

Human Hepatocyte – A Model For Toxicological Studies. Functional And Biochemical Characterization

M. MODRIANSKÝ^{1,2}, J. ULRICOVÁ¹, P. BACHLEDA³, P. ANZENBACHER⁴,
E. ANZENBACHEROVÁ⁴, D. WALTEROVÁ¹ AND V. ŠIMÁNEK^{1,2}

¹ Department of Medical Chemistry and Biochemistry, Medical Faculty,
Palacký University, Olomouc, Czech Republic

² Center for Analytical Chemistry of Molecular Structures,
Palacký University, Olomouc, Czech Republic

³ Transplantation Center, Faculty Hospital, Olomouc, Czech Republic

⁴ Institute of Experimental Biopharmaceutics, PRO.MED.CS. Praha
‡ Academy of Sciences, Hradec Králové, Czech Republic

Abstract. Isolated human hepatocytes (HH) are an accepted model for *in vitro* experiments for testing liver function and xenobiotic metabolism. Preferred over more traditional animal hepatocyte model used in toxicological studies, it is the model of choice when substances undergoing biotransformation in man are investigated. The aim of this study was to optimize isolation and culture conditions for HH primary culture with regard to cell yield, viability, and metabolic activity, and to evaluate the suitability of donor samples for toxicology experiments. Cell viability, total cytochrome P450 (CYP) content, CYP3A4, CYP1A2 activity, and finally mixed ethoxycoumarin-*O*-deethylase (ECOD) activity were parameters measured in order to characterize the isolated HH. The quality of the primary cultures, stable and functional for a seven-day period following 24 hour stabilization, was assessed by lactate dehydrogenase (LDH) leakage and response to the model toxin *tert*-butylhydroperoxide (tBH) and to silybinin, a model cytoprotective substance. Based on HH obtained from livers of five multiorgan donors (average age 44.8 years, three males and two females), the individual variability of donors needs to be considered in evaluating cultures focussing on clinical liver tests. Greater sensitivity to toxins and silybinin was found in the hepatocyte culture from one donor with higher aminotransferase activity. In another case, higher serum bilirubin appeared to be linked to higher ECOD activity. Our conclusion is that values of clinical liver tests ought to suggest a healthy organ thus eliminating previous hepatocyte damage, the crucial factor of primary culture stability and functioning.

Correspondence to: Martin Modrianský, PhD, Department of Medical Chemistry and Biochemistry, Hněvotínská 3, 775 15 Olomouc, Czech Republic.
E-mail: oregon@tunw.upol.cz

Key words: Human hepatocytes — Cell culture — Cytochrome P450 — Toxicity — Silybinin

Introduction

The liver is a vital organ performing and maintaining multiple important physiological functions such as plasma protein secretion, cholesterol synthesis, drug metabolism, and glycogen storage. Specific liver functions however are difficult to study *in vivo* as they are obfuscated by the activity of other organs. This obstacle is overcome by using isolated organ, tissue slices, isolated cells, or even subcellular fractions and certain particles, e.g. lipoproteins. Although perfused whole liver is the model closest to the *in vivo* situation, it can only be viably maintained for a relatively brief period. On the other hand, isolated cells as primary cell culture offer experimental material for several days and the experimental data obtained are easily extrapolated to the whole organ (Moshage and Yap 1992).

Receiving more attention in the last decade, the cell culture model has been used mainly in four types of experiments: drug metabolism (Bort et al. 1996), influences on the expression of biotransformation enzymes (Till et al. 1999; Kostrubsky et al. 1999), toxicity of xenobiotics (Rakba et al. 1999), and evaluation of the genotoxic potential of chemicals for the liver (Bellamy et al. 1997). Many studies use hepatocytes isolated from laboratory animals, especially rats (Till et al. 1999). Extrapolation from animals to humans is sometimes problematic owing to different specificities of biotransformation enzymes, especially cytochrome P450 (CYP) monooxygenases (Shih et al. 1999). However, serious ethical issues aside, human tissue poses a major problem due to its limited and irregular availability as a source of research material. Most human liver samples come from partial hepatectomies. Other sources are adult liver wedge for use in child recipient and livers from multi-organ donors (Bayliss and Skett 1996). The latter also offers sufficient material for the preparation of microsomes, mitochondria, and purification of specific enzymes. Even though no completely reliable protocol exists to date, advances in cryopreservation hold promise of the more extended use of isolated cells (Coundouris et al. 1993).

A wide spectrum of methods has been devised and used to characterize primary culture hepatocyte viability and function. Trypan blue, neutral red, and crystal violet are classical viability tests now accompanied by fluorometric and spectrophotometric methods utilizing other dyes and chromogenic chemicals, e.g. tetrazolium salts (Doyle et al. 1996). Monitoring cytosolic enzyme activity and protein synthesis are two examples of functional markers (Kosina et al. 1999). 7-Ethoxycoumarin-*O*-deethylase (ECOD) activity is a mixed CYP activity widely used for biochemical characterization along with two specific activities: 7-ethoxyresorufin-*O*-deethylation (EROD) for CYP1A2 and nifedipine oxidation for CYP3A4 isoforms. The first mentioned CYP isoenzyme is of interest due to its established activation of procarcinogens, polyaromatic hydrocarbons in particular. The latter represents activity of a human liver specific CYP isoform responsible for biotransformation of more

than 60% of xenobiotics metabolized in the liver.

Human hepatocyte primary culture has several disadvantages, however. The first and well-known is the variability of donors stemming from either genetic disposition, prior liver injury, or both. As a consequence responses to substances of interest may vary widely between donors. The same applies to methods for isolated cell seeding to culture dishes and the establishment of cultures assessed from the formation of confluent monolayer of cells. A second consideration is the viability of cultures that can extend over weeks (Ferrini et al. 1997) but should be at least one week to allow for 96 hours protein expression experiments. Third is a well documented decrease in CYP expression over time (Moshage and Yap 1992). Possible solutions such as the presence of extracellular matrix, cell density, culture media and supplements, and co-cultures have been reviewed elsewhere (Skett 1994). Ideal conditions for human hepatocyte isolation and cultivation do not exist and available protocols have to be evaluated for pertinent purposes (Bayliss and Skett 1996).

The aim of this study was to establish a protocol for human hepatocyte isolation and cultivation for evaluating the effects of natural substances of plant origin and compare them with a model toxin. The original samples were characterized using clinical and serological tests, and their biotransformation capacity was assessed using ECOD and EROD. Prepared primary cultures were characterized functionally using the cytosolic enzyme activity test. Nifedipine oxidation, a CYP3A4 specific activity, and rifampicin induction of CYP3A4 served as markers for the maintenance of xenobiotic metabolism and enzyme inducibility. A link between routine clinical tests and the sensitivity of cultures to a generally non-toxic compound silybinin is drawn.

Materials and Methods

Chemicals

Tert-butylhydroperoxide (tBH) as an 80 % solution in di-*tert*-butylperoxide for synthesis was purchased from Merck (Darmstadt, Germany). Rifampicin, 7-ethoxycoumarin, 7-hydroxycoumarin, ethylresorufin, resorufin, and all culture media and supplements were purchased from Sigma (St. Louis, USA). LDH assay kit was purchased from Lachema (Brno, Czech Republic). Silybinin (lot No. Cv 090495/2) was a kind gift from Galena Opava a.s. Collagenase Cruda was purchased from Sevac (Czech Republic). All other chemicals were of the highest grade commercially available.

Clinical tests

All tests were performed at the Department of Clinical Biochemistry, Faculty Hospital Olomouc according to protocols routinely used.

Hepatocyte isolation and culture

The livers, designated throughout the text as LH1 thru LH5 with the numeral corresponding to the order in which each specimen was received, were obtained from multiorgan donors who met an accidental death. The hepatocytes were isolated from the HTK pre-washed liver using two-step collagenase perfusion (Berry and Friend 1969; Seglen 1976; Strom et al. 1982; Guguen-Guillouzo et al. 1982). The liver segment was washed sequentially with HEPES1, EGTA, and HEPES 2 media, followed by 20 minutes perfusion with HEPES 3 medium containing Ca^{2+} ions and collagenase.

The following was the composition of media: HEPES 1 consisted of HEPES (20 mmol.l^{-1}), NaCl (120 mmol.l^{-1}), KCl (5 mmol.l^{-1}), glucose (28 mmol.l^{-1}), mannitol (100 $\mu\text{mol.l}^{-1}$), sorbitol (100 $\mu\text{mol.l}^{-1}$), glutathione (100 $\mu\text{mol.l}^{-1}$), penicillin G (10 U/ml), streptomycin (100 $\mu\text{mol.l}^{-1}$), and amphotericin B (25 $\mu\text{g/ml}$), pH 7.4. HEPES 2 consisted of HEPES (20 mmol.l^{-1}), NaCl (120 mmol.l^{-1}), KCl (5 mmol.l^{-1}), glucose (28 mmol.l^{-1}), penicillin (10 U/ml), streptomycin (100 $\mu\text{mol.l}^{-1}$), and amphotericin B (25 $\mu\text{g/ml}$), pH 7.4. HEPES 3 had the same composition as HEPES 2 plus CaCl_2 (0.7 mmol.l^{-1}) and 500 mg collagenase (530 U/g). HEPES 4 had the same composition as HEPES 2 plus 1 % fetal calf serum (FCS). EGTA medium consisted of EGTA (0.5 mmol.l^{-1}), KCl (5.4 mmol.l^{-1}), KH_2PO_4 (0.44 mmol.l^{-1}), NaCl (140 mmol.l^{-1}), Na_2HPO_4 (0.34 mmol.l^{-1}), and Tricine (25 mmol.l^{-1}), pH 7.2. Culture medium (LHM) consisted of Williams medium E and HAM F12 in a 1:1 ratio including the following additives: glucose (7 mmol.l^{-1}), glutamine (2.4 mmol.l^{-1}), penicillin (100 U/ml), streptomycin (10 $\mu\text{mol.l}^{-1}$), sodium pyruvate (0.4 mmol.l^{-1}), dexamethasone (1.8 $\mu\text{mol.l}^{-1}$), holo-transferin (5 mg.l^{-1}), ethanolamine (1 $\mu\text{mol.l}^{-1}$), insulin (350 nmol.l^{-1}), glucagon (0.2 mg.l^{-1}), linolic acid (11 $\mu\text{g.l}^{-1}$), and amphotericin B (1.4 mg.l^{-1}), pH 7.2.

The Glisson capsule was disintegrated and cells were released into HEPES 4 medium containing 1 % FCS (Pichard et al. 1990; Ferrini et al. 1997). Isolated cells were separated from larger pieces of unperturbed tissue by filtering through sterile gauze and then centrifuged for 3 minutes at $50 \times g$ at room temperature. The cells were washed three times in the LHM culture medium (Isom et al. 1985). Centrifugation for 3 minutes at $50 \times g$ at room temperature followed each wash.

To allow cell attachment and formation of a monolayer, hepatocytes were diluted in LHM culture medium containing 5% FCS and seeded onto collagen coated culture dishes (Berry et al. 1991). The resulting cell concentration was 1.25×10^5 cells/ cm^2 . Hepatocytes were incubated for a minimum of 4 hours at 37°C in an atmosphere containing 5% CO_2 (Kiso et al. 1983; Kiso and Hikino 1991). The culture medium was then exchanged for a serum free, and the primary cultures were stabilized prior to experiments for a minimum of 24 hours under conditions as above.

Cultures were routinely checked under an Olympus CK-2 microscope with Olympus PM-PBK-6 camera adapter and PM-C 35B camera (all Olympus, Japan)

to detect any dramatic changes in culture appearance pre- and post- xenobiotic application.

Toxicity tests

Following pre-incubation the culture media were exchanged for the same volume of identical media lacking serum. All experiments were performed in triplicate. All substances tested were dissolved or diluted in dimethylsulfoxide (DMSO) prior to application with the concentration of DMSO not exceeding 1% of the culture medium volume. To measure LDH activity released into the culture medium a 50 μl aliquot was pipetted out of the culture plate after 4 and 21 hours in case of tBH and silybinin, respectively. Addition of 0.5% Triton X-100 served as a control for total cell destruction where contents of the cytosol were released into the culture medium.

Activity of LDH (EC 1.1.1.27) was measured spectrophotometrically (Bergmeyer 1962; King 1965) using commercially available sets on UV 1601 spectrophotometer (Shimadzu, Japan).

Preparation of microsomes

Previously published protocols were the basis for our approach (Haraguchi et al. 1995; Mathiesen et al. 1995). A liver segment was cut into smaller parts approximately 10 g a piece. Individual pieces were cut using scissors in a beaker and then homogenized in 3 mmol.l^{-1} Tris/HCl buffer, pH 7.4, containing 0.25 mol.l^{-1} sucrose and 0.1 mmol.l^{-1} EDTA. The final concentration of the homogenate was 20% (w/w). It was centrifuged for 15 minutes at $800 \times g$ at 4°C . The supernatant was poured into fresh tubes and centrifuged for 20 minutes at $10,000 \times g$ at 4°C . The resulting supernatant was centrifuged for 1 hour at $105,000 \times g$ at 4°C . The pellet after ultracentrifugation was resuspended in 0.1 mol.l^{-1} phosphate buffer, pH 7.4, containing 20% glycerol and 1 mmol.l^{-1} EDTA. Following protein (Bradford 1976) and total CYP (Omura and Sato 1964) estimations, the prepared microsomes were aliquoted into Eppendorf tubes and stored at -80°C for future use. Any liver pieces and segments unused for microsomal preparation were stored at -80°C as well. To prepare microsomes from the primary cultures, the cells were scraped off the culture dishes into 0.1 mol.l^{-1} phosphate buffer, pH 7.4. The suspension was subjected to sonication at 80 Watts for 45 seconds on ice to disrupt the cells. Starting with centrifugation at $10,000 \times g$ for 20 minutes the remaining steps were the same as above.

ECOD activity

A modification of the methods (Guengerich 1994; Yamazaki et al. 1996) devised for cytochrome P450 activity measurement involving multiple P450 isoforms was used for the initial biotransformational characterization of liver samples. Briefly, microsomes ($\sim 300 \mu\text{g}$ protein) were added to tubes containing 1 ml of 0.1 mol.l^{-1} phosphate buffer, pH 7.4, 0.5 mmol.l^{-1} NADPH, and 0.5 mmol.l^{-1} 7-ethoxycoumarin. The reaction was allowed to proceed for 15 minutes at 37°C in the dark. It was

stopped by adding 50 μl of 25% TCA. After brief vortex 2 ml of chloroform were added to tubes and vortexed again. One ml of the lower organic phase was transferred into clean tubes after 5 minutes centrifugation at $3,000 \times g$. 2 ml of 30 mmol.l^{-1} borate buffer, pH 9.0, were mixed with the transferred organic phase, followed by 5 minutes centrifugation at $3,000 \times g$. Approximately 1.8 ml of the upper aqueous phase was transferred into a fluorescence cuvette and fluorescence was measured using $\lambda_{\text{Ex}} = 338 \text{ nm}$ and $\lambda_{\text{Em}} = 456 \text{ nm}$ on RF1501 spectrofluorometer (Shimadzu, Japan). The calibration curve was constructed from the fluorescence readings obtained from samples prepared as above containing the same amount of heat-inactivated microsomes and 10, 5, 2, and 1 nmol of 7-hydroxycoumarin which is the product of CYP catalyzed 7-ethoxycoumarin-*O*-deethylation.

EROD activity

A modification of methods published for the measurement of CYP1A activity (Donato et al. 1992; Chang and Waxman 1998; Shimada and Yamazaki 1998) was used to estimate the CYP1A2 activity in the liver samples. Briefly, microsomes ($\sim 300 \mu\text{g}$ protein) were added to tubes containing 2 ml of 0.1 mol.l^{-1} HEPES buffer, pH 7.8, 0.5 mmol.l^{-1} NADPH, and 0.5 $\mu\text{mol.l}^{-1}$ 7-ethoxycoumarin. The reaction was allowed to proceed for 15 minutes at 37°C in the dark and was stopped by adding 2.5 ml of methanol. After 10 minutes centrifugation at $2,000 \times g$ the top 2.6 ml of solution were discarded by pipetting and the remaining solution was poured into a fluorescence cuvette. Fluorescence was measured using $\lambda_{\text{Ex}} = 530 \text{ nm}$ and $\lambda_{\text{Em}} = 585 \text{ nm}$ RF1501 spectrofluorometer (Shimadzu, Japan). The calibration curve was constructed from fluorescence readings obtained from samples prepared as above and containing the same amount of heat-inactivated microsomes and 100, 75, 50, and 25 pmol of resorufin which is the product of CYP1A catalyzed 7-ethoxyresorufin-*O*-deethylation.

Nifedipine oxidation (CYP3A4)

Nifedipine activity was assessed according to procedures described in Guengerich et al. (1986). Typically, a sample containing 100 pmol of CYP, 0.5 mmol.l^{-1} NADP⁺, 3.7 mmol.l^{-1} isocitrate, 0.5 IU of isocitrate dehydrogenase, and 5 mmol.l^{-1} MgSO₄ was diluted up to 1 ml by 50 mmol.l^{-1} Tris/HCl buffer with 0.15 KCl (pH 7.4). After preincubation, 200 nmol of nifedipine was added and the reaction mixture was incubated for 20 minutes at 37°C . Then, the reaction was stopped by addition of 1 mol.l^{-1} disodium carbonate in 2 mol.l^{-1} NaCl. Nifedipine metabolites were extracted to dichloromethane with subsequent centrifugation to separate the organic layer. This layer was pipetted to a glass vial and evaporated under a stream of dry nitrogen. The solid residue was dissolved in 500 μl of the mobile phase and injected directly onto a LiChrospher 100 RP-18 column with a LiChro CART precolumn with the same stationary phase (MERCK, Darmstadt, Germany). The mobile phase used was 58% methanol with a flow rate of 1 ml/min and detection at 254 nm. HPLC apparatus used consisted of a quaternary P 4000 pump, SCM 400

degasser, AS 3500 autosampler, and Spectra Focus detector (all Thermo Separation Products, San Jose, USA).

Results and Discussion

The clinical data of the five donors are summarized in Table 1. None of the criteria influenced the initial viability, ranging from 64.7% to 77.4% with cell yield from 2.3×10^8 to 2.5×10^9 cells, and the functional state of the isolated hepatocytes as no differences were observed. With time, however, the primary culture LH1 appeared more stable and viable despite prior drug intoxication suggesting age as a factor to consider in case of long-term primary cultures (> 2 weeks). The aminotransferase activities, bilirubin levels, and serological tests did reflect some characteristics of hepatocytes and the corresponding culture as discussed below.

Table 1. Clinical characteristics of liver donors including results of routine liver tests and selected serological tests performed

Donor	Sex	Age	ALT* ($\mu\text{kat.l}^{-1}$)	AST ($\mu\text{kat.l}^{-1}$)	ALP ($\mu\text{kat.l}^{-1}$)	Bi ($\mu\text{mol.l}^{-1}$)	HIV	HCV	CMV	EBV
LH1	F	24	1.55	1.65	1.40	18.00	N	N	N	N
LH2	F	48	6.64	4.66	0.66	14.48	N	N	n/a [#]	n/a
LH3	M	56	0.97	1.20	n/a	19.30	N	N	N	N
LH4	M	43	1.23	2.68	0.82	25.20	N	N	N	N
LH5	M	52	0.90	2.52	1.29	63.00	N	N	IgM ⁻ IgG ⁺	N

*Abbreviations used: ALT, alanine aminotransferase; AST, aspartate aminotransferase; ALP, alkaline phosphatase; Bi, bilirubin; HIV, human immunodeficiency virus; HCV hepatitis C virus; CMV, cytomegalovirus; EBV, Epstein-Barr virus; N, negative.

[#]n/a, not available

A typical hepatocyte culture after 24 hours of stabilization is shown in Fig.1. Any differences observed at this stage of the culture were interpreted as a variation in cell density which was based on the number of live cells calculated from the trypan blue exclusion test. The cell densities can be easily overestimated if a high concentration of trypan blue is used. Cells formed a single layer with a characteristic floor tile pattern thus maintaining mutual contact with each other (Berry et al. 1991). Only the cell nucleus was clearly discernible when observing the culture under a low magnification microscope. The unfocussed bodies were cells floating in the medium. Occasionally these cells formed local multi-layered blobs that could not be washed away with medium change, but we had no reason to believe that these had any more than minimal effects on the behavior of the whole culture. Their

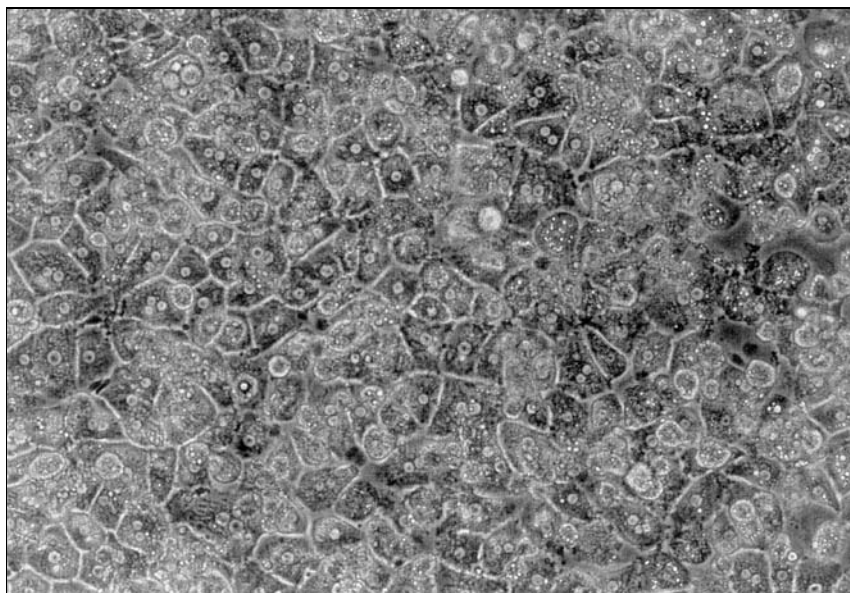


Figure 1. Primary human hepatocyte culture after 24 hours stabilization.

appearance was likely caused by cell clusters unperturbed by collagenase and untrapped by filter gauze. Another explanation was individual fibroblasts inseparable during hepatocyte isolation as fibroblast colonies sometimes appeared in the later stages of the culture (> 4 days).

Like tetrachlormethane and allyl alcohol, *tert*-butylhydroperoxide (tBH) is slowly becoming a standard for toxicity tests. It inflicts serious damage on cell membranes by the known mechanism of lipid peroxidation (Masaki et al. 1989). Because such a damage is readily detected as cytosolic enzyme leakage, it is a valuable tool for routine comparative substance toxicity tests. The presumed active substance of a *Silybum marianum* extract, silybinin, was chosen to represent a generally non-toxic substance with known hepatoprotective properties (Flora et al. 1998).

All cultures were subjected to 0.5 mmol.l^{-1} tBH, and two concentrations of silybinin (0.5 mmol.l^{-1} and 0.05 mmol.l^{-1}). The cell damage caused by tBH after 4 hours of incubation is shown in Fig. 2. Cell debris appeared as randomly distributed dots of undefined matter. The floor tile pattern of hepatocyte monolayer was destroyed and the more resistant cells assumed spherical form. Essentially the same appearance of the culture with fewer intact cells remaining was observed after addition of 0.5% Triton X-100 (not shown). By contrast, silybinin had minute effects on the culture appearance which largely resembled the control culture (Fig. 1). At this stage an aliquot of the culture medium was transferred to tubes and LDH activity in the medium was measured.

The results of LDH leakage measurements are summarized in Table 2. The

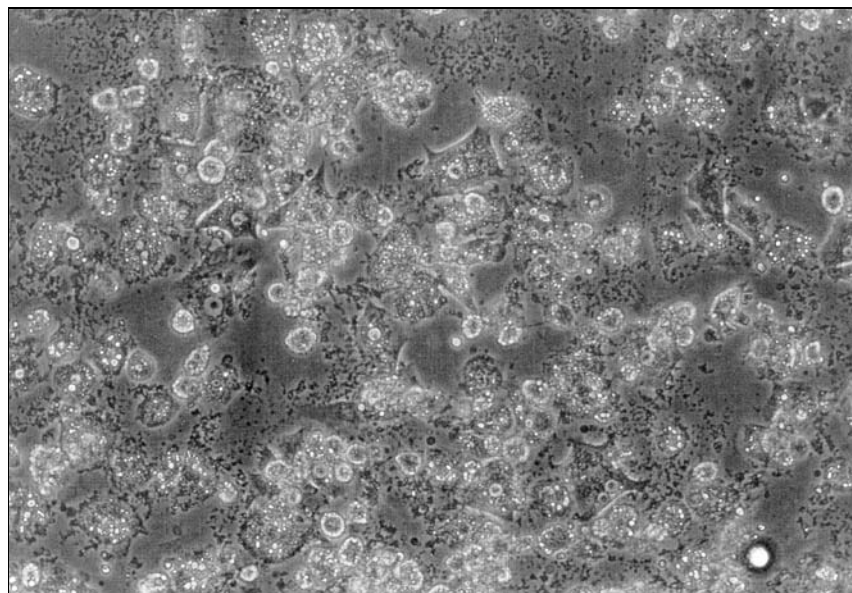


Figure 2. Primary human hepatocyte culture after 4 hours incubation with 0.5 mmol.l^{-1} *tert*-butylhydroperoxide.

Table 2. Toxic reaction to xenobiotics monitored as LDH* activity. Incubation times were 21.5 and 4 hours for silybinin and tBH, respectively

	Concentration ($\mu\text{mol.l}^{-1}$)	LDH activity ($\mu\text{kat.l}^{-1}$)#				
		LH1	LH2	LH3	LH4	LH5
control	0	8.80 ± 1.66	1.81 ± 0.49	2.46 ± 0.22	1.86 ± 0.59	1.53 ± 0.25
silybinin	50	7.61 ± 0.50	1.73 ± 0.44	3.15 ± 0.36	1.07 ± 0.25	0.98 ± 0.35
silybinin	500	10.72 ± 2.61	5.18 ± 0.14	2.92 ± 0.84	0.36 ± 0.07	4.10 ± 0.21
tBH	500	38.20 ± 2.70	5.13 ± 0.41	5.96 ± 0.77	2.21 ± 0.88	3.69 ± 0.32
Triton X-100	0.5% (v/v)	41.9	7.7	10.4	4	7.5

*Abbreviations used: LDH, lactate dehydrogenase; tBH, *tert*-butylhydroperoxide.

#Values are means \pm S.D. for three experiments.

control LDH activity in all cases was in the order of 20–25% of maximum activity reached after Triton X-100 lysis. Comparison of LDH activity in the presence of tBH and 0.5 mmol.l^{-1} silybinin samples LH2 and LH5 displayed higher values in the presence of the latter substance contradicting its general non-toxicity. The con-

centration of silybinin used was comparable to that in patients with severe *Amanita phalloides* poisoning, where silybinin is used as a rescue agent. Further comparison with clinical data suggested that chronic or past liver injury, observed as higher aminotransferases (LH2) or higher bilirubin values (LH5), but not a lethal acute liver intoxication (LH1), is the cause of a higher sensitivity of the cultures. A possible cause for the higher bilirubin level in LH5 sample was prior cytomegalovirus (CMV) infection evidenced as the presence of IgG antibodies (Table 1). It implies that caution has to be exercised when evaluating data obtained on primary hepatocyte cultures prepared from donors with prior liver damage. Moreover, the past donor health condition may affect the overall culture characteristics as e.g. the sensitivity to xenobiotics, cell viability, etc.

For further characterization, the biotransformation activity of liver samples (Table 3) and of selected hepatocyte primary cultures was evaluated in the respective microsomal fractions. ECOD and EROD activities were estimated in all samples where ECOD is a mixed type activity representing chiefly CYP1A2 and 2E1 isoforms (Yamazaki et al. 1996) and EROD is CYP1A2 specific activity (Chang and Waxman 1998). Combined, these two activities give an initial orientation regarding the biotransformation ability of the sample. The CYP3A4 specific nifedipine oxidation in conjunction with tBH and rifampicin treatment was used as an indicator for maintenance of biotransformation properties in selected cultures.

Selected CYP activities (Table 3) by all but one sample were in the range reported in the literature (Yamazaki et al. 1996). We associate the higher ECOD activity in LH5 with a higher bilirubin level in that liver specimen. This may have been the outcome of previous CMV infection as other possible causes were not documented. Judging from the EROD results, CYP1A2 activity in LH5 was the lowest among the samples tested suggesting an increased CYP2E1 activity. Because the most common inducer of CYP2E1 is ethanol, prior alcohol consumption by the donor is likely but not evidenced. The high sensitivity of LH5 sample to silybinin (Table 2) originated in a previous liver injury as deduced from three pieces of

Table 3. Total CYP* content and selected activities in the microsomal fraction of liver samples

Donor	Total CYP (pmol/mg protein)	ECOD (nmol/min nmol P450) [#]	EROD (nmol/min nmol P450)
LH1	426	0.901 ± 0.164	0.024 ± 0.001
LH2	500	0.848 ± 0.192	0.035 ± 0.016
LH3	435	0.969 ± 0.041	0.052 ± 0.015
LH4	420	0.397 ± 0.077	0.072 ± 0.003
LH5	693	1.538 ± 0.230	0.018 ± 0.004

*Abbreviations used: CYP, cytochrome P450; ECOD, ethoxycoumarin-*O*-deethylase; EROD, ethoxyresorufin-*O*-deethylase.

[#]Values are means ± S.D. for minimum of eight experiments.

evidence: past infection, high bilirubin level, and high ECOD activity.

Microsomal fractions from the LH2 primary culture displayed nifedipine oxidation (CYP3A4) activity comparable to the original microsomes. The activities were 3.05 nmol/min nmol P450 and 3.6 nmol/min nmol P450. This activity was only slightly decreased to 2.4 nmol/min nmol P450 when tBH was present in the culture medium under conditions used for toxicity tests (see Materials and Methods). The values obtained are in good correlation with previously published data (Guengerich et al. 1986; Anzenbacher et al. 1998). The culture therefore maintained its biotransformational properties for the duration of the toxicity experiment.

In a similar test, microsomal fractions prepared from LH3 primary culture exposed to 25 $\mu\text{mol.l}^{-1}$ rifampicin exhibited even higher CYP3A4 activity than the original microsomes; 5.88 and 1.39 nmol/min nmol P450 respectively. The increase further marked a functional culture because it was capable of responding to extrinsic stimulus as demonstrated by the induction of CYP3A4 by rifampicin.

Conclusion

The protocol for human hepatocyte isolation and culturing compiled from the available literature and described in the Materials and Methods section yields in our hands a fully functional primary culture. The culture is suitable for toxicological as well as other types of studies, e.g. xenobiotic metabolism and protein expression, as judged from the evidence presented. However, we stress the importance of functional and serological tests for cell culture evaluation.

Acknowledgements. We would like to thank the following institutions for financial support: the Grant Agency of the Czech Republic (grants 303/98/0414 and 303/99/P002), the Ministry of Education of the Czech Republic (grants VS 96021 and J14/98:15110004), and the Internal Grant Agency of the Ministry of Health of the Czech Republic (grant NL5267-3/1999).

References

- Anzenbacher P., Soucek P., Anzenbacherova E., Gut I., Hruby K., Svoboda Z., Kvetina J. (1998): Presence and activity of cytochrome P450 isoforms in minipig liver microsomes. Comparison with human liver samples. *Drug. Metab. Disposition* **26**, 56—59
- Bayliss M. K., Skett P. (1996): Isolation and culture of human hepatocytes. In: *Human Cell Culture Protocols* (Ed. G. E. Jones), pp. 369—390, Humana Press, Totowa, New Jersey
- Bellamy C. O., Prost S., Wyllie A. H., Harrison D. J. (1997): UV but not gamma-irradiation induces specific transcriptional activity of p53 in primary hepatocytes. *J. Pathol.* **183**, 177—81
- Bergmeyer H. U. (1962): *Methoden der enzymatischen Analyse*, Verlag Chemie, Weinheim
- Berry M. N., Friend D. S. (1969): High-yield preparation of isolated rat liver parenchymal cells: a biochemical and fine structural study. *J. Cell. Biol.* **43**, 506—520
- Berry M. N., Edwards A. M., Barritt G. J. (1991): *Isolated Hepatocytes, Preparation, Properties and Application*. Elsevier, New York

- Bort R., Ponsoda X., Carrasco E., Gomez-Lechon M. J., Castell J. V. (1996): Metabolism of aceclofenac in humans. *Drug. Metab. Disposition* **24**, 834—841
- Bradford M. M. (1976): A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal. Biochem.* **72**, 248—254
- Chang T. K. H., Waxman D. J. (1998): Enzymatic analysis of cDNA-expressed human CYP1A1, CYP1A2, and CYP1B1 with 7-ethoxyresorufin as substrate. In: *Methods in Molecular Biology, Vol. 107: Cytochrome P450 Protocols* (Eds. I. R. Phillips and E. A. Shephard) pp. 103—109, Humana Press, Inc., Totowa, New Jersey
- Countouris J. A., Grant M. H., Engeset J., Petrie J. C., Hawkworth G. W. (1993): Cryopreservation of human adult hepatocytes for use in drug metabolism and toxicity studies. *Xenobiotica* **23**, 1399—1409
- Donato M. T., Gomez-Lechon M. J., Castell J. V. (1992): Biotransformation of drugs by cultured hepatocytes. In: *In Vitro Alternatives to Animal Pharmacotoxicology* (Eds. J. V. Castell and M. J. Gomez-Lechon) pp. 149—178, Farmaindustria, Madrid
- Doyle A., Griffiths J. B., Newell D. G. (1996): *Cell & Tissue Culture: Laboratory Procedures*, John Wiley & Sons, Ltd., Chichester
- Ferrini J. B., Pichard L., Domerque J., Maurel P. (1997): Long-term primary cultures of adult human hepatocytes. *Chem-Biol. Inter.* **107**, 31—45
- Flora K., Hahn M., Rosen H., Benner K. (1998): Milk thistle (*Silybum marianum*) for the therapy of liver disease. *Amer. J. Gastroenterol.* **93**, 139—143
- Guengerich F. P. (1994): Analysis and characterization of enzymes In: *Principles and Methods of Toxicology*, 3rd edition (Ed. W. Hayes) p. 1271, Raven Press, Ltd. New York
- Guengerich F. P., Martin M. V., Beaune P. H., Kremers P., Wolff T., Waxman D. J. (1986): Characterization of rat and human liver microsomal cytochrome P-450 forms involved in nifedipine oxidation, a prototype for genetic polymorphism in oxidative drug metabolism. *J. Biol. Chem.* **261**, 5051—5060
- Guguen-Guillouzo C., Campion J. P., Brissot P., Glaise D., Launois B., Bourel M., Guillouzo A. (1982): High yield preparation of isolated human adult hepatocytes by enzymatic perfusion of the liver. *Cell. Biol. Int. Rep.* **6**, 625—628
- Haraguchi H., Saito T., Okamura N., Yagi A. (1995): Inhibition of lipid peroxidation and superoxide generation by diterpenoids from *Rosmarinus officinalis*. *Planta Med.* **61**, 333—336
- Isom H. C., Secott T., Georgoff I., Woodworth C., Mummaw J. (1985): Maintenance of differentiated rat hepatocytes in primary culture. *Proc. Natl. Acad. Sci. USA* **82**, 3252—3256
- King J. (1965): *Practical Clinical Enzymology*, D. Van Nostrand Comp., Ltd., London
- Kiso Y., Hikino H. (1991): *Methods in Plant Biochemistry Vol. 6*, Academic Press, New York
- Kiso Y., Tohkin M., Hikino H. (1983): Assay method for antihepatotoxic activity using carbon tetrachloride induced cytotoxicity in primary cultured hepatocytes. *Planta Med.* **49**, 222—225
- Kosina P., Dvořák Z., Walterová D. (1999): Human hepatocyte: I. Model for the study of xenobiotic metabolism and toxicity. *Čes. Slov. Farm.* **48**, 65—71 (in Czech)
- Kostrubsky V. E., Ramachandran V., Venkataramanan R., Dorko K., Esplen J. E., Zhang S., Sinclair J. F., Wrighton S. A., Strom S. C. (1999): The use of human hepatocyte cultures to study the induction of cytochrome P-450. *Drug. Metab. Disposition* **27**, 887—894
- Masaki N., Kyle M. E., Farber J. L. (1989): *tert*-Butyl hydroperoxide kills cultured hepatocytes by peroxidizing membrane lipids. *Arch. Biochem. Biophys.* **269**, 390—399

- Mathiesen L., Malterud K. E., Sund R. B. (1995): Antioxidant activity of fruit exudate and C-methylated dihydrochalcones from *Myrica gale*. *Planta Med.* **61**, 515—518
- Moshage H., Yap S. H. (1992): Primary cultures of human hepatocytes: a unique system for studies in toxicology, virology, parasitology and liver pathophysiology in man. *J. Hepatol.* **15**, 404—413
- Omura T., Sato R. (1964): The carbon monoxide-binding pigment of liver microsomes. I. Evidence for its hemoprotein nature. *J. Biol. Chem.* **239**, 2370—2378
- Pichard L., Fabre I., Fabre G., Domergue J., Aubert B. S., Mourad G., Maurel P. (1990): Cyclosporin A drug interactions. Screening for inducers and inhibitors of cytochrome P-450 (cyclosporin A oxidase) in primary cultures of human hepatocytes and in liver microsomes. *Drug. Metab. Disposition* **18**, 595—606
- Rakba N., Melhaoui A., Loyer P., Guy Delcros J., Morel I., Lescoat G. (1999): Bgugaine, a pyrrolidine alkaloid from *Arisarum vulgare*, is a strong hepatotoxin in rat and human liver cell cultures. *Toxicol. Lett.* **104**, 239—48
- Seglen P. O. (1976): In: *Methods in Cell. Biology*, Vol. 13 (Ed D. M. Prescott) pp. 29—83, Academic Press, New York
- Shih H., Pickwell G. V., Guenette D. K., Bilir B., Quattrochi L. C. (1999): Species differences in hepatocyte induction of CYP1A1 and CYP1A2 by omeprazole. *Hum. Exp. Toxicol.* **18**, 95—105
- Shimada T., Yamazaki H. (1998): Cytochrome P450 reconstitution systems. In: *Methods in Molecular Biology*, Vol. 107: *Cytochrome P450 Protocols* (Eds. I. R. Phillips and E. A. Shephard) pp. 85—93, Humana Press, Inc., Totowa, New Jersey
- Skett P. (1994): Problems in using isolated and cultured hepatocytes for xenobiotic metabolism/metabolism based toxicity testing – solutions? *Toxicol. Vitro.* **8**, 491—504
- Strom S. C., Jirtle R. L., Jones R. S., Novicki D. L., Rosenberg M. R., Novotny A., Irons G., McLain J. R., Michalopoulos G. (1982): Isolation, culture, and transplantation of human hepatocytes. *J. Natl. Cancer Inst.* **68**, 771—778
- Till M., Riebniger D., Schmitz H. J., Schrenk D. (1999): Potency of various polycyclic aromatic hydrocarbons as inducers of CYP1A1 in rat hepatocyte cultures. *Chem-Biol. Interact.* **117**, 135—150
- Yamazaki H., Inoue K., Mimura M., Oda Y., Guengerich F. P., Shimada T. (1996): 7-Ethoxycoumarin *O*-deethylation catalyzed by cytochromes P450 1A2 and 2E1 in human liver microsomes. *Biochem. Pharmacol.* **51**, 313—319