Cryopreservation of rat precision-cut liver slices by ultrarapid freezing
Influence on phase I and II metabolism and on cell viability upon incubation for 24 hours

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Abstract

Several cryopreservation methods for precision-cut rat liver slices (PCLS) have been proposed, allowing a short-term (a few hours) maintainance of viability and functionnality upon thawing. The aim of the present study was to test the metabolic capacity of PCLS cryopreserved by an ultrarapid method. The biotransformation of paracetamol to its glucuronide and sulfate conjugates and of midazolam to its hydroxylated metabolites was studied in thawed PCLS incubated for 24 hours at 37°C in Williams' medium E. In addition, protein levels of the key enzymes involved in these metabolic reactions, i.e. UGT1A1, ST1A1, CYP2E1 and CYP3A2 were determinated. In addition, biological markers of cell function (ATP and glycogen levels) and toxicity (LDH leakage in the medium) were also measured. Compared to controls (non cryopreserved PCLS), CYP3A2 activity and content and CYP2E1 content were maintained at the same level all along the incubation, whereas paracetamol glucuronidation and sulfation dropped to 24 and 21 % of the control value, respectively, immediately after thawing. Freezing-thawing conditions also modified cell functionnality, leading to a lower intracellular ATP and glycogen content, and an increase in cell lysis, as shown by LDH released in the medium. The results of this study suggest that cryopreserved PCLS are able to maintain some phase I activities for 24 hours after thawing whereas some phase II metabolic capacities are not maintained. © 2001 Elsevier Science Inc. All rights reserved.

Keywords: Cryopreservation; Rat; Liver slices

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Introduction

Human and rat PCLS are described as valuable tools for *in vitro* metabolic studies of drug candidates at least after a short term incubation [1,2]. As compared to hepatocytes, this model offers the advantages of preserving the tissue architecture and the proportion of the different cell types [3]. Our laboratory has recently shown, by using PCLS, that Kupffer cells can be involved in regulating phase II metabolism in the adjacent hepatocytes [4]. Cryopreservation of cells or PCLS would facilitate a broader and more efficient utilization of the tissue, and moreover, allow their use at any desired time [5].

Cryopreservation of hepatocytes is widely described. A recent review reports that the successful cryopreservation conditions for hepatocytes take into account the freezing protocol, the concentration and the procedure of addition of cryoprotectant such as DMSO, and the removal of unvital hepatocytes after thawing [6]. Only few papers report successful cryopreservation of PCLS. Different methods of cryopreservation are described in the literature. Human PCLS cryopreserved by a slow freezing method, which is achieved by using a computer-controlled cooling device, maintain protein synthesis and K^+ content at 89% and 54% of the control values (non cryopreserved slices) within 4 hours of incubation [7]. The application of a rapid freezing method (direct immersion in liquid nitrogen) allows to maintain urea synthesis, sulfoconjugation and CYP-dependent oxidation of ethoxycoumarin, testosterone hydroxylation and N-deethylation of lidocaine at the same level as non-cryopreserved rat or human PCLS after 2 or 3 hours of incubation [5,8,9]. Madan *et al* [10] have also shown that cytochrome P-450 induction could be maintained in cryopreserved cultured rat hepatocytes. More recently, it has been shown that 1-β-naphtoflavone was able to induce CYP1A1 mRNA in human fresh PCLS after 6 hours of exposure. In cryopreserved human slices after thawing, CYP1A1 mRNA induction occurred, but at a very low level [11]. Most of the data relative to long term (24 hours and more) maintenance of metabolic activities after cryopreservation have been obtained on rat or human hepatocytes [12,13] whereas no studies have been reported concerning analysis of long-term metabolic capacity of cryopreserved rat PCLS.

In the present study we propose to analyse phase I and II metabolic capacity of cryopreserved PCLS towards paracetamol and midazolam as model substances during 24 hours of incubation after thawing. Paracetamol is metabolized in the liver tissue, namely into glucurono- and sulfoconjugates, that constitute the major phase II metabolites, at least in rats. The UDP-glucuronosyltransferase 1A1 (UGT1A1) and sulfotransferase 1A1 (ST1A1) are the most important isoforms implicated in the glucuronidation and sulfation of paracetamol in rats [14, 15]. This allows rat liver tissue to be incubated with high concentration of paracetamol (5 mM), without exhibiting any sign of cell toxicity up to 6 hours of incubation [16]. Midazolam is mainly metabolized by CYP3A isoforms (CYP3A2 in majority), leading to 1′-hydroxy-, 4-hydroxy- and 1′,4-dihydroxymidazolam, the later representing a minor metabolite, as phase 1 metabolites [17].

In the present study, we have adapted an ultrarapid method described by Glöckner *et al* [11] allowing to freeze rat PCLS, and we have tested the capacity, after 2, 4, and 24 hours of incubation, of thawed PCLS to produce paracetamol glucuronide and sulfate, and 1′-hydroxy- and 4-hydroxymidazolam. The results have been correlated 1. to the content of protein isoforms involved in xenobiotic metabolism and 2. to the maintenance of ATP and glycogen levels in the PCLS and 3. to the LDH leakage in the medium as 'cytotoxic marker'.

Methods

Chemicals

Williams' medium E and fetal calf serum (FCS) were purchased from Gibco BRL (Middlesex, UK). Dimethyl sulfoxide (DMSO), gentamicin sulfate, leupeptine, PMSF, antipain, dithiothreitol (DTT), EDTA, triton and enzymes (the purest grade available) were obtained from Sigma chemicals (St Louis, USA). L-Glutamine was purchased from ICN Biomedicals Inc (Ohio, USA), paracetamol from Janssens Pharmaceutica (Beerse, Belgium), flunitrazepam from Roche (Grenzach-Wyhlen, Germany) and 2-acetamidophenol from Aldrich Chemicals Co (Dorset, UK). Midazolam (Dormicum®, Roche, Grenzach-Wyhlen, Germany), insulin (Actrapid HM®, Novo Nordisk, Bagsvaerd, Danemark) and dexamethasone phosphate (Decadron®, MSD, New Jersey, USA) were commercialy available. Rabbit antibody to rat CYP3A2, rabbit antibody to rat CYP2E1, goat anti-rabbit IgG HRP and rabbit anti-sheep IgG HRP were purchased from Chemicon (Temecula, USA). Sheep antibodies to rat ST1A1 was generously provided by Dr MWH Coughtrie (University of Dundee, Scotland, UK) and rabbit antibody to rat UGT1A6 by Dr Shin-ichi Ikushiro (Himeji Institute of Technology, Hyogo, Japan). Anti-rabbit IgG-POD was purchased from Boehringer® (Germany). Western immunobloting kit employing enhanced chemiluminescence detection and nitrocellulose membranes were obtained from Amersham (UK). All other chemicals used were of the purest grade commercially available.

Preparation of PCLS

The liver from adult male Wistar rat from Iffa Credo (Brussel, Belgium) (280–320 g) was perfused *in situ* with ice-cold Krebs-Ringer solution prior to liver removal. The liver was kept in ice-cold oxygenated Krebs-Ringer solution before slicing. Slices (250 μm thickness) were prepared in oxygenated ice-cold Krebs-Ringer buffer using a Krumdieck slicer according to a procedure previously described [3]. Fresh slices were stored (30 min – 1 h) in ice-cold Williams' Medium E supplemented with glutamine (2 mM), insuline (100 nM), dexamethasone (10 nM) (hereafter referred to as "preincubation medium") and 10% FCS (v/v) until incubation.

Slice cryopreservation and thawing

Slice cryopreservation and thawing were performed according to the method described by Glöckner *et al* [11]. Prior to freezing, the slices were stored in ice-cold "preincubation medium" supplemented with 10% DMSO (v/v) for 30 min. The slices were then put into cryogenic vials (five to six slices per tube) without any medium, followed by ultrarapid freezing by immersion in liquid nitrogen. The slices were kept in liquid nitrogen for at least 10 min before thawing.

Two ml "preincubation medium" supplemented with 10% FCS (39°C) were added on frozen slices and the vials were placed into a water bath at 39°C. After thawing, the slices were washed in "preincubation medium" (see above) containing 10% FCS at 4°C for 5 min and then incubated as the fresh ones.

Slice incubation

Slices were placed into vials containing "preincubation medium" supplemented with glucose to reach a final concentration of 25 mM and gentamicine (5.10^{-3} % w/v) (2 ml medium

per slice). The vials were saturated with a mixture of 95% O_2 / 5% CO_2 and placed in a shaking water bath at 37°C. Following 1 hour of preincubation (0 h- time), allowing fresh slices to recover ATP and glycogen content, the slices were transferred to other vials containing fresh medium for 2 to 24 hours. At various time points, incubation medium samples and slices were taken for analysis.

LDH release

The viability of liver slices was estimated by measuring the activity of lactate dehydrogenase (LDH) in the culture medium, according to the procedure of Wroblesky and Ladue [18]. The results are expressed as international units (IU) LDH per mg of protein. The amount of protein was determined by the method of Lowry [19] using BSA as standard.

ATP content

Liver slices were taken, washed twice in saline and sonicated in 1 ml of 2% perchloric acid. The intracellular ATP content was measured on neutralized perchloric acid extracts using ATP Bioluminescence Assay Kit CLS II from Boehringer (Germany).

Glycogen content

Liver slices were taken, washed twice in saline and sonicated in 1 ml of 1 M KOH. They were further heated at 100°C for 10 min. After neutralisation with acetic acid and centrifugation, the supernatant was incubated in the presence of α-amylo glucosidase in pH 5 acetate buffer. The glucose produced was quantified by an enzymatic reaction as previously described [20].

Paracetamol metabolism

Paracetamol glucuronide and paracetamol sulfate were quantified by using reverse-phase HPLC according to the procedure of Lau and Critchley [21]. Fresh and cryopreserved liver slices were incubated during 2 hours in medium supplemented with 5 mM paracetamol. At the end of the incubation, aliquots of incubation medium were stored at −20°C for further analysis. After centrifugation, 25 μl of samples together with 50 μl of internal standard 2-acetamidophenol (400 μg/ml) were injected on a Nova-Pak C18 column. The mobile phase (0.1M KH_2PO_4/0.1% acetic acid/0.75% propane-2-ol) was delivered to the column at a flow rate of 1.50 ml/min. The results were expressed as μg metabolites/mg protein.

Midazolam metabolism

1′-OH midazolam and 4-OH midazolam were quantified by using a method described by Eeckhoudt *et al* [22]. Fresh and cryopreserved liver slices were incubated during 30 min in medium supplemented with midazolam (0.125 mg/2 ml). Aliquots of incubation medium were stored at −20°C for further analysis. 300 μl of an aliquot of medium were treated with 1 ml of 0.3 M sodium acetate buffer (pH 5) containing 5000 IU/ml β-glucuronidase and 38 μl flunitrazepam (5 μg/ml). After incubation for 2 hours at 37°C, 200 μl of 2% NaOH and 4.5 ml of cyclohexane-diethyl ether (31:69) were added and the mixture was extracted on a rotary mixer for 10 min at 4°C and then centrifuged at 2000g for 10 min at 4°C. The organic

phase was transferred to a clean tube and evapored to dryness under a stream of nitrogen. The residue was dissolved in water-acetonitrile (95:5) and 20μl were injected onto the HPLC column. The results were expressed as μg metabolites/mg protein.

Western immunoblotting

Liver slices were sonicated in Tris HCl buffer (pH 7.4) containing PMSF 0.1 mM, leupeptine (2.10^{-4} % w/v), antipain (2.10^{-4} % w/v), DTT 2 mM, EDTA 0.1 mM and triton 0.4 %. After centrifugation, the supernatant was stored at $-30°C$. The proteins were subjected to (SDS)-polyacrylamide gel electrophoresis (10 % separating gel) following by electroblot to nitrocellulose or PVDF membrane. The membrane was blocked 1 hour at 25°C in TBS buffer (pH 7.4) containing 5% (w/v) powdered milk protein followed by an incubation with diluted antibodies at 25°C or 4°C (CYP2E1) in a fresh solution of powdered milk protein (1% w/v) in TBS buffer. The membrane was washed and incubated with a dilution of secondary antibody at 25°C. The primary and secondary antibodies were diluted respectively 1/8000 and 1/10000 for CYP3A2, 1/2500 and 1/5000 for CYP2E1, 1/4000 and 1/10000 for UGT1A1, and 1/10000 and 1/15000 for ST1A1. Immunodetection was performed using the ECL[TM] detection kit (Amersham). The film was scanned and the density of the bands was calculated using the program Image Master (Pharmacia Biotech Benelux, Roosendaal, The Netherlands).

Histological study

Slices were fixed in 10% formalin for histopathological analysis. Paraffin sections (5 μm) were stained by haematoxylin and eosin.

Statistical analysis

All the parameters were measured at least in duplicate on two PCLS obtained from the same rat. A mean value has been calculated for each experiment. The results presented in the tables and figures correspond to the mean $\pm$ SEM of at least 4 experiments (it means experiments performed on at least 4 different rats). A paired student-t test was performed for the statistical comparison of AUC_{fresh} vs $AUC_{cryopreserved}$ in all the curves presenting the evolution of the parameters with time according to statistical guidelines [23] or comparison of the value of fresh vs cryopreserved PCLS at each incubation time.

Results

Viability of fresh PCLS

LDH leakage into the incubation medium and tissue glycogen and ATP levels were measured over a 24 hr incubation time period to assess the integrity and viability of fresh liver slices (Table 1).

LDH leakage gradually increased during the incubation. The ATP level slighty increased as a function of time of incubation whereas the glycogen level decreased by 55% during the first 4 hours of incubation. After 24 hours, it returned to 66% of the value measured at time 0.

The capacity of fresh PCLS to metabolize paracetamol and midazolam after 0, 2, 4 or 24 hours of incubation is shown in table 2.

Table 1
LDH leakage into the medium and glycogen and ATP content of fresh liver slices

Incubation time	Medium-LDH	Tissue-glycogen	Tissue-ATP
h	IU/mg protein	μg/mg protein	nmol/mg protein
0	ND	190.7 ± 25.1	12.1 ± 2.6
2	0.028 ± 0.008	116.5 ± 23.7	14.7 ± 2.0
4	0.101 ± 0.038	85.8 ± 17.2	14.2 ± 2.6
24	0.765 ± 0.209	125 ± 26.5	19.1 ± 1.9

Data are expressed as the mean ± SEM (n=4 or 5). ND = not determined.

Paracetamol metabolites were measured in the medium after a 2-hr incubation time in the presence of paracetamol. This period (2 h) ensures the linearity in the formation of the glucuronide and sulfate conjugates (data not shown). After 4 hours of incubation, no significant modification of metabolite production occurred. From this time on, the concentration of paracetamol glucuronide decreased with time of incubation, and represented 28% of time 0 - value, after 24 hours of incubation. On the other hand, paracetamol sulfate decreased from 4 hours, but to a lower extent (paracetamol sulfate represented 55% of the initial value after 24 hours of incubation).

Midazolam metabolites were measured in the medium after a 30-min incubation time in presence of midazolam. This period (30 min) ensures the linearity in the formation of the 4-hydroxy and 1′-hydroxy conjugates (data not shown). Under these conditions, the production of 4-hydroxy and 1′-hydroxymidazolam started to decrease after 2 hours of incubation to reach approximately 40% of the initial value after 24 hours.

Cryopreserved PCLS

LDH leakage in the medium of cryopreserved PCLS is presented in fig 1A. It increased with time of incubation and is at all time points higher than the value measured in fresh slices.

Glycogen and ATP content of cryopreserved PCLS are presented in fig 1B and 1C. At time 0, both glycogen and ATP content were lower than the values obtained in fresh slices. Glycogen gradually decreased with time of incubation. The absolute concentration being 10.6 μg/

Table 2
Release of paracetamol and midazolam metabolites in the culture medium by fresh PCLS

Incubation time	Paracetamol metabolite		Midazolam metabolite	
	glucuronide	sulfate	4-hydroxy	1′-hydroxy
h	μg/mg protein		μg/mg protein	
0	8.2 ± 1.4	8.6 ± 0.9	0.471 ± 0.045	0.337 ± 0.057
2	6.3 ± 1.1	7.7 ± 0.8	0.465 ± 0.046	0.349 ± 0.093
4	6.1 ± 1.1	7.1 ± 1.6	0.295 ± 0.043	0.192 ± 0.044
24	2.3 ± 1	4.7 ± 1.4	0.177 ± 0.033	0.133 ± 0.033

Paracetamol and midazolam metabolites released in the culture medium by fresh PCLS after 2 h incubation in presence of paracetamol or 30 min in presence of midazolam at various incubation time. Data are expressed as the mean ± SEM (n=5 or 6).
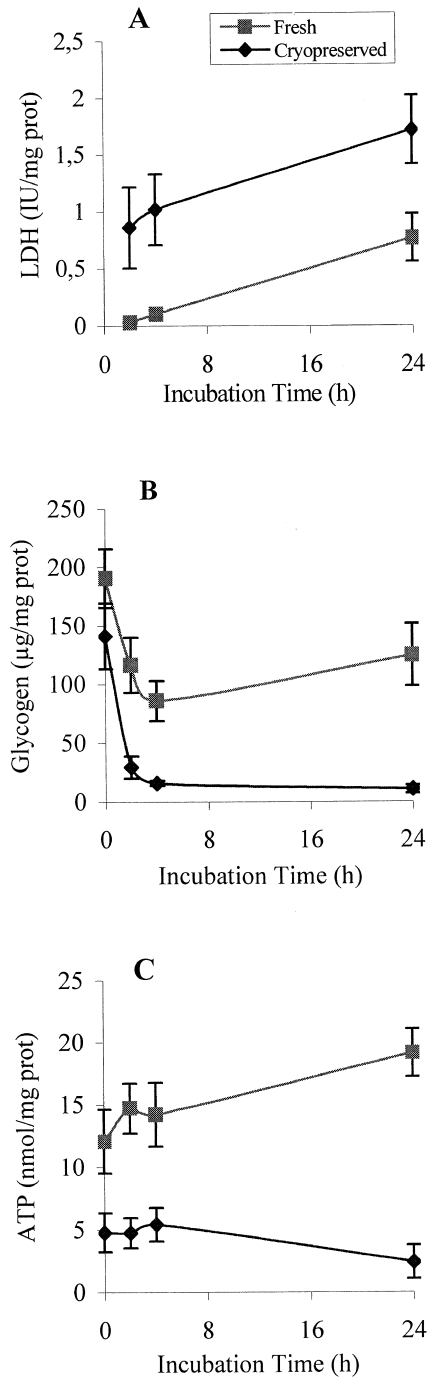
Fig. 1. LDH leakage into the medium (A) and glycogen (B) and ATP content (C) of fresh and cryopreserved PCLS after thawing at various incubation time. Data are expressed as the mean $\pm$ SEM (n=4 or 5). Paired student-t test: $p<0.05$ for comparison of AUC_{fresh} vs $AUC_{cryopreserved}$ or comparison of the value of fresh vs cryopreserved at each incubation time.
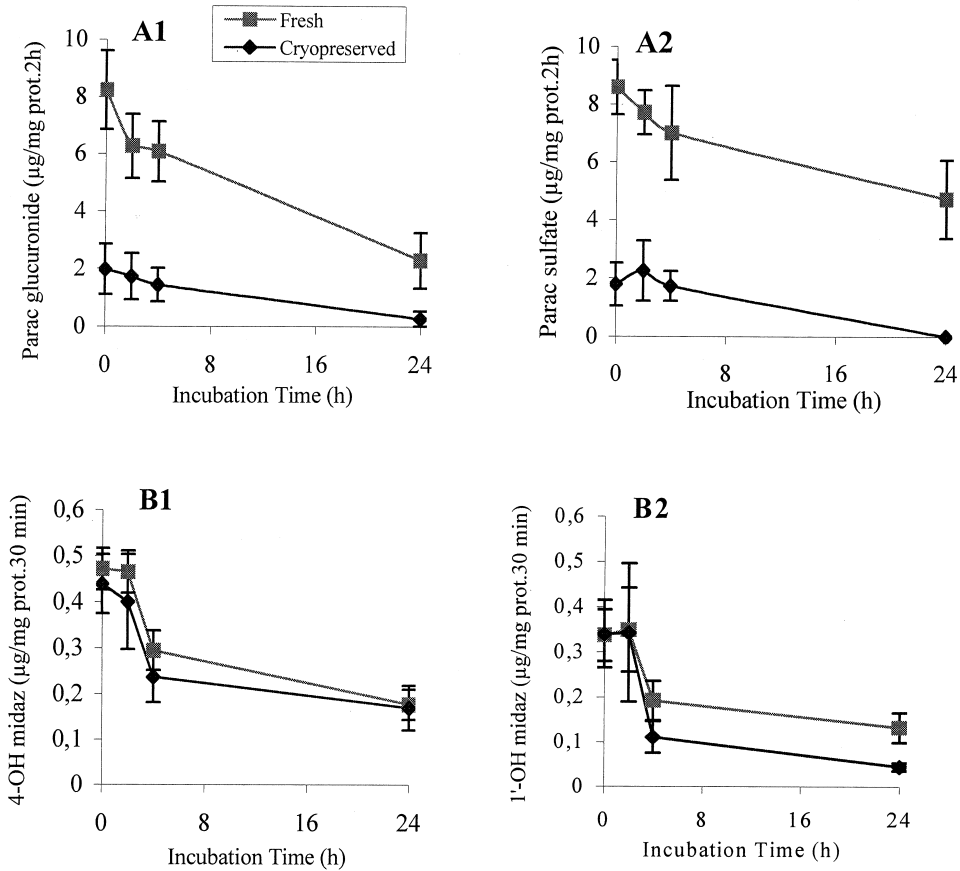
Fig. 2. Paracetamol glucuronide (A1) and sulfate (A2) formation during 2 h incubation in the presence of parace-tamol and 4-hydroxy- (B1) and 1′-hydroxymidazolam (B2) formation during 30 min incubation in the presence of midazolam at various incubation times in fresh and cryopreserved slices.

mg protein after 24 hours. ATP was maintained at approximately 35 % of the control value at least until 4 hours, reaching 13% after 24 hours.

The amount of paracetamol and midazolam metabolites in the medium of fresh and cryo-preserved slices is presented in fig. 2. Paracetamol glucuronide and sulfate amount released in the medium of cryopreserved PCLS were strongly decreased immediately at time 0 as compared with values obtained in fresh slices.

4-Hydroxy- and 1′-hydroxymidazolam concentration were maintained at relatively high levels all along the incubation as compared to fresh PCLS and reach, respectively, 96 and 34 % of the control value after 24 hours of incubation.

Western immunobloting

The protein levels of CYP3A2, CYP2E1, UGT1A1 and ST1A1 are presented in fig. 3. Cryopreservation of liver slices did not alter the amount of CYP3A2, CYP2E1 and UGT1A1
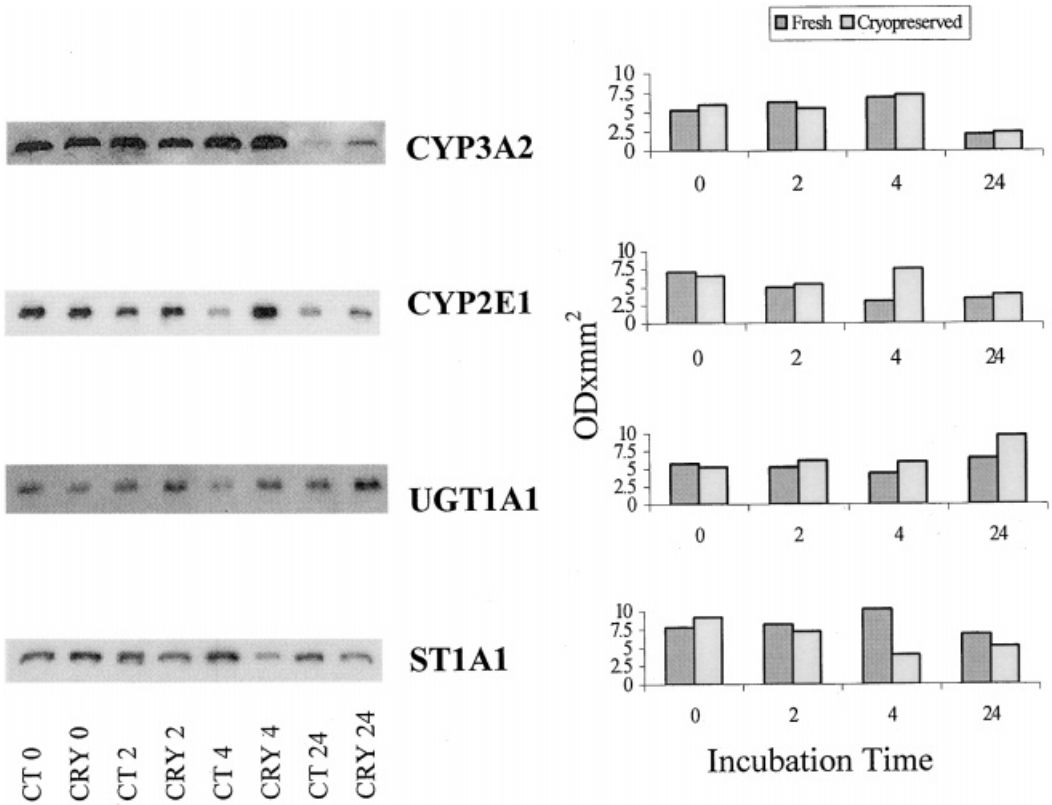
Fig. 3. Immunoblot of fresh and cryopreserved PCLS after 0, 2, 4 and 24 hours incubation. Gels were loaded with 10 μg protein/lane (CYP3A2), 2.5 μg protein/lane (CYP2E1 and ST1A1) or 2 μg protein/lane (UGT1A1). Photographs of the developed films were densitometrically quantified and data were expressed as ODxmm^2 for the various incubation times.

as compared to fresh slices. However, we observed a slight decrease in the amount of ST1A1 from 2 hours incubation onwards.

Histological study

We observed in cryopreserved slices, even at time 0, some picnosis in hepatocytes evoluting into cell death that was seen after 24 hours of incubation (necrosis foci disseminated in the whole slices). Despite this fact, cryopreserved PCLS maintain some intact cells (foci) all along the incubation. Only some cells evoluting to picnosis could be histologically detected in fresh PCLS after 24 hours of incubation.

Discussion

In the present study, we have analysed the time course of the metabolic capacity and the functionnality of cryopreserved PCLS, previously cryopreserved by using an ultrarapid method, adapted from Glöckner *et al* [11]. Most of the studies published until now compared

thawed versus fresh PCLS, by presenting data obtained at one short-term (2 to 6 hours) time point of incubation. The originality of the present study was to compare the time course during 24 hours of incubation of parameters measured in parallel in fresh and cryopreserved PCLS. Moreover, we have chosen to measure parameters which are very sensitive to metabolic alterations due to mechanical or oxidative stress such as ATP and glycogen level.

The concentration of the phase I metabolites of midazolam, namely 4-hydroxy and 1′-hydroxymidazolam, released in the medium was maintained at the maximum level for 2 hours of incubation, then decreased with time to the same extent in both fresh and thawed PCLS. The decrease of total metabolites concentration reached, after 24 hours of incubation, about 40% of the value measured at time 0, and was correlated to the decrease of CYP3A2 protein content in PCLS, this isoform being one of the major involved in midazolam hydroxylation in rat liver [17]. It has already been described that the regulation of CYP3A enzymes may occcur at several levels of gene expression but the catalytic activity is often related to the protein level [24]. However, in our study, we have shown that a decrease in the production of 1′-hydroxy and 4-hydroxymidazolam occurred after 4 hours of incubation in fresh and cryopreserved PCLS whereas CYP3A2 content was maintained at the initial level.

Since we have analyzed the phase II metabolism by using paracetamol as a model substrate, it seemed interesting to measure also time course of CYP2E1 tissue levels, this isoform being one of the major cytochrome P450 isoforms responsible for the oxidative reaction of paracetamol to n-acetylparabenzoquinoneimine (NAPQI), a reactive metabolite. In fresh PCLS, there was a progressive decrease of CYP2E1 content, reaching 50% of the initial value after 24 hours of incubation. Cryopreservation seemed to maintain a better CYP2E1 level, at least until 4 hours of incubation. The decrease in total CYP content during incubation of fresh PCLS has already been described by several authors. Wright and Paine [25] have shown that CYP level represented only 26% of the initial value after 24 hours and Lake *et al* [26] have demonstrated that total CYP reached about 40% of the time 0 value after 48 hours incubation. In fresh human PCLS incubated for 24 hours, CYP2E1 activity reached 10% of the time 0 value whereas CYP3A activity was not longer detectable [27].

The amount of paracetamol glucuronide and sulfate released in the medium of fresh PCLS only slightly decreased after 4 hours of incubation but decreased to approximately 30% and 55% of the time 0-value after 24 hours of incubation, respectively. The decrease in UDP-glucuronosyltransferase and sulfotransferase activity after 24 hours of incubation in cultured rat PCLS has already been described by Hashemi *et al* [28]. As compared to the values obtained with fresh slices, the release of those phase II metabolites drastically dropped at each incubation time. After 24 hours of incubation, almost no paracetamol sulfate and paracetamol glucuronide could be measured in the medium of thawed slices. Despite this fact, cryopreservation did not significanlty affect the amount of UGT1A1 content in PCLS, even after 24 hours of incubation. ST1A1 content was relatively constant in fresh PCLS but a slight decrease is observed in cryopreserved PCLS that can not explain the lower amount in sulfoconjugates measured in the medium.

As illustrated in the present study, conjugation reactions may be strongly affected despite the fact that the levels of the corresponding transferase isoforms are maintained in the tissue. Glucuronidation and sulfation may be dependent on the availability of their cofactors, respectively UDP-glucuronic acid (UDPGA) and 3′-phosphoadenosine-5′-phosphosulfate (PAPS).

Could it be influenced by cryopreservation ? Glucuronidation is dependent to carbohydrate metabolism. UDPGA is derived from glucose-1-phosphate and UTP in a two step reaction requiring NAD^+. Glucose-1-phosphate for the formation of UDPGA required for glucuronidation comes from glycogenolysis [29] and the addition of paracetamol in the incubation medium of PCLS leads to a decrease in glycogen content in the slice [4]. *In vivo*, the administration of a high dose of acetaminophen also leads to a dramatic fall in the glycogen level in fed rats [30] and mice [31]. Since, before paracetamol addition to the medium, the glycogen level decreased more rapidly and was much lower in thawed than in fresh slices, this lower avaibility of glucose moieties for UDPGA synthesis could contribute to the lower glucuronidation capacity. This process is also dependent on ATP levels [32] which are lower in thawed than fresh PCLS. The dependence of PAPS synthesis on ATP level is supported by many *in vitro* studies in which several substances leading to a decrease of ATP level (through glycolysis inhibitors like 2-deoxyglucose, mitochondrial electron transport inhibitors, uncouplers of oxidative phosphorylation and addition of fructose) strongly decreased PAPS level and/or the sulfation process [33]. Thus it is possible that the decrease in ATP levels, occurring in the present study immediately after thawing, could be involved in the lower sulfoconjugation activity of cryopreserved PCLS towards paracetamol. However, this hypothesis does not explain the decrease in paracetamol and midazolam metabolites observed in fresh PCLS after 2 hours of incubation. The synthesis of UDPGA and PAPS is also dependent of several enzymes (UTP-glucose pyrophosphorylase and UDP-glucose dehydrogenase for UDPGA and ATP-sulfurylase and APS-kinase for PAPS). A decrease in the activity and/or content of this enzymes could also explain a decrease in the metabolites measured in the medium of both fresh and cryopreserved PCLS.

Cell lysis, demonstrated here through the LDH leakage in the medium, occurs at the beginning of the incubation. Those results are in accordance with the ones recently published by Day *et al* [8], who have shown that the LDH and ALT retention in cryopreserved rat PCLS decreased at 70% of the content measured in fresh slices after 2 hours of incubation. It seems that, depite this fact, some metabolic activity, like urea synthesis and 7-ethoxycoumarin sulfate formation may be maintained, at least for a short period of time (2 hours), in cryopreserved slices. We have also observed a decrease in glycogen content in cryopreserved PCLS as compared to fresh ones, which dropped dramatically within 2 hours following the start of incubation. The ATP level was also much lower in cryopreserved than in fresh PCLS, but was maintained at the same level (35 % of the fresh values) at least for 4 hours of incubation. Those parameters indicate a loss of functionnality and viability of cryopreserved PCLS but despite this fact, those PCLS maintain CYP-dependent activity at the same level than fresh ones. In our study, we have also performed an histological examination of PCLS. We have shown, in cryopreserved PCLS, even at time 0, some picnosis in hepatocytes, evoluting into cell death (necrosis) foci disseminated in the whole slices that is seen after 24 hours of incubation. However PCLS maintained some intact hepatocytes even after 24 hours of incubation. Only some cells evoluting to picnosis could be histologically detected in fresh PCLS after 24 hours of incubation. The results thus show that the decrease in ATP and glycogen level together with an increase in LDH released in the medium are in accordance with the histological examinations.What is surprising is the fact that, despite those alterations, cryopreservation of slices allows, after thawing, the maintenance of CYP dependent activity and content.

ATP and glycogen levels are lower in cryopreserved compared to fresh slices directly upon incubation. This suggests that all events responsible for the lack of maintenance of metabolic activities could occur during the time of preincubation at 37°C following thawing. We are now concentrating our efforts to analyse the metabolic behaviour of cryopreserved PCLS during this preincubation period in order to try to restore the ATP and glycogen content before starting the incubation period.

Acknowledgments

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