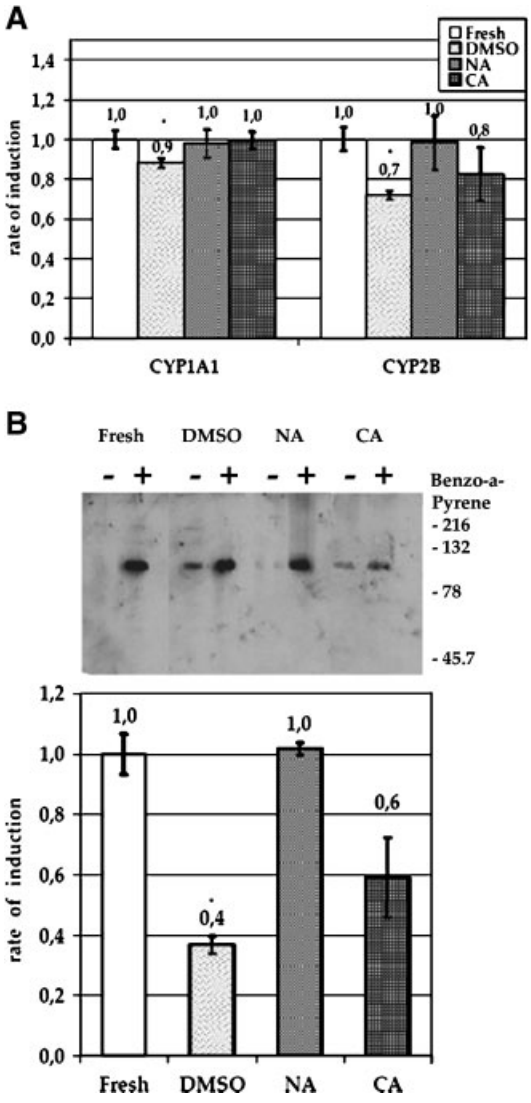

Figure 5. Activity and expression of cytochrome P450 isoenzymes in cryopreserved hepatocytes: effect of WPEs. **A**: Activity of cytochrome P450 isoforms CYP1A1 and CYP2B and (**B**) expression of CYP1A1, 48 h after seeding thawed rat hepatocytes following cryopreservation for 7 days in WME supplemented with 15% DMSO and 50% FBS (DMSO), WPEs NA and CA. Freshly isolated hepatocytes supplemented with WPEs (Fresh) served as reference. A: The induction rate of cytochrome P450 isoforms was measured by EROD (CYP1A1) and PROD (CYP2B) assays after 24 h induction with benzo-a-pyrene. B: Immunodetection of CYP1A1 after 24 h induction with benzo-a-pyrene (+) and quantification by densitometry. Rate of induction is the ratio between the density of the non-induced (−) versus induced (+) lane. Membrane staining (Coomassie blue) was used to confirm protein loading (data not shown). Freshly isolated hepatocytes (Fresh) served as reference. Data (mean ± SEM) represent triplicate measurements from four independent experiments ($n = 12$). Immunodetection (B) of proteins is shown from one representative experiment, which was repeated at least in triplicate. $^*P < 0.05$.

DISCUSSION

The major problems with classical methods of hepatocyte cryopreservation are low survival rate in culture and poor metabolic activities and functional integrity. Unlike cell lines, hepatocytes do not replicate in culture. Thus an efficient method of cryopreservation is necessary to reduce

cellular and functional damage incurred in hepatocytes during freezing.

To find an alternative to currently available cryoprotectants, we explored the use of substances made by organisms that survive freezing conditions. Overwintering plants such as winter wheat accumulate compounds that ensure survival of the plant during freezing. These compounds include sugars, amino acids, and proteins such as AFPs and dehydrins. These proteins have the potential to protect cells against dehydration caused by freezing. These properties could be advantageous for cryopreservation of hepatocytes. We therefore investigated whether protein extracts from cold tolerant wheat could act as cryopreservation agents of rat hepatocytes. Our results clearly demonstrated that 20 mg of NA WPE (68.4% viability) was as efficient as 15% DMSO supplemented with 50% FBS (62.5% viability) for the cryopreservation of hepatocytes. However, 20 mg of CA WPE gave a lower viability of 35.8%, which could be due to certain phenolic compounds that are known to accumulate during CA of wheat, as well as the increase in osmolarity of the CA WPE. Other cold tolerant plants such as barley, rye, alfalfa, and spinach also possessed cryoprotective activity for hepatocytes (data not shown). Cryoprotective activity is specific to plant extracts, since BSA and *E. coli* proteins did not show any activity.

Eukaryotic cell models are essential in most laboratories that study or utilize proteomics, genomics, microarray-, and RNA-based technologies, and consequently the long-term cryostorage of these cell cultures is required. The relevance of the cryopreservation technology with WPEs to other eukaryotic cell types was also investigated. There was a 2.3, 1.7, 1.6, and 1.3-fold increase in the viability of the Caco-2, HIEC, Jurkat, and A549 cell lines cryopreserved with NA WPE compared to that with DMSO. The viability of the CHO-B1 and HeLa cell lines cryopreserved with NA WPE was similar to that of DMSO. This suggests that WPE is a better cryoprotective agent than DMSO for cryopreservation of eukaryotic cells and thus could be used as a universal cryoprotectant.

The LDH test further demonstrated that hepatocytes cryopreserved with NA and CA WPEs maintained better viability in culture than those that were cryopreserved with DMSO. Furthermore, the viability of WPE-cryopreserved hepatocytes was similar to that of fresh hepatocytes, suggesting that WPEs are less toxic and more efficient as cryopreservation agents than DMSO.

To further determine the capacity of hepatocytes to survive in culture after cryopreservation, the plating efficiency of cells was compared after 24 h in culture. Both the WPE-cryopreserved hepatocytes and the optimized DMSO standard performed well with similar attachment efficiencies in the range of 50%, relative to fresh cells. These results for optimized DMSO-cryopreserved hepatocytes were similar to those obtained in other studies (Silva et al., 1999; Sosef et al., 2005). Microscopic analysis of post-thaw hepatocytes demonstrated their ability to attach to collagen-coated dishes and to restore near-normal morphology with cell to cell contacts, following cryopreservation with WPEs. The attachment properties and cellular morphology were better conserved in hepatocytes that had been cryopreserved with WPEs, rather than with DMSO, demonstrating again the higher efficiency of WPEs to cryopreserve hepatocytes. Cell to cell contacts were present in WPE-cryopreserved hepatocytes, suggesting better conservation of membrane integrity.

A major interest of the pharmaceutical industry during the development of new drugs for clinical use relies on short-term assays for determining metabolic profiles and rates of metabolic clearance, in either freshly isolated or cryopreserved hepatocytes. In our study, the rate of albumin secretion by fresh and DMSO-cryopreserved hepatocytes shows the typical pattern of albumin synthesis in primary hepatocytes in vitro (Guillouzo et al., 1999; Kim et al., 2001; Son et al., 2004). Rates of albumin secretion by DMSO-cryopreserved hepatocytes during 4 days were lower than those of fresh hepatocytes and this is likely attributed to damage caused by freezing. The albumin secretion rates of NA and CA WPE-cryopreserved hepatocytes also showed a similar pattern of albumin synthesis to that of fresh hepatocytes in vitro (Guillouzo et al., 1999; Kim et al., 2001; Son et al., 2004; Sosef et al., 2005), again demonstrating the advantage of WPEs as cryoprotective agents.

Urea synthesis requires a high energy demand from the cell and is a sensitive index of energy metabolism in hepatocytes (Zaleski and Bryla, 1978). DMSO-cryopreserved hepatocytes synthesized urea at lower rates compared to fresh cells, in agreement with Silva et al. (1999) and Sosef et al. (2005). In contrast, after 3 and 4 days post-seeding, WPE-cryopreserved hepatocytes maintained their ammonium detoxification function at a similar level to that of fresh hepatocytes. These results show the potential of WPEs to maintain intact the primary metabolic activities of protein synthesis, liver-specific gene expression, translational, and secretory pathways.

Finally, cryopreserved hepatocytes were challenged to determine if they could retain their capacity to respond to CYP inducers. DMSO-cryopreserved hepatocytes retain most of the drug-metabolizing activities (Li et al., 1999), as well as their ability to induce the drug-metabolizing enzymes (Hengstler et al., 2000; Silva et al., 1999; Sosef et al., 2005). We demonstrated that DMSO-cryopreserved cells responded to CYP1A1 and CYP2B inducers in a similar manner to fresh cells, although with a slightly lower induction rate. Benzo-a-pyrene induction of CYP1A1 activity correlated with an increase in the rate of CYP1A1 protein synthesis, in agreement with other studies (Madan et al., 1999; Silva et al., 1999). For NA and CA WPE-cryopreserved hepatocytes, we observed the same induction in rate of activity as fresh cells, and a lower protein synthesis induction rate only in CA WPE-cryopreserved hepatocytes. These results suggest that WPE-cryopreserved hepatocytes retained their metabolic activity and their capacity to respond to CYP inducers more efficiently than DMSO-cryopreserved cells. This confirms that WPEs are better cryopreservative agents

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than DMSO for primary rat hepatocytes. Moreover, we demonstrated that the metabolic activities of the cryopreserved hepatocytes are maintained for at least 4 days, which fulfills the requirements of the pharmaceutical industry for short-term assays to determine the metabolic profile and rates of metabolic clearance.

The NA and CA WPEs are composed mostly of proteins and traces of other compounds such as sugars (glucose, fructose, sucrose, and trehalose), glycine betaine (an organic osmolyte), antioxidants, and phenolic compounds. When the extract was boiled, there was a significant loss of cryoprotective activity suggesting that the active cryoprotective substances are of a protein nature. In addition, supplementation of the media with different sugars, glycine betaine, and trehalose, did not significantly improve the cryopreservation of hepatocytes, confirming that the active compounds are indeed the wheat proteins. These active proteins are likely to be a mixture of the freezing associated proteins present in winter wheat extract. The freezing associated proteins have the properties of protecting cell membranes against dehydration caused by extracellular ice formation. It is also possible that the wheat proteins interact with the membrane bilayers and reduce the elevation of membrane transition temperature and thus reduce the occurrence of non-lamellar phases (Breton et al., 2000). However, the exact function of the winter WPE remains unknown.

In conclusion, this study clearly demonstrates that the WPE is an efficient cryopreservation agent of hepatocytes and other cell types. There was a minimal loss of cells and hepatocytes retained their metabolic activities in a similar manner to fresh cells. The WPE is a non-toxic natural product, which is economic and easy to prepare. It is an efficient universal cryoprotectant that could replace DMSO, which has severe limitations due to cellular toxicity. However, it will be of interest to identify specific protein(s) that confer these beneficial cryoprotective properties and to elucidate their functions.

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References

Billings RE, McMahon RE, Ashmore J, Wagle SR. 1977. The metabolism of drugs in isolated rat hepatocytes. A comparison with *in vivo* drug metabolism and drug metabolism in subcellular liver fractions. Drug Metab Dispos 5:518–526.

Breton G, Danyluk J, Ouellet F, Sarhan F. 2000. Biotechnological applications of plant freezing associated proteins. Biotechnol Annu Rev 6:59–101.

Canadian Council on Animal Care. 1993. Guide to the care and use of expetimental animals. Canadian council on animal care.

Clément B, Guillouzo A. 1992. Cellular and molecular aspects of cirrhosis. Paris, France: INSERM Ed. 394p.

Danyluk J, Perron A, Houde M, Limin A, Fowler B, Benhamou N, Sarhan F. 1998. Accumulation of an acidic dehydrin in the vicinity of the plasma membrane during cold acclimation of wheat. Plant Cell 10: 623–638.

Fahy GM. 1986. The relevance of cryoprotectant "toxicity" to cryobiology. Cryobiology 23:1–13.

Gerlach J, Kloppel K, Schauwecker HH, Tauber R, Muller C, Bucherl ES. 1989. Use of hepatocytes in adhesion and suspension cultures for liver support bioreactors. Int J Artif Organs 12:788–792.

Gerlach J, Trost T, Ryan CJ, Meissler M, Hole O, Muller C, Neuhaus P. 1994. Hybrid liver support system in a short term application on hepatectomized pigs. Int J Artif Organs 17:549–553.

Glicklis R, Shapiro L, Agbaria R, Merchuk JC, Cohen S. 2000. Hepatocyte behavior within three-dimensional porous alginate scaffolds. Biotechnol Bioeng 67:344–353.

Guillemette J, Marion M, Denizeau F, Fournier M, Brousseau P. 1993. Characterization of the *in vitro* hepatocyte model for toxicological evaluation: Repeated growth stimulation and glutathione response. Biochem Cell Biol 71:7–13.

Guillouzo A, Rialland L, Fautrel A, Guyomard C. 1999. Survival and function of isolated hepatocytes after cryopreservation. Chem Biol Interact 121:7–16.

Guy CL. 1990. Cold acclimation and freezing stress tolerance: Role of protein metabolism. Annu Rev Plant Physiol Plant Mol Biol 41:187–223.

Hengstler JG, Ringel M, Biefang K, Hammel S, Milbert U, Gerl M, Klebach M, Diener B, Platt KL, Bottger T, Steinberg P, Oesch F. 2000. Cultures with cryopreserved hepatocytes: Applicability for studies of enzyme induction. Chem Biol Interact 125:51–73.

Houston JB. 1994. Utility of *in vitro* drug metabolism data in predicting *in vivo* metabolic clearance. Biochem Pharmacol 47:1469–1479.

Kedderis GL. 1997. Extrapolation of *in vitro* enzyme induction data to humans *in vivo*. Chem Biol Interact 107:109–121.

Kim SK, Yu SH, Lee JH, Lee JY, Rademacher A, Lee DH, Park JK. 2001. Effect of collagen concentration on the viability and metabolic function of encapsulated hepatocytes. Microbiol Biotechnol 11:423–427.

Knight CA, Duman JG. 1986. Inhibition of recrystallization of ice by insect thermal hysteresis proteins: A possible cryoprotective role. Cryobiology 23:256–262.

Knight CA, DeVries AL, Oolman LD. 1984. Fish antifreeze protein and the freezing and recrystallization of ice. Nature 308:295–296.

Laemmli UK. 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature 227:680–685.

Li AP, Gorycki PD, Hengstler JG, Kedderis GL, Koebe HG, Rahmani R, de Sousas G, Silva JM, Skett P. 1999. Present status of the application of cryopreserved hepatocytes in the evaluation of xenobiotics: Consensus of an international expert panel. Chem Biol Interact 121:117–123.

Madan A, DeHaan R, Mudra D, Carroll K, LeCluyse E, Parkinson A. 1999. Effect of cryopreservation on cytochrome P-450 enzyme induction in cultured rat hepatocytes. Drug Metab Dispos 27:327–335.

Marshall CB, Fletcher GL, Davies PL. 2004. Hyperactive antifreeze protein in a fish. Nature 429:153.

Meeks RG, Harrison SD, Bull RJ. 1991. Hepatotoxicology. Boca Raton, FL: CRC Press. 712p.

Moffatt P, Plaa GL, Denizeau F. 1996. Rat hepatocytes with elevated metallothionein expression are resistant to N-methyl-N′-nitro-N-nitrosoguanidine cytotoxicity. Toxicol Appl Pharmacol 136:200–207.

Moldeus P, Hogberg J, Orrenius S. 1978. Isolation and use of liver cells. Methods Enzymol 52:60–71.

Powis G, Santone KS, Melder DC, Thomas L, Moore DJ, Wilke TJ. 1987. Cryopreservation of rat and dog hepatocytes for studies of xenobiotic metabolism and activation. Drug Metab Dispos 15:826–832.

Reader S, Marion M, Denizeau F. 1993. Flow cytometric analysis of the effects of tri-n-butyltin chloride on cytosolic free calcium and thiol levels in isolated rainbow trout hepatocytes. Toxicology 80:117–129.

Santone KS, Melder DC, Powis G. 1989. Studies of chemical toxicity to fresh and cryopreserved rat hepatocytes. Toxicol Appl Pharmacol 97:370–376.

Schneider U, Mazur P. 1984. Osmotic consequences of cryoprotectant permeability and its relation to the survival of frozen-thawed embryos. Theriogenology 21:68–79.

Seglen PO. 1976. Preparation of isolated rat liver cells. Methods Cell Biol 13:29–83.

Silva JM, Day SH, Nicoll-Griffith DA. 1999. Induction of cytochrome-P450 in cryopreserved rat and human hepatocytes. Chem Biol Interact 121:49–63.

Son JH, Kim KH, Nam YK, Park JK, Kim SK. 2004. Optimization of cryoprotectants for cryopreservation of rat hepatocyte. Biotechnol Lett 26:829–833.

Sosef MN, Baust JM, Sugimachi K, Fowler A, Tompkins RG, Toner M. 2005. Cryopreservation of isolated primary rat hepatocytes: Enhanced survival and long-term hepatospecific function. Ann Surg 241:125–133.

Takahashi M, Ishikura H, Takahashi C, Nakajima Y, Matsushita M, Matsue H, Sato K, Sato K, Noto H, Taguchi K. 1993. Immunologic considerations in the use of cultured porcine hepatocytes as a hybrid artificial liver. Anti-porcine hepatocyte human serum. ASAIO J 39: M242–M246.

Thomashow MF. 1990. Molecular genetics of cold acclimation in higher plants. Adv Genet 28:99–131.

Uchino J, Matsue H, Takahashi M, Nakajima Y, Matsushita M, Hamada T, Hashimura E. 1991. A hybrid artificial liver system. Function of cultured monolayer pig hepatocytes in plasma from hepatic failure patients. ASAIO Trans 37:M337–M338.

Uotila M, Ruoslahti E, Engvall E. 1981. Two-site sandwich enzyme immunoassay with monoclonal antibodies to human alpha-fetoprotein. J Immunol Methods 42:11–15.

Wan X, Lachapelle M, Marion M, Fournier M, Denizeau F. 1993. Recovery potential of hepatocytes from inhibition of albumin secretion by cadmium. J Toxicol Environ Health 38:381–392.

Zaleski J, Bryla J. 1978. Effect of alloxan-diabetes on gluconeogenesis and ureogenesis in isolated rabbit liver cells. Biochem J 176:563–568.

Zaleski J, Richburg J, Kauffman FC. 1993. Preservation of the rate and profile of xenobiotic metabolism in rat hepatocytes stored in liquid nitrogen. Biochem Pharmacol 46:111–116.

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