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RESEARCH ARTICLE

Plateable cryopreserved human hepatocytes for the assessment of cytochrome P450 inducibility: experimental condition-related variables affecting their response to inducers

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Abstract

1. *Rationale*: The aim of the present study was to assess the stability of cryopreserved human hepatocytes over 5 years and to explore experimental condition-related variables such as seeding density, culture matrix and medium, start and duration of treatment that could potentially affect the quality of cultures and their response to cytochrome P450 (CYP) inducers.
2. *Results*: 63/125 batches of cryopreserved human hepatocytes were plateable after thawing. Of those, 17 batches showed reproducible recovery, viability and plateability (less than 5% intra-batch variability) up to 5 years. When cultured in collagen home-coated 48-well plates at a seeding density allowing 70% confluence, cryopreserved human hepatocytes display activities equivalent to fresh counterparts. Their response to CYP inducers is maximal and equivalent to fresh counterpart for an incubation of 72 h starting at Day 2 or Day 3 after plating when cultured in modified Hepatocyte Maintenance Medium (HMM). The number of cryopreserved human hepatocytes can be further reduced by using a cocktail of CYP substrates for the assessment of their inducibility.
3. *Conclusions*: Experimental condition-related variables, such as seeding density, culture matrix and medium, start and duration of treatment, affecting the response of plateable thawed cryopreserved human hepatocytes to cytochrome P450 inducers can be reduced by optimizing critical steps of the protocols.

Keywords: Cryopreserved human hepatocytes, optimal response to CYP induction, culture format, medium, matrix, seeding density, stability during cryopreservation

Introduction

Over the last 20 years, *in vitro* drug metabolism, kinetic and drug–drug interaction data have been used with increasing refinement to predict *in vivo* clearance and drug–drug interactions and this focus may account for improvements in late stage attrition statistics (Soars et al. 2007). Tools like hepatic microsomes, isolated hepatocytes and heterologously expressed recombinant cytochrome (CYP)s have been incorporated into automated drug discovery methodologies in order to

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generate the data required for new molecular entity optimization. Availability of good quality, fresh liver tissue has long limited human hepatocyte experiments but the progress in cryopreservation (Li et al. 1999b; Ostrowska et al. 2000; Alexandre et al. 2002; Bi et al. 2006; Kafert-Kasting et al. 2006; Hewitt et al. 2007; Stephenne et al. 2010) over the last decade has seen an explosion in the use of human hepatocytes within pharmaceutical industry and academia allowing hepatocytes to become an "off-the-shelf" reagent. Indeed, hepatocytes provide the most physiologically relevant model with which to measure qualitative and quantitative aspects of hepatic metabolism since they contain the full complement of enzymes a compound is likely to encounter during first pass metabolism. Additionally, interactions with transporter proteins present in hepatocyte membranes can be key determinants of hepatic clearance.

One of the most common use of human hepatocytes is in the prediction of metabolic clearance (CL_{int}). Recent work has shown that differences in experimental conditions can clearly confound quantitative clearance predictions and any conclusions drawn from compiling datasets from a range of laboratories (Miners et al. 2006). The systematic under-prediction of *in vivo* CL_{int} from *in vitro* CL_{int} using hepatocytes (Blanchard et al. 2005; Foster et al. 2011) could be overcome by optimizing incubation conditions such as volume, medium composition, agitation rate of hepatocytes in suspension that have been shown to strongly influence rates of drug metabolism *in vitro* (Blanchard et al. 2006; Jouin et al. 2006).

Human hepatocytes are also routinely used to assess drug-drug interactions through both reversible and time-dependent inhibition of CYPs (Zhao et al. 2005; Soars et al. 2007; Gomez-Lechon et al. 2008; 2010) as well as for uptake and drug-drug interactions involving hepatic transporters (Soars et al. 2007; Badolo et al. 2011; De Bruyn et al. 2011). Again, it has been suggested that although polymorphism largely contributes to the inter-individual variability observed in uptake rates *in vitro*, their frequency might involve other factors, such as differences in experimental conditions (Soars et al. 2007; Badolo et al. 2011). We have reported that cryopreservation does not affect drug metabolizing enzyme expression (Richert et al. 2006) and activity (Hengstler et al. 2000; Alexandre et al. 2002; Jouin et al. 2006; Abadie-Viollon et al. 2010; Swift et al. 2010) in human hepatocytes in suspension, but both passive diffusion and activity of uptake transporters have been reported to be decreased in a batch-dependent way (Badolo et al. 2011), despite their maintenance of expression at the RNA level (Richert et al. 2006) suggesting that preservation of cytoplasmic membrane structure is essential for the activity of these proteins.

Integrity of cell membrane is also required for attachment and culture of human hepatocytes, a prerequisite for the study of the regulation of drug metabolizing enzymes such as CYP and UGTs and the study of hepatobiliary transport and regulation. Culture conditions of hepatocytes such as seeding density, extracellular matrix and media can markedly affect expression and functions of these proteins (Lecluyse 2001; Richert et al. 2002; 2010; Turncliff et al. 2006; Soars et al. 2007; Swift and Brouwer 2010; Nishimura et al. 2011). This is of particular importance for the use of human hepatocytes able to attach after cryopreservation (i.e. plateable), since huge batch-batch variability in the attachment rates and capacity to make monolayers have been observed (Li et al. 1999b; Alexandre et al. 2002; Illouz et al. 2008; Richert et al. 2010). Human hepatocytes in primary culture are widely recognized as being the "gold standard" for predicting *in vivo* CYP induction (Maurel 1996; Lecluyse 2001; Richert et al. 2006; 2009; 2010; Gomez-Lechon et al. 2007; 2010; Hewitt et al. 2007; Abadie-Viollon et al. 2010). Due to the donor-donor variability in the response to inducers, it is widely accepted by regulation agencies (FDA 2006) that more than one human hepatocyte culture needs to be assessed for potential drug-drug interactions through CYP induction. There is a clear need for limiting other experimental condition-related variables possibly affecting the response to inducers by harmonization of the protocols to be used, as confirmed by our previous reports on an ECVAM-funded pre-validation study on the use of human hepatocytes to assess cytochrome P450 induction (Abadie-Viollon et al. 2010; Richert et al. 2010).

In a first follow-up study (Abadie-Viollon et al. 2010; Richert et al. 2010) we have shown that cryopreserved human hepatocytes that retain their plateability after thawing may represent an alternative to freshly isolated human hepatocytes for the ECVAM-coordinated validation study for the assessment of cytochrome P450 inducibility. Here we report our results on plateability of cryopreserved human hepatocytes batches and stability during cryopreservation time up to 5 years, the effects of seeding density, culture format and extracellular matrix on monolayer confluency. We also report experimental conditions being able to affect CYP activities in addition to inter-individual drug metabolizing enzyme variability. Finally, we describe a protocol for assessing CYP induction using human hepatocytes after cryopreservation with equivalent responses to fresh counterparts.

Materials and methods

Reagents and materials

All culture media used for the hepatocyte isolation, fetal calf serum (FCS), including Hank's balanced salt solution (HBSS), all supplements, insulin, insulin-transferrin-selenium (ITS) were purchased from Invitrogen (Fisher Scientific, France). Collagenase and all chemical compounds were purchased from Sigma-Aldrich (St Quentin Fallavier, France) were of analytical grade. Collagen and all culture material were from Becton Dickinson (Le Pont de Claix, France). The cryopreservation solution Cryostor® was obtained from Biolife Solutions (BioLife Solutions, Inc., Bothell, WA).

Human livers

Human liver tissue (n = 125), was obtained from resections from patients undergoing partial liver hepatectomy for therapy of hepatic tumors, with permission of the national ethics committees and regulatory authorities. Biopsies (20–100 g) were removed from the safety margin of the tissue resected near the tumor. Biopsies were flushed with ice-cold organ preservation solution (Viaspan® (Bristol-Myers Squibb Pharmaceuticals Ltd, Woerden, Belgium), Celsior® (Genzyme, Saint Germain en Laye, France) or SCOT 15 (MacoPharma, Mouvaux, France)) and transferred to the laboratory for hepatocyte isolation. The donor demographics were as follows: 58 caucasian males and 67 caucasian females with a mean age of 57 ± 16 and 61 ± 13 years, respectively. The pathologies of the patients were either metastasis from colorectal (40.3%) and breast origin (5.6%), hepatocellular carcinomas (6.5%), adenomas (4.0%), cholangiocarcinomas (4.0%), other pathologies (12.9%) or from unknown origin (26.6%).

Human hepatocyte isolation

Human hepatocytes were isolated as previously described (Richert et al. 2004; Lecluyse et al. 2005; Lecluyse and Alexandre 2010) by a two-step perfusion technique. Briefly, two to four hepatic vessels were cannulated and perfused with 0.5 mM EGTA-containing buffer for 10 min and then with a 0.02% collagenase-containing buffer for 20 min at 37°C. At the end of the perfusion, the biopsy was removed from the perfusion system and immersed into warm HBSS containing 10% FCS (suspension buffer). Using tissue forceps and scissors, the Glisson's capsule was gently teared open and the hepatocytes release into the medium following gentle shaking and passing the tissue between the tissue forceps, leaving behind the connective tissue and any undigested material. The cells were filtered through 850, 400 and 100 μm meshes and then centrifuged at 50 g for 5 min at room temperature (RT). Cells were washed once in suspension buffer and once in Percoll-containing buffer (final concentration 28.8%) at 168 g for 20 min at RT. The hepatocytes were washed again in suspension buffer at 50 g for 5 min at RT. Cells were counted and their viability was determined by Trypan blue (0.4%) exclusion and expressed as the percentage of viable cells over total cells. Only the preparations with more than 70% of viability were used for further experiments.

Human hepatocyte cryopreservation and thawing

Cells were cryopreserved as described by Alexandre et al. (2002) with few modifications. Briefly, the cell pellet was resuspended in ice-cold Cryostor® solution containing 10% FCS and 10% DMSO at 10 × 10^6 viable cells/mL and aliquoted into 2 mL cryotubes. Cryotubes were transferred into Mr Frosty® isopropanol containers (Nalgene) and stored overnight at −80°C (freezing rate −1°C/min) and then frozen at −150°C for long term storage.

For the cell thawing, the vials were placed in a 37°C water bath. As soon as the content was thawed (60–90 s), the hepatocyte suspension was transferred into a 50 mL centrifuge tube containing a pre-warmed Percoll-containing buffer (final concentration 28.8%) and centrifuged at 168 g for 20 min at RT. The resulting pellet was resuspended in adequate culture medium and viability was determined by Trypan blue exclusion method.

Human hepatocyte culture

Cell suspension was seeded at different cell densities (0.170, 0.226 and 0.282 × 10^6 cells/cm^2) in two different plate formats (24- and 48-well plates) in Williams'E medium containing Glutamax I (WE) supplemented with 10% FCS, insulin (4 μg/mL), dexamethasone (1 μM) and antibiotics (Penicilline/Streptomycine or Gentamycine). Cells were plated either onto Biocoat®-collagen I plates or onto home-coated collagen type I (Becton Dickinson) plates at 5 μg/cm^2 as recommended by the manufacturer. Unattached cells were removed after overnight seeding and replaced with fresh serum-free medium.

Determination of cell recovery after thawing, attachment rate and confluency

Viable cell recovery was calculated by comparing the viable cell number recovered after thawing and Percoll purification to the viable cell number originally frozen. Viable cell recovery was calculated as follows: viable cell recovery (%) = (thawed viable cell number/original viable cell number frozen) × 100 (Swales et al. 1996; Alexandre et al. 2002).

After overnight culturing (12–16 h), at the first medium change, the protein content of adherent and non-adherent cells was determined. The plating efficiency was expressed as a percentage of initially plated cells = (protein of the cell monolayer/protein of initially plated cells) × 100 and confluency was estimated both by visual observation and monolayer protein content (Alexandre et al. 2002).

Induction protocols

Induction was started at day 1, day 2 or day 3 after seeding and induction media were WE or Hepatocyte Maintenance Medium (HMM, Lonza) with or without some modifications, supplemented with ITS (1%), dexamethasone (100 nM) and antibiotics. Reference inducers used were β-naphthoflavone (βNF, 25 μM), rifampicin (RIF, 10 μM) and phenobarbital (PB, 500 or 1000 μM) for CYP1A2, 3A4/5 and 2C9, and 2B6, respectively (Abadie-Viollon et al. 2010). Control cultures were treated with the solvent DMSO (0.1% v/v final concentration). The cells were then incubated under air/CO_2 (95/5%) at 37°C for 72 h up to 120 h with every day renewal of medium containing or not reference inducers.

Determination of CYP1A2, 3A4/5, 2B6 and 2C9 activities

Cell monolayers were directly incubated either with single substrates as previously described by Abadie-Viollon et al. (2010) or with a cocktail of substrates as

described by Kanebratt et al. (2008) with some modifications. Monolayers were washed with warm phosphate buffered saline (PBS) during 15 min at 37°C, and then incubated for 30–45 min, depending on the substrate, at 37°C under air/CO2 (95/5%) in DMEM with Glutamax I supplemented with insulin (4 µg/mL), dexamethasone (1 µM) and antibiotics, in presence or absence of salicylamide (1 mM), and containing the substrates in single or as a cocktail: phenacetin or ethoxyresorufin 10 µM, midazolam 3 µM or testosterone 100 µM, bupropion 100 or 500 µM and diclofenac 10 µM or tolbutamide 100 µM for CYP1A2, 3A4/5, 2B6 and 2C9, respectively. At the end of the incubation period, supernatants were collected and stored at –80°C until analysis.

Protein determination

The hepatocyte monolayer was dissolved in 0.1 M sodium hydroxide at RT and the protein content was determined in each well using a Pierce BCA (bicinchoninic acid) Protein Assay Kit (Sigma) using bovine serum albumin as a standard.

Analytical methods

The formation of resorufin (CYP1A2) was determined by spectrofluorimetry Ex 530/Em 580 nm (Burke et al. 1985). Before the introduction of the cocktail, testosterone-6β-hydroxylation (CYP3A4/5), tolbutamide-hydroxylation (CYP2C9) and bupropion-hydroxylation (CYP2B6) activities were determined by HPLC according to previously described methods (Faucette et al. 2000; Richert et al. 2002). Analysis of CYP activities after incubating the monolayers with the cocktail of substrates, acetaminophen (CYP1A2), midazolam 1′-hydroxylation (CYP3A4/5), diclofenac 4-hydroxylation (CYP2C9) and bupropion-hydroxylation (CYP2B6) activities were determined by LC-MS/MS. Briefly, LC-MS/MS analyses were performed with a API2000 triple quadrupole (Applied Biosystems/Sciex) coupled to Agilent 1200 HPLC system consisting of degasser G1379B, binary pump G1312A, and a well-plate sampler G1367B equipped with a 100 µl syringe. The software Analyst 1.4.2 (which controls the LC system and the mass spectrometer), including IntelliQuan (quantification) and a Gemini C18, (3 µm, 2.1 × 30 mm, Phenomenex, Torrance, CA) analytical column were used. Chromatography was performed using a generic gradient at a flow rate of 0.7 ml min^{-1}. The mobile phases consisted of A: 10 mM ammonium acetate supplemented with a total content of 0.1% acetic acid (v/v) and B: acetonitrile supplemented with a total content of 0.1% acetic acid (v/v). The gradient conditions were as follows: 0–1.0 min 0% B, 1.0–2.0 min 0–95% B, 2.0–3.0 min 95% B, 3.0–3.1 min 95–0% B, 3.1–7.0 min 0% B. The total time between injections was 1 min. Electrospray ionization in positive mode (ESI+) with multiple reaction monitoring (MRM) using the following conditions: curtain gas, 28.00; CAD gas, 11.00; GS1, 40; GS2, 70; ion spray, 5000 eV; temperature, 500°C; EP, 8.50 eV. The mass spectrometer was operated under open resolution (Q1 and Q3). LC–MS/MS MRM conditions for the individual probe metabolites and associated internal standards were monitored as follows: acetaminophen m/z 152→110, collision energy 23 eV; 4-OH-diclofenac m/z 311.9→229.9, collision energy 43 eV; 1′-OH-midazolam m/z 342.0→203.0 and 342.0→323.7, collision energy 21 eV; OH-bupropion m/z 256.0→238.0 and 256.0→139.0, collision energy 21 eV; and DDIBA m/z 336.0→91.9, collision energy 63 eV. The dwell time for each transition was 50 ms.

Statistical analysis

Results were expressed as individual data or as mean ± SD or SEM. Differences among means were assessed using computer-aided ANOVA paired or unpaired t-test (Statview® 4.5 software, Abacus Concept, Berkeley). Values of $p < 0.05$ were considered significant.

Results and discussion

Plateability of cryopreserved human hepatocytes

Plateability after thawing of cryopreserved human hepatocytes was assessed by measuring the confluency of cell monolayers 24 h after seeding (Biocoat® 24wp at 0.226 or 0.282 × 10^6 cells/cm^2), with a given batch being considered plateable when over 70% confluency was attained at first thawing. We found that 63 of the 125 batches cryopreserved over a 5-year period were plateable. These results further confirm our previous results (Alexandre et al. 2002) with a restricted number of 28 batches for which we obtained a 50% attachment rate. Several authors have also reported that a maximum of half of the lots yielded over 50% confluent monolayer cultures when plated onto collagen coated plates. (Loretz et al. 1989; Ruegg et al. 1997; Li 2007).

Stability during cryopreservation time of human hepatocytes was assessed with 17 cryopreserved human hepatocyte batches over a 5-year period following their freezing. Cell viability, recovery as well as plateability after overnight seeding (Biocoat® 24wp at 0.226 or 0.282 × 10^6 cells/cm^2) were determined (Figure 1). The mean

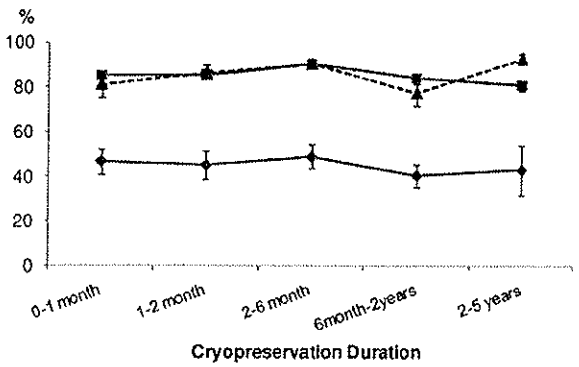

Figure 1. Effect of long term cryopreservation (up to 5 years), of 17 batches of cryopreserved human hepatocytes, on cell viability (■) and recovery (◆) after thawing, and plateability (▲) after overnight seeding in WE medium (Biocoat® 24-well plates at 0.226 or 0.282 × 10^6 cells/cm^2). Results are expressed as mean percentages ± SEM.

recovery of viable cell number per batch after thawing was 44.9 ± 16.2% and the intra-batch variability over 5 years was 3.8%. The mean viability of batches was 85.4 ± 7.0% and intra-batch variability over 5 years was 0.4%. The mean plateability of batches was 83.7 ± 11.5% and intra-batch variability over 5 years was 4.4%. Two groups have previously reported for a single donor that yield and viability remained stable, respectively up to 120 days (Li et al. 1999b) and 1 year (McGinnity et al. 2004), and another group (Terry et al. 2010) reported a stability over 3 years for 5 donors, but the present study is, to our knowledge, the first report on between-batch reproducible stability of recovery, viability and plateability up to 5 years of cryopreservation on a large panel of cryopreserved human hepatocytes.

Plating efficiency onto collagen matrix. It has long been understood that plating hepatocytes onto collagen was superior to other liver-specific extracellular matrix proteins (Bissell et al. 1986). Seeding onto thin-layer collagen home-coated plates or onto commercially available collagen-coated Biocoat® plates was compared using 48-well plates. Figure 2 shows an example of the morphology of the cell monolayer 24 h after plating (A and C) and after 5 days of culture (B and D). Monolayer appeared visually more confluent after 24 h seeding in home-coated (C and D) versus Biocoat® (A and B) plates, a result further confirmed by the cell monolayer protein content significantly higher ($p < 0.05$) in home-coated (0.072 ± 0.006 mg/cm²) vs. Biocoat plates (0.055 ± 0.004 mg/cm²). After 5 days of culture, less debris were seen on top of human hepatocyte monolayers cultured on home-coated plates (D) compared to Biocoat® plates (B), and the typical shape and hepatocyte-hepatocyte type of contacts more apparent in the former cultures. Thus home-coated plates appear superior for allowing cryopreserved human hepatocytes to form a high quality monolayer.

Seeding density and Plate format. As cell density has been shown to influence morphology of human hepatocytes in culture and is important for cell-cell contact (Hamilton et al. 2001; Richert et al. 2010), the confluency of 14 plateable cryopreserved human hepatocyte batches was tested using 24- and 48-well home coated plates at three different cells densities (0.170, 0.226 and 0.282 × 10^6 cells/cm²). After 24 h of culture, monolayer protein content (mg/cm²) was measured and confluency was also visually asssessed (%) (Figure 3). For a given cell seeding density there was no significant difference in the monolayer protein content and visual confluency between the two plate formats. The monolayer protein content and visual confluency overall increased as a function of cell seeding density, although only visual confluency was

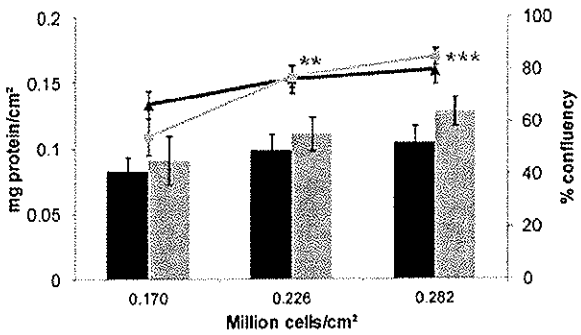

Figure 3. Plating efficiency after overnight plating in WE medium of 14 batches of cryopreserved/thawed human hepatocytes seeded in 24-well and 48-well home coated plates at different cell densities. Protein content (in bars) in 24-well plates (■) and in 48-well plates (▩) is expressed in mg/cm² as the mean ± SEM. The confluency of the monolayer (in lines) in 24-well plates (▲) and in 48-well plates (◉) is expressed in percentage as the mean ± SEM. **$p < 0.01$, different from 0.170 million cells/cm² in 48-well plates. ***$p < 0.001$, different from 0.170 million cells/cm² in 48-well plates.

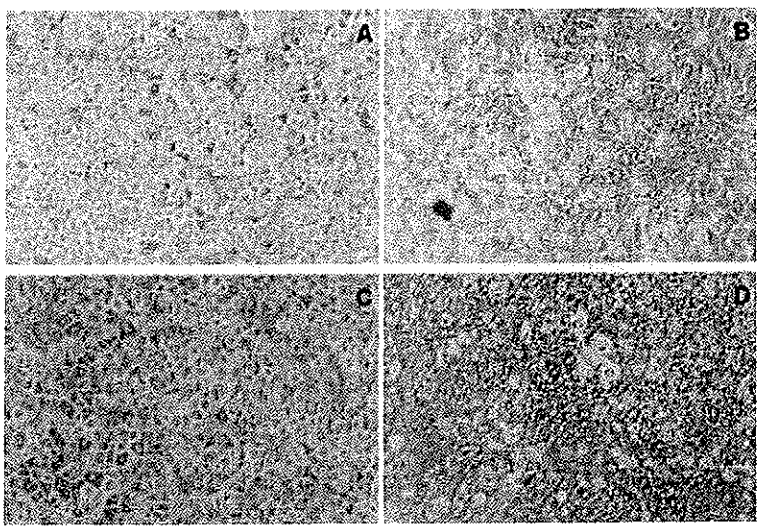

Figure 2. Phase contrast micrographs of cryopreserved human hepatocytes after thawing. Cells were plated onto 48-well Biocoat plates (A, B) or onto 48-well home-coated plates (C, D) at a seeding density of 0.282 × 10^6 cells/cm² and cultured for 24 h (A, C) and 5 days (B, D) in WE medium.

statistically significantly lower in 48-well plates at the lowest seeding density tested. Thus refinement of optimal seeding density for a given batch can be helpful with regard to saving these precious cells.

Variability in CYP activities

Seeding density and Plate format. CYP3A4/5 and CYP2B6 activities were lower at suboptimal seeding density leading to <70% confluency. Lecluyse (2001) also reported that at suboptimal confluency, not only significant alterations in the normal morphology and integrity of the monolayer were observed, even in cultures at 50% normal plating density, but this was accompanied by decreases in CYP expression. It is noteworthy however that whenever 70% confluency of the monolayer was reached, activities were equivalent, further suggesting some flexibility in the seeding density to be used. This was also evidenced by the results of the 14 batches of thawed human hepatocytes seeded at 0.226 and 0.282 million cells/cm² in 24- and 48-well plates: indeed, Figure 4 depicts that there was no significative difference in CYP1A2 (Figure 4A) and CYP3A4/5 (Figure 4B) activities 24 h after plating between the two plate formats and the two seeding densities which allowed confluency ranging from 70 to 100%. It has been previously suggested that quality and function of cells in confluent monolayer is independent of culture format (Zhang et al. 2005; Turncliff et al. 2006; Abadie-Viollon et al. 2010). The crucial importance of confluency of human hepatocytes in culture has been specially emphasized in the context of optimal response to CYP expression (Lecluyse 2001; Richert et al. 2010), CYP induction (Hamilton et al. 2001; Lecluyse 2001; Abadie-Viollon et al. 2010) as well as Mrp2 and Mdr1a/b transporter expression and/or function (Turncliff et al. 2006).

Inter-individual variability in CYP activities. It is well accepted that large differences in drug-metabolizing enzymes are evident in cultured human hepatocytes from various donors and appear to reflect the heterogeneity in the human liver (Gomez-Lechon et al. 2008). Given the great inter-individual variability of P450 patterns in humans, investigation in hepatocytes from more than one donor is necessary for the prediction of drug metabolic profiles, drug-drug interactions, and toxicity of new drugs in the human population (Lecluyse 2001; Aueviriyavit et al. 2007; Richert et al. 2010). A major advantage of cryopreserved hepatocytes is that the experiment can be repeated using the same conditions with the result that the data is reproducible (Hewitt et al. 2007). When CYP1A2 (n = 36) (Figure 5A) and CYP3A4/5 (n = 37) (Figure 5B) activities of hepatocytes after cryopreservation were compared to their freshly isolated counterparts, 24 h after seeding, we found indeed that

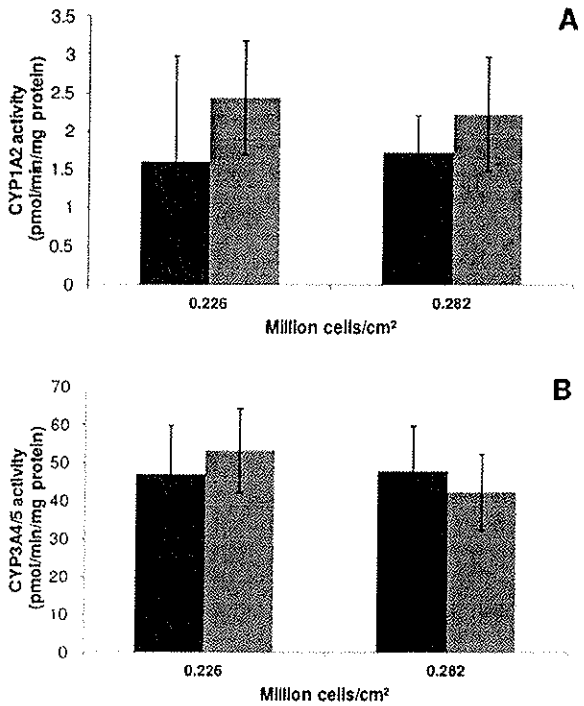

Figure 4. CYP1A2 (A) and CYP3A4/5 (B) basal activities of 14 batches of cryopreserved/thawed human hepatocytes after thawing. Cells were seeded at two different cell densities in 24-(■) and 48-well plates (▩) in WE medium onto home coated plates. Visual confluency ranged from 70 to 100% in all groups. Results are expressed as mean activities (pmol/min/mg protein) ± SD.

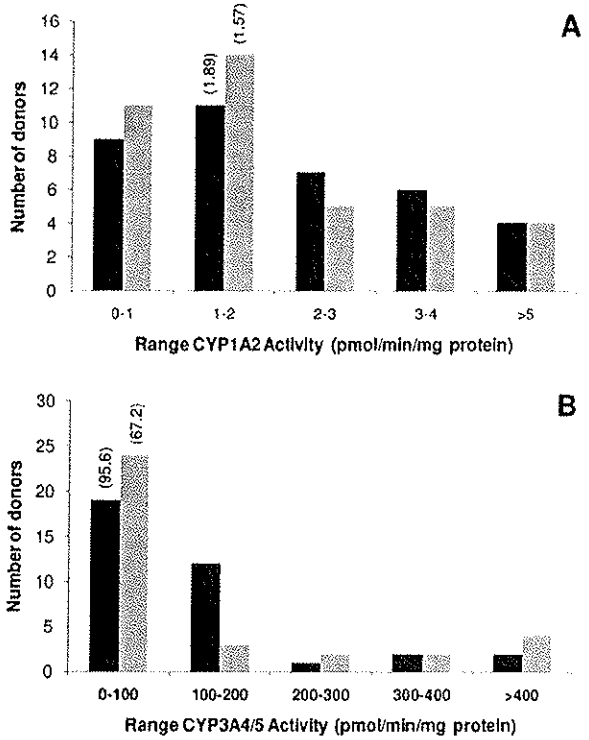

Figure 5. Distribution of CYP1A2 (A) (n = 36) and CYP3A4/5 (B) (n = 37) activities in freshly isolated (■) and their counterpart cryopreserved/thawed (▩) human hepatocytes, seeded onto 24/48-well plates, after 24 h in culture in WE medium. Results are expressed in pmol/min/mg protein. Values in brackets represent the median of the activities.

median values (pmol/min/mg cell protein) were equivalent, respectively 1.57 vs. 1.89 (p = 0.8473) for CYP1A2 and 67.2 vs. 95.6 (p = 0.5236) for CYP3A4/5. The donor-to-donor variability can result from both genetic make up of the donor, its medication, operative procedure and even the underlying disease process (Alexandre et al. 2002; Lloyd et al. 2003). As pointed out by Roymans et al. 2005, the high variability in basal CYP activity seen in cryopreserved hepatocytes (see also Figure 7), as in fresh hepatocytes or *in vivo* is not disadvantageous since the population of the hepatocytes can be carefully selected by function of the experimental aim.

Single substrates versus cocktail of substrates were compared for the determination of CYP activities in 48 h cultures of cryopreserved human hepatocytes. Various probe cocktails have been successfully proposed for the *in vitro* assessment of CYP activities (Kanebratt et al. 2008; Lahoz et al. 2008a,b). Cultured cells were directly incubated with a cocktail of substrates. The corresponding metabolites formed and released into the incubation medium are further quantified by a direct injection of an aliquot of cell supernatants by using mass spectrometry. This procedure markedly reduces the number of hepatocytes needed for each CYP450 activity measurement, a fact that is particularly critical concerning human hepatocytes. The results from substrate incubations in a cocktail of phenacetin (CYP1A2), bupropion (CYP2B6), midazolam (CYP3A4) and diclofenac (CYP2C9), at the same concentration and incubation time than as single incubations provided equivalent CYP1A2 activities, CYP2B6 was decreased in 2/3 and equivalent in 1/3 donors, CYP3A4/5 was equivalent in 2/3 and increased in 1/3 donors and CYP2C9 was decreased in 2/3 and equivalent in 1/3 donors (Figure 6). Some drawbacks have indeed been addressed for incubations with cocktail mixtures, such as potential substrate interactions and the inhibition of a particular CYP activity by other substrates present during incubation (Palmer et al. 2001; Lahoz et al. 2008b).

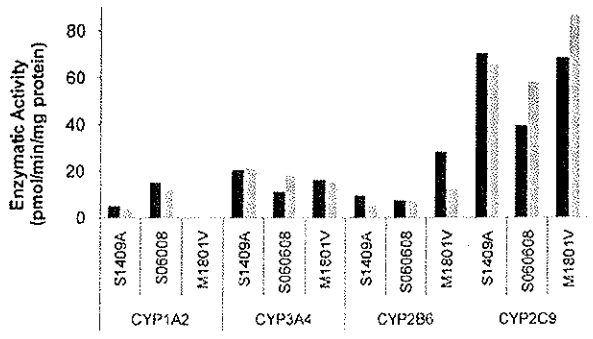

Figure 6. CYP activities in cryopreserved/thawed human hepatocytes (batches S1409A, S060608 and M1801V) after 24 h of culture. Cells were seeded onto home-coated plates at a density of 0.282×10^6 cells/cm^2 in WE medium. Monolayers were incubated either with single substrates (■) or with a cocktail of substrates(▩). Results are expressed as pmol/min/mg protein.

CYP inducibility

Inter-individual variability in the response to reference inducers is shown in Figure 7. Control activities of CYP1A2 in 32 donors ranged from 0.029 to 9.35 and induced activities by βNF ranged from 0.866 to 83.4 pmol/min/mg protein (Figure 7A), fold over control ranged 5–150 (Figure 7B). Control activities of CYP3A4/5 in 32 donors ranged from 0.417 to 39.1 and induced activities by RIF ranged from 2.70 to 175 pmol/min/mg protein (Figure 7C), fold over control ranged 2–60 (Figure 7D). Control activities of CYP2B6 in 20 donors ranged from 0.151 to 31.9 and induced activities by PB ranged from 0.313 to 183 pmol/min/mg protein (Figure 7E) with fold over control ranged 2–13 (Figure 7F). These results clearly confirm and extent previous observations that plateable cryopreserved human hepatocytes are inducible as are freshly isolated cells (Li et al. 1999a,b; Lu and Li 2001; Roymans et al. 2005; Hewitt et al. 2007; Abadie-Viollon et al. 2010), and that the donor-to-donor variation in fold induction is independent on basal CYP activity as we previously reported (Abadie-Viollon et al. 2010). The variability can again result from both genetic make up of the donor, its medication, operative procedure and even the underlying disease process (Alexandre et al. 2002; Lloyd et al. 2003; Richert et al. 2010).

Substrate incubations. Incubation of monolayers with either single substrates or using cocktail strategy was compared for the evaluation of CYP inducibility by specific inducers (βNF for CYP1A2, RIF for CYP3A4/5 and CYP2C9, and PB for CYP2B6). Figure 8 shows that fold over controls were equivalent when using or not the cocktail strategy. Consequently the between-substrate interferences observed that accounted for differences in activities when using cocktail vs. individual substrates does not hamper the evaluation of fold induction by a given inducer. Lyon et al. (2010) also concluded that a cocktail of substrates allows assessment of the inducing potential of drug candidates with limiting the number of plateable cryopreserved human hepatocytes to be used.

It has been reported that sensitivity of LC-MS/MS can be rate-limiting in the detection of the metabolite acetaminophen formed by CYP1A2 conversion of phenacetin. Therefore we compared CYP1A2 activity when incubating the monolayer of a given culture with ethoxyresorufin only to CYP1A2 activity when monolayer was incubated first with the cocktail without phenacetin, and thereafter with the single substrate ethoxyresorufin. Not only equivalent control activities i.e. 0.418 and 0.611 pmol/min/mg protein, respectively but also similar response to βNF 25 μM induction, i.e. 47-fold and 66-fold were obtained. Donato et al. (1993) have reported that hepatocyte monolayers can be successfully successively incubated with substrates for enzyme activity measurements.

Induction media. The effect of two different induction media, HMM and WE, were evaluated for the CYP3A4/5 inducibility following a 72 h induction period with RIF 10 μM. The results, depicted in Figure 9, show a higher fold

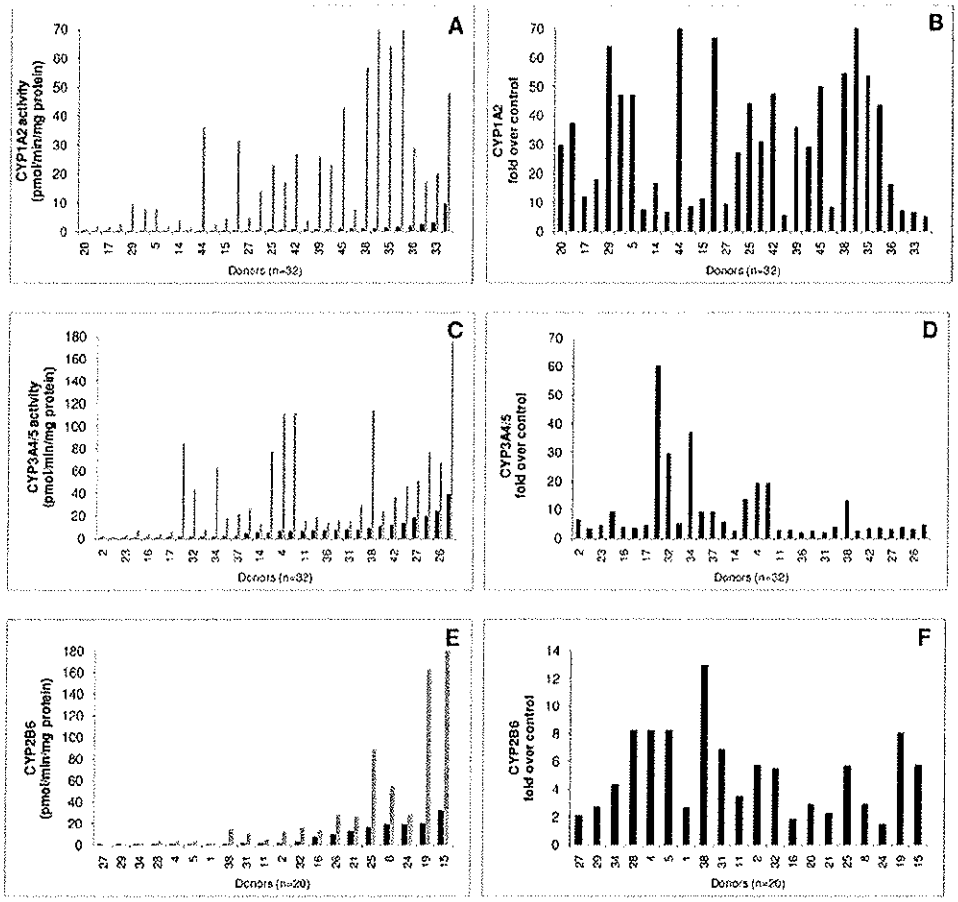

Figure 7. Interindividual variability of cytochrome P450 activities (pmol/min/mg protein) in cryopreserved/thawed human hepatocyte cultures from different donors after a 72 h period of induction (▩) in HMM medium with specific inducers. CYP activities were plotted and sorted in an increasing order of controls (■). A) CYP1A2 activity (ethoxyresorufin O-deethylation), C) CYP3A4/5 activity (testosterone 6β-hydroxylation), E) CYP2B6 (bupropion-hydroxylation). Figures B), D), and F) represent the fold over control.

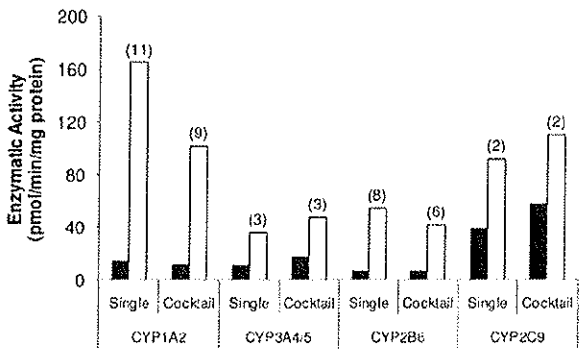

Figure 8. CYP activities measured with single substrates or using the cocktail strategy. Cells from cryopreserved/thawed human hepatocytes (batch S060608) were seeded onto home-coated plates at a density of 0.282 × 10^6 cells/cm^2 in WE medium. After a 2-day culture period in HMM medium, cell monolayers were cultured during 72 h in the absence (■) or in the presence of βNF (25 μM), RIF (10 μM) or PB (500 μM) (□). After the induction period in HMM medium, monolayers were incubated with single substrates or a cocktail of substrates as described in M & M. Results are expressed as pmol/min/mg protein with fold over control represented in brackets.

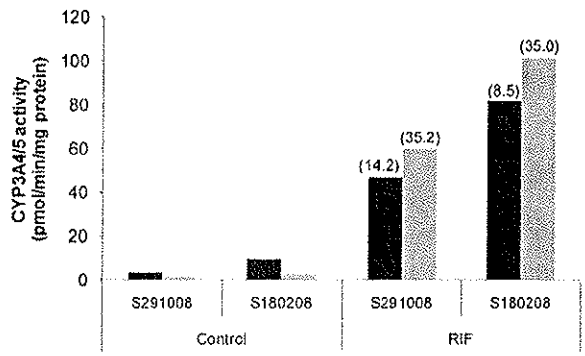

Figure 9. Effect of two different induction media (HMM ■ and WE ▩) on CYP3A4/5 activity of cryopreserved/thawed human hepatocytes seeded onto Biocoat® 24-well plates at 0.282 × 10^6 cells/cm^2. At day 2 of culture cells were induced for 72 h with RIF 10 μM. Activities are expressed as pmol/min/mg protein with fold over control represented in brackets.

over control in the two cryopreserved/thawed human hepatocyte batches cultured in WE compared to HMM induction medium (batch S291008: 35.2 vs. 14.2, batch

S180208: 35.0 vs. 8.5, respectively). It is noteworthy that the "induced" activities in the two media were not very different, confirming previous observations that basal activities are medium-dependent (Schuetz et al. 1993; Lecluyse 2001). However, the higher control activities observed in HMM compared to the probably less rich WE medium could be of interest during analysis to avoid control values being under the Lowest Limit Of Quantification.

Duration of induction. The activities of CYP1A2, 2B6, 3A4/5 and 2C9 were assessed in one batch of cryopreserved human hepatocytes following, respectively a 72 h or a 120 h induction period with the specific inducers βNF (25 μM), PB (1000 μM) and RIF (10 μM). Fold inductions were, respectively 9 vs. 11 for CYP1A2, 25 vs. 18 for CYP3A4/5, 5 vs. 4 for CYP2B6 and 2 vs. 2 for CYP2C9. These results suggest that a 72 h induction duration is sufficient for the assessment of reponse of CYP-dependent activities to inducers, a result in accordance with results obtained previously by us (Abadie-Viollon et al. 2010) and others (Maurel 1996; Donato et al. 1999).

Start of induction. Induction was started 24 h (Day 1) and 48 h (Day 2) after seeding of thawed human hepatocytes. The activities and the fold over control of CYP1A2, 2B6 and 3A4/5 were determined following a 72 h induction period with specific inducers (i.e. at Day 4 and Day 5 of culture). As shown in Figure 10, all CYP activities decreased with time in culture for HHC170407 while were equivalent for N2309VT at the two culture times. It has been previously reported that CYP activities in freshly isolated and plateable cryopreserved

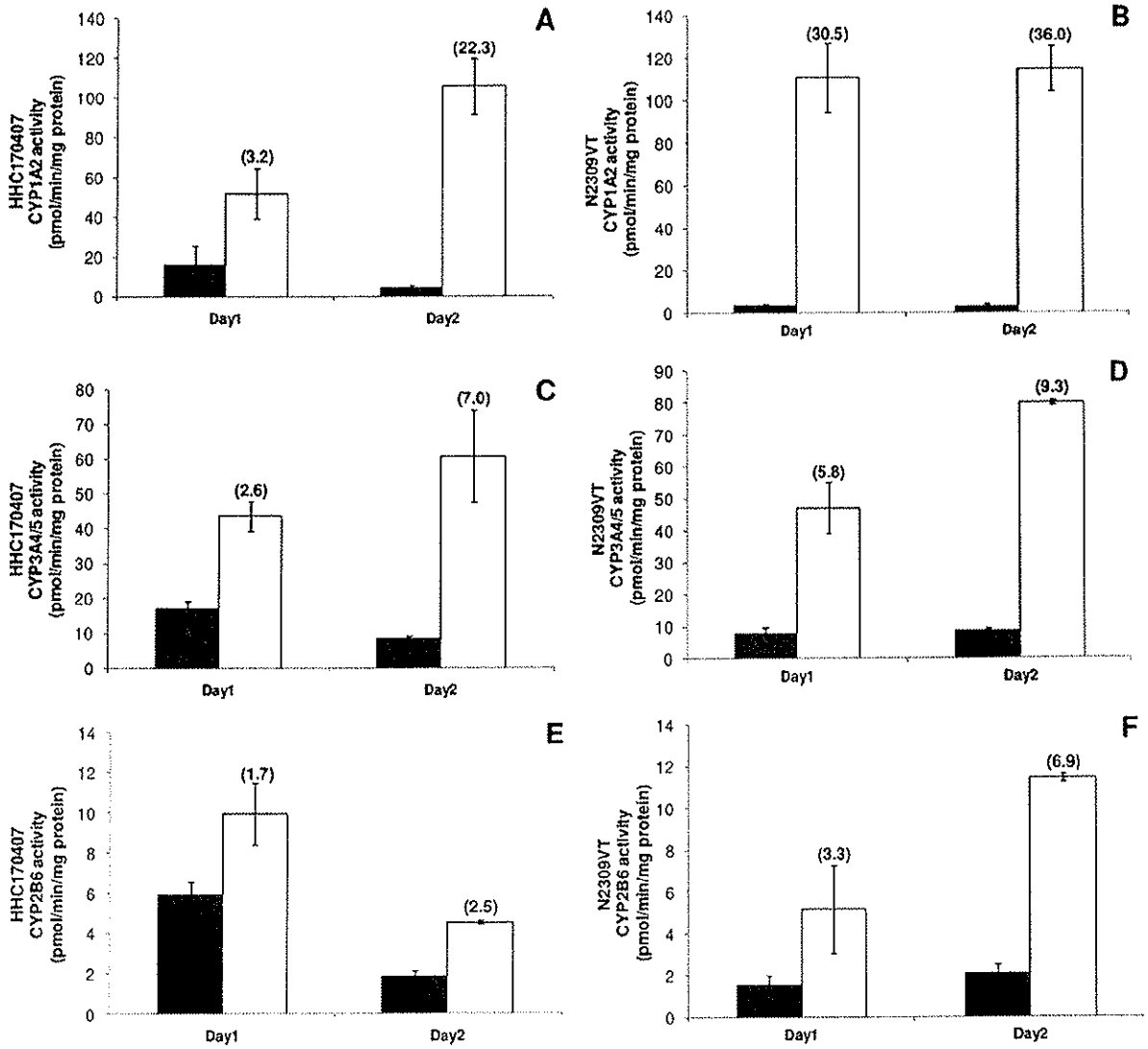

Figure 10. Effect of start of induction (Day 1 or Day 2 after plating) on CYP activities (control (■) and induced (□)) in cryopreserved/thawed human hepatocytes (batch HHC170407 (A, C, E) and batch N2309VT (B, D, F)). Cells were seeded onto home-coated plates at 0.282×10^6 cells/cm^2 in WE medium. At day 2 of culture cells were induced for 72 h in HMM medium with specific inducers. Results are expressed as pmol/min/mg protein ± SD with the fold over control in brackets.

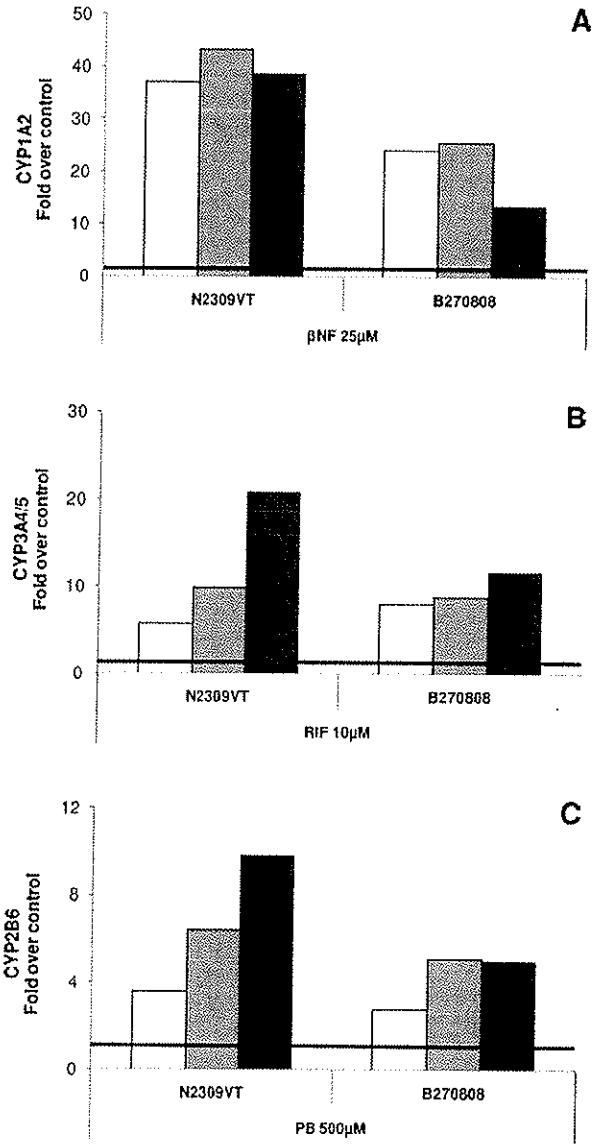

Figure 11. Fold induction of CYP1A2 (A), CYP3A4/5 (B), and CYP2B6 (C) in cryopreserved/thawed human hepatocytes (batch N2309VT and batch B270808) with start of 72 h induction period at Day 1 (□), Day 2 (▩) and Day 3 (■) after seeding. Induction medium was HMM in the two former and modified HMM in the latter case. Results are expressed as fold over control.

human hepatocytes drop during the first days in culture (Lecluyse 2001; Abadie-Viollon et al. 2010; Richert et al. 2010). Our results suggest a donor-donor variability in the evolution of basal CYP activities during time in culture. Fold inductions were higher in these two cultures when the induction was started at Day 2. When comparing N2309VT to its fresh counterpart it appeared that control activities were lower after cryopreservation, being respectively 3.3 and 2.5 vs. 13.7 and 6.2 for CYP1A1/2-, 9.3 and 8.1 vs. 14.6 and 11 for CYP3A4/5-, 1.9 and 1.8 vs. 3.9 and 2.4 for CYP2B6-dependent activities (pmol/min/mg cell protein). It has been previoulsy shown that activities in cultured plateable cryopreserved/thawed human hepatocytes are lower compared to the native freshly isolated (Hewitt et al. 2001; Kafert-Kasting et al. 2006). Interestingly, CYP activities reached in induced cells were equivalent in freshly isolated cells and after cryopreservation when induction was started at Day 2 (respectively 106 vs. 107 for CYP1A2, 90 vs. 79 for CYP3A4/5 and 2.4 vs. 1.8 for CYP2B6). Equivalence in the response to inducers of human hepatocytes freshly isolated and after cryopreservation has been reported (Silva et al. 1999; Abadie-Viollon et al. 2010). Although in our previous study, we found comparable induction of CYP1A2, CYP2C9 and CYP3A4/5, as measured by mRNA expression or CYP activities, using freshly isolated human hepatocytes pre-cultured for 24 h and 48 h prior to addition of the respective inducers (Abadie-Viollon et al. 2010), this was not the case for N2309VT, suggesting that for both freshly isolated and thawed human hepatocytes longer pre-culture time cells, allowing stabilisation of basal CYP activities, give an optimal response to CYP inducers, as also suggested by Dou et al. (1992). Figure 11 shows that when using a modified HMM medium, thawed human hepatocytes can be induced for 72 h from Day 3 with equivalent or higher fold induction of CYPs by specific inducers than when starting induction at Day 2. A similar protocol has also been recently described by (Kamiguchi et al. 2010).

Conclusion

In conclusion, the present work shows stability in recovery, viability and plateability of cryopreserved human hepatocytes during cryopreservation time up to 5 years. In addition to inter-individual related variables, experimental condition-related variables, such as seeding density, culture matrix and medium, start and duration of treatment, affect the response of plateable thawed cryopreserved human hepatocytes to cytochrome P450 inducers and that they can be reduced by optimizing critical steps of the protocols. We report that when cultured in collagen home-coated 48-well plates at a density allowing 80% confluence, cryopreserved human hepatocytes display activities equivalent to fresh counterparts. Their response to inducers is maximal and equivalent to fresh counterpart for an incubation of 72 h starting at Day 2 or Day 3 after plating when cultured in modified HMM. A cocktail of substrates can be used to assess the induction potential, further allowing to reduce the number of cells to be used.

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Declaration of interest

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References

Abadie-Viollon C, Martin H, Blanchard N, Pekthong D, Bachellier P, Mantion G, Heyd B, Schuler F, Coassolo P, Alexandre E, Richert L. (2010). Follow-up to the pre-validation of a harmonised protocol for assessment of CYP induction responses in freshly isolated and cryopreserved human hepatocytes with respect to culture format, treatment, positive reference inducers and incubation conditions. Toxicol *In Vitro* 24:346-356.

Alexandre E, Viollon-Abadie C, David P, Gandillet A, Coassolo P, Heyd B, Mantion G, Wolf P, Bachellier P, Jaeck D, Richert L. (2002). Cryopreservation of adult human hepatocytes obtained from resected liver biopsies. Cryobiology 44:103-113.

Aueviriyavit S, Furihata T, Morimoto K, Kobayashi K, Chiba K. (2007). Hepatocyte nuclear factor 1 alpha and 4 alpha are factors involved in interindividual variability in the expression of UGT1A6 and UGT1A9 but not UGT1A1, UGT1A3 and UGT1A4 mRNA in human livers. Drug Metab Pharmacokinet 22:391-398.

Badolo L, Trancart MM, Gustavsson L, Chesné C. (2011). Effect of cryopreservation on the activity of OATP1B1/3 and OCT1 in isolated human hepatocytes. Chem Biol Interact 190:165-170.

Bi YA, Kazolias D, Duignan DB. (2006). Use of cryopreserved human hepatocytes in sandwich culture to measure hepatobiliary transport. Drug Metab Dispos 34:1658-1665.

Bissell DM, Stamatoglou SC, Nermut MV, Hughes RC. (1986). Interactions of rat hepatocytes with type IV collagen, fibronectin and laminin matrices. Distinct matrix-controlled modes of attachment and spreading. Eur J Cell Biol 40:72-78.

Blanchard N, Alexandre E, Abadie C, Lavé T, Heyd B, Mantion G, Jaeck D, Richert L, Coassolo P. (2005). Comparison of clearance predictions using primary cultures and suspensions of human hepatocytes. Xenobiotica 35:1-15.

Blanchard N, Hewitt NJ, Silber P, Jones H, Coassolo P, Lavé T. (2006). Prediction of hepatic clearance using cryopreserved human hepatocytes: a comparison of serum and serum-free incubations. J Pharm Pharmacol 58:633-641.

Burke MD, Thompson S, Elcombe CR, Halpert J, Haaparanta T, Mayer RT. (1985). Ethoxy-, pentoxy- and benzyloxyphenoxazones and homologues: a series of substrates to distinguish between different induced cytochromes P-450. Biochem Pharmacol 34:3337-3345.

De Bruyn T, Ye ZW, Peeters A, Sahi J, Baes M, Augustijns PF, Annaert PP. (2011). Determination of OATP-, NTCP- and OCT-mediated substrate uptake activities in individual and pooled batches of cryopreserved human hepatocytes. Eur J Pharm Sci 43:297-307.

Donato MT, Castell JV, Gómez-Lechón MJ. (1999). Characterization of drug metabolizing activities in pig hepatocytes for use in bioartificial liver devices: comparison with other hepatic cellular models. J Hepatol 31:542-549.

Donato MT, Gómez-Lechón MJ, Castell JV. (1993). A microassay for measuring cytochrome P450IA1 and P450IIB1 activities in intact human and rat hepatocytes cultured on 96-well plates. Anal Biochem 213:29-33.

Dou M, de Sousa G, Lacarelle B, Placidi M, Lechene de la Porte P, Domingo M, Lafont H, Rahmani R. (1992). Thawed human hepatocytes in primary culture. Cryobiology 29:454-469.

Faucette SR, Hawke RL, Lecluyse EL, Shord SS, Yan B, Laethem RM, Lindley CM. (2000). Validation of bupropion hydroxylation as a selective marker of human cytochrome P450 2B6 catalytic activity. Drug Metab Dispos 28:1222-1230.

FDA. (2006). Guidance for Industry. Drug Interaction Studies - Study Design, Data Analysis, and Implications for Dosing and Labeling (Draft Guidance), U.S. Department of Health and Human Services, Food and Drug Administration.

Foster JA, Houston JB, Hallifax D. (2011). Comparison of intrinsic clearances in human liver microsomes and suspended hepatocytes from the same donor livers: clearance-dependent relationship and implications for prediction of *in vivo* clearance. Xenobiotica 41:124-136.

Gómez-Lechón MJ, Castell JV, Donato MT. (2007). Hepatocytes-the choice to investigate drug metabolism and toxicity in man: *in vitro* variability as a reflection of in vivo. Chem Biol Interact 168:30-50.

Gómez-Lechón MJ, Castell JV, Donato MT. (2008). An update on metabolism studies using human hepatocytes in primary culture. Expert Opin Drug Metab Toxicol 4:837-854.

Gómez-Lechón MJ, Castell JV, Donato MT. (2010). The use of hepatocytes to investigate drug toxicity. Methods Mol Biol 640:389-415.

Hamilton GA, Jolley SL, Gilbert D, Coon DJ, Barros S, LeCluyse EL. (2001). Regulation of cell morphology and cytochrome P450 expression in human hepatocytes by extracellular matrix and cell-cell interactions. Cell Tissue Res 306:85-99.

Hengstler JG, Utesch D, Steinberg P, Platt KL, Diener B, Ringel M, Swales N, Fischer T, Biefang K, Gerl M, Böttger T, Oesch F. (2000). Cryopreserved primary hepatocytes as a constantly available *in vitro* model for the evaluation of human and animal drug metabolism and enzyme induction. Drug Metab Rev 32:81-118.

Hewitt NJ, Bühring KU, Dasenbrock J, Haunschild J, Ladstetter B, Utesch D. (2001). Studies comparing in vivo:in vitro metabolism of three pharmaceutical compounds in rat, dog, monkey, and human using cryopreserved hepatocytes, microsomes, and collagen gel immobilized hepatocyte cultures. Drug Metab Dispos 29:1042-1050.

Hewitt NJ, Lechón MJ, Houston JB, Hallifax D, Brown HS, Maurel P, Kenna JG, Gustavsson L, Lohmann C, Skonberg C, Guillouzo A, Tuschl G, Li AP, LeCluyse E, Groothuis GM, Hengstler JG. (2007). Primary hepatocytes: current understanding of the regulation of metabolic enzymes and transporter proteins, and pharmaceutical practice for the use of hepatocytes in metabolism, enzyme induction, transporter, clearance, and hepatotoxicity studies. Drug Metab Rev 39:159-234.

Illouz S, Alexandre E, Pattenden C, Mark L, Bachellier P, Webb M, Berry D, Dennison A, Richert L. (2008). Differential effects of curcumin on cryopreserved versus fresh primary human hepatocytes. Phytother Res 22:1688-1691.

Jouin D, Blanchard N, Alexandre E, Delobel F, David-Pierson P, Lavé T, Jaeck D, Richert L, Coassolo P. (2006). Cryopreserved human hepatocytes in suspension are a convenient high throughput tool for the prediction of metabolic clearance. Eur J Pharm Biopharm 63:347-355.

Kafert-Kasting S, Alexandrova K, Barthold M, Laube B, Friedrich G, Arseniev L, Hengstler JG. (2006). Enzyme induction in cryopreserved human hepatocyte cultures. Toxicology 220:117-125.

Kamiguchi N, Aoyama E, Okuda T, Moriwaki T. (2010). A 96-well plate assay for CYP4503A induction using cryopreserved human hepatocytes. Drug Metab Dispos 38:1912-1916.

Kanebratt KP, Diczfalusy U, Bäckström T, Sparve E, Bredberg E, Böttiger Y, Andersson TB, Bertilsson L. (2008). Cytochrome P450 induction by rifampicin in healthy subjects: determination using the Karolinska cocktail and the endogenous CYP3A4 marker 4beta-hydroxycholesterol. Clin Pharmacol Ther 84: 589-594.

Lahoz A, Donato MT, Montero S, Castell JV, Gómez-Lechón MJ. (2008a). A new *in vitro* approach for the simultaneous determination of phase I and phase II enzymatic activities of human hepatocyte preparations. Rapid Commun Mass Spectrom 22: 240-244.

Lahoz A, Donato MT, Picazo L, Castell JV, Gómez-Lechón MJ. (2008b). Assessment of cytochrome P450 induction in human hepatocytes using the cocktail strategy plus liquid chromatography tandem mass spectrometry. Drug Metab Lett 2:205-209.

LeCluyse EL. (2001). Human hepatocyte culture systems for the *in vitro* evaluation of cytochrome P450 expression and regulation. Eur J Pharm Sci 13:343-368.

Lecluyse EL, Alexandre E. (2010). Isolation and culture of primary hepatocytes from resected human liver tissue. Methods Mol Biol 640:57-82.

LeCluyse EL, Alexandre E, Hamilton GA, Viollon-Abadie C, Coon DJ, Jolley S, Richert L. (2005). Isolation and culture of primary human hepatocytes. Methods Mol Biol 290:207-229.

Li AP. (2007). Human hepatocytes: isolation, cryopreservation and applications in drug development. Chem Biol Interact 168:16-29.

Li AP, Gorycki PD, Hengstler JG, Kedderis GL, Koebe HG, Rahmani R, de Sousas G, Silva JM, Skett P. (1999a). 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.

Li AP, Lu C, Brent JA, Pham C, Fackett A, Ruegg CE, Silber PM. (1999b). Cryopreserved human hepatocytes: characterization of drug-metabolizing enzyme activities and applications in higher throughput screening assays for hepatotoxicity, metabolic stability, and drug-drug interaction potential. Chem Biol Interact 121:17-35.

Lloyd TD, Orr S, Skett P, Berry DP, Dennison AR. (2003). Cryopreservation of hepatocytes: a review of current methods for banking. Cell Tissue Bank 4:3-15.

Loretz LJ, Li AP, Flye MW, Wilson AG. (1989). Optimization of cryopreservation procedures for rat and human hepatocytes. Xenobiotica 19:489-498.

Lu C, Li AP. (2001). Species comparison in P450 induction: effects of dexamethasone, omeprazole, and rifampin on P450 isoforms 1A and 3A in primary cultured hepatocytes from man, Sprague-Dawley rat, minipig, and beagle dog. Chem Biol Interact 134:271-281.

Lyon K, Amunom I, Neat JN, Holsapple J, Buckley D, Parkinson A. (2010). In situ evaluation of CYP1A2, CYP2B6 and CYP3A4/5 induction in cultured human hepatocytes with a cocktail of probe substrates: A comparison of percent positive control values. Drug Metab. Rev. 42:134-135.

Maurel P. (1996). The use of adult human hepatocytes in primary culture and other in vitro systems to investigate drug metabolism in man. Advan Drug Delivery Rev 22:105-132.

McGinnity DF, Soars MG, Urbanowicz RA, Riley RJ. (2004). Evaluation of fresh and cryopreserved hepatocytes as *in vitro* drug metabolism tools for the prediction of metabolic clearance. Drug Metab Dispos 32:1247-1253.

Miners JO, Knights KM, Houston JB, Mackenzie PI. (2006). In vitro-*in vivo* correlation for drugs and other compounds eliminated by glucuronidation in humans: pitfalls and promises. Biochem Pharmacol 71:1531-1539.

Nishimura M, Ejiri Y, Kishimoto S, Suzuki S, Satoh T, Horie T, Narimatsu S, Naito S. (2011). Expression levels of drug-metabolizing enzyme, transporter, and nuclear receptor mRNAs in a novel three-dimensional culture system for human hepatocytes using micro-space plates. Drug Metab Pharmacokinet 26:137-144.

Ostrowska A, Bode DC, Pruss J, Bilir B, Smith GD, Zeisloft S. (2000). Investigation of functional and morphological integrity of freshly isolated and cryopreserved human hepatocytes. Cell Tissue Bank 1:55-68.

Palmer JL, Scott RJ, Gibson A, Dickins M, Pleasance S. (2001). An interaction between the cytochrome P450 probe substrates chlorzoxazone (CYP2E1) and midazolam (CYP3A). Br J Clin Pharmacol 52:555-561.

Richert L, Abadie C, Bonet A, Heyd B, Mantion G, Alexandre E, Bachellier P, Kingston S, Pattenden C, Illouz S, Dennison A, Hoffmann S, Coecke S. (2010). Inter-laboratory evaluation of the response of primary human hepatocyte cultures to model CYP inducers - a European Centre for Validation of Alternative Methods (ECVAM) - funded pre-validation study. Toxicol *In Vitro* 24:335-345.

Richert L, Alexandre E, Lloyd T, Orr S, Viollon-Abadie C, Patel R, Kingston S, Berry D, Dennison A, Heyd B, Mantion G, Jaeck D. (2004). Tissue collection, transport and isolation procedures required to optimize human hepatocyte isolation from waste liver surgical resections. A multilaboratory study. Liver Int 24: 371-378.

Richert L, Binda D, Hamilton G, Viollon-Abadie C, Alexandre E, Bigot-Lasserre D, Bars R, Coassolo P, LeCluyse E. (2002). Evaluation of the effect of culture configuration on morphology, survival time, antioxidant status and metabolic capacities of cultured rat hepatocytes. Toxicol *In Vitro* 16:89-99.

Richert L, Liguori MJ, Abadie C, Heyd B, Mantion G, Halkic N, Waring JF. (2006). Gene expression in human hepatocytes in suspension after isolation is similar to the liver of origin, is not affected by hepatocyte cold storage and cryopreservation, but is strongly changed after hepatocyte plating. Drug Metab Dispos 34:870-879.

Richert L, Tuschl G, Abadie C, Blanchard N, Pekthong D, Mantion G, Weber JC, Mueller SO. (2009). Use of mRNA expression to detect the induction of drug metabolising enzymes in rat and human hepatocytes. Toxicol Appl Pharmacol 235:86-96.

Roymans D, Annaert P, Van Houdt J, Weygers A, Noukens J, Sensenhauser C, Silva J, Van Looveren C, Hendrickx J, Mannens G, Meuldermans W. (2005). Expression and induction potential of cytochromes P450 in human cryopreserved hepatocytes. Drug Metab Dispos 33:1004-1016.

Ruegg CE, Silber PM, Mughal RA, Ismail J, Lu C, Bode DC, Li AP. (1997). Cytochrome P-450 induction and conjugated metabolism in primary human hepatocytes after cryopreservation. In Vitro Toxicol. 10:217-222.

Schuetz EG, Schuetz JD, Strom SC, Thompson MT, Fisher RA, Molowa DT, Li D, Guzelian PS. (1993). Regulation of human liver cytochromes P-450 in family 3A in primary and continuous culture of human hepatocytes. Hepatology 18:1254-1262.

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

Soars MG, McGinnity DF, Grime K, Riley RJ. (2007). The pivotal role of hepatocytes in drug discovery. Chem Biol Interact 168:2-15.

Stéphenne X, Najimi M, Sokal EM. (2010). Hepatocyte cryopreservation: is it time to change the strategy? World J Gastroenterol 16:1-14.

Swales NJ, Luong C, Caldwell J. (1996). Cryopreservation of rat and mouse hepatocytes. I. Comparative viability studies. Drug Metab Dispos 24:1218-1223.

Swift B, Brouwer KL. (2010). Influence of seeding density and extracellular matrix on bile Acid transport and mrp4 expression in sandwich-cultured mouse hepatocytes. Mol Pharm 7: 491-500.

Swift B, Pfeifer ND, Brouwer KL. (2010). Sandwich-cultured hepatocytes: an *in vitro* model to evaluate hepatobiliary transporter-based drug interactions and hepatotoxicity. Drug Metab Rev 42:446-471.

Terry C, Dhawan A, Mitry RR, Lehec SC, Hughes RD. (2010). Optimization of the cryopreservation and thawing protocol for human hepatocytes for use in cell transplantation. Liver Transpl 16:229-237.

Turncliff RZ, Tian X, Brouwer KL. (2006). Effect of culture conditions on the expression and function of Bsep, Mrp2, and Mdr1a/b in sandwich-cultured rat hepatocytes. Biochem Pharmacol 71:1520-1529.

Zhang P, Tian X, Chandra P, Brouwer KL. (2005). Role of glycosylation in trafficking of Mrp2 in sandwich-cultured rat hepatocytes. Mol Pharmacol 67:1334-1341.

Zhao P, Kunze KL, Lee CA. (2005). Evaluation of time-dependent inactivation of CYP3A in cryopreserved human hepatocytes. Drug Metab Dispos 33:853-861.