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Use of Fresh and Cryopreserved Human Liver Slices in Toxicology with Special Reference to *In Vitro* Induction of Cytochrome P450*

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Abstract—Human liver slices were prepared with a Krumdieck slicer from macroscopically healthy surgical waste after partial hepatectomy. They were incubated without or with the addition of the inducer β-naphthoflavone (BNF) (25 μM) either immediately after preparation (fresh slices) for up to 24 hours or after cryopreservation in liquid nitrogen (thawed slices) for up to 6 hours. Potassium concentration was well maintained in fresh and thawed slices over 24 and 6 hours, respectively, but at lower levels than in rat liver slices. Albumin secretion showed relatively large interindividual differences. Both parameters were lower in thawed slices than in fresh ones, but indicated a certain number of viable cells. In untreated fresh slices CYP1A1-mRNA was not detectable; however, it increased distinctly within 6 hours of exposure to BNF. The amounts of induced CYP1A1-mRNA differed by a factor of more than 100 among six human livers and were lower than in fresh rat liver slices. Even in thawed human slices, CYP1A1-mRNA expression could be induced *in vitro* by BNF, although at a very low level and preferentially in those specimens with comparably high inducibility already before freezing.

Keywords: precision-cut liver slices; human cryopreservation; CYP1A1-mRNA; RT-PCR; β-naphthoflavone; *in vitro* induction; albumin secretion; potassium.

Abbreviations: BNF = β-naphthoflavone; CYP = cytochrome P450; DMSO = dimethyl sulfoxide.

INTRODUCTION

Precision-cut liver slices are used increasingly for several investigations including studies on xenobiotic metabolism. Recent reviews summarize the current state of this model system (Ekins, 1996; Olinga, *et al*., 1997; Parrish *et al*., 1995), pointing out the advantages of tissue slices over isolated cells, applications as well as open questions. In our laboratory, fresh rat liver slices have been incubated for up to 48 hr with high viability and relatively stable functions including stable potassium content, high rate of albumin secretion, maintained 7-ethoxycoumarin *O*-deethylation activity or maintained pattern of testosterone metabolites (Müller *et al*., 1998). Furthermore, in the same paper we demonstrated the *in vitro* inducibility of CYP1A1-mRNA expression in rat liver slices by exposure to BNF for 6 hr. The amounts of CYP1A1-mRNA could be quantified by competitive RT-PCR and were found to be in the range of about 10^{-16} mol/μg total RNA after BNF exposure *in vitro*. In untreated fresh rat liver slices this specific mRNA was not detectable (concentrations below 1.36×10^{-19} mol/μg total RNA were not searched for), thus the induction factor was at least 1000. Immunohistochemical proof of *in vitro*-induced CYP1A1 24 hr after BNF exposure revealed enzyme induction preferentially in the superficial layers of rat liver slices (Neupert *et al*., 1998).

The expression of several CYP forms and its inducibility by xenobiotics has toxicologic relevance, and species differences have to be expected. Therefore, the use of human tissue is necessary to prove the suitability of this *in vitro* model. In this paper our first results with fresh human liver slices are shown. As rare human material should be optimally utilized, the possibility of cryopreserving remaining parts of tissue would be of advantage. In principle, cryopreservation of tissue slices and their use after thawing is possible, although followed by functional loss (cf. Olinga *et al*., 1997). Our own results from cryopreserved rat liver slices have been

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recently summarized (Glöckner *et al.*, 1998), demonstrating reduced retention of potassium compared to the high levels in fresh rat liver slices. Nevertheless, parts of cryopreserved slices survived, and even albumin secretion was detectable during 2–4 hr of incubation after thawing. Additionally, *in vitro* exposure of cryopreserved rat liver slices to BNF induced the expression of CYP1A1-mRNA within 6 hr, but to a lower extent than in fresh slices, as proved by simple or competitive RT-PCR. Up to now long-term incubation of cryopreserved rat liver slices was not successful, thus limiting their application to experiments for up to 6 hr so far. Now, cryopreservation of human liver slices was performed to test viability and *in vitro* CYP inducibility during short-term incubation after thawing.

MATERIAL AND METHODS

Human liver tissue was obtained from macroscopically healthy surgical waste after partial hepatectomy (for details see Table 1). The tissue was stored in ice-cold carbogen-saturated Krebs–Henseleit HEPES buffer immediately after resection. Preparation of tissue cores (diameter 10 mm) and slicing in ice-cold carbogen-saturated Krebs–Henseleit HEPES buffer using a Krumdieck slicer (slice thickness approx. 250 μm) was performed within about 1 hr. Fresh slices were incubated immediately in 50-ml Erlenmeyer flasks containing 10 ml Williams' Medium E with insulin (1 μM) and gentamicin (50 mg/litre) at 37°C and gassed with carbogen in a bidirectionally shaking water-bath (untreated slices). For CYP induction, BNF was added to the incubation medium [finally 25 μM, dissolved in dimethyl sulfoxide (DMSO), finally 0.2%]. The procedure of cryopreservation has been described recently (for details see Glöckner *et al.*, 1998). The slices were exposed to ice-cold Sack's solution containing 10% DMSO for 15 min, put into plastic cryopreservation tubes (four to five slices per tube) without any medium, followed by rapid freezing and storing in liquid nitrogen. For thawing, the tubes were placed into a water-bath (37°C), and immediately 2 ml Sack's solution (37°C, DMSO-free) were added. After thawing the slices were washed twice in Sack's solution at room temperature and then incubated in the same manner as fresh ones.

The determination of potassium content, albumin secretion and CYP1A1-mRNA expression was performed as described for fresh rat liver slices (Müller *et al.*, 1998) with modifications of RT-PCR and competitive RT-PCR: The primers for PCR were targeted to sites in the c-DNA corresponding to genomic exon 7 producing amplicons of 277 bp. Forward primer hum CYP1A1: 5′-TAGACACTGATCTGGCTGCAG-3′ (Omiecinski *et al.*, 1990); reverse primer hum CYP 1A1: 5′-CTGAAGCCAGTCAGCACCCTCA-3′. 35 - 45 PCR cycles (20″ 94°C, 20″ 59°C, 30″72°C) were performed. The RNA used as internal standard was prepared as follows: The amplicons of 277 bp were used as template for PCR (40 cycles each consisting of 20″ 94°C, 20″ 59°C, 30″ 72°C) with the following composite primers: forward: 5′-TAATACGACTCACTATAGGTAGACACTGATCTGGCTGCAG-3′; reverse: 5′-TTTTTTTTTTTTTTTCTGAAGCCAGTCAGCACCCTCATCTTCAGAGCTTCTCAG-AGGCCT-3′. The amplicons were purified by agarose electrophoresis and were used as template for RNA synthesis by T7 RNA-polymerase (RIBOMAX-T7 system, PROMEGA). With the above mentioned primer pair, these RNA produced RT-PCR amplicons of 246 bp with a sequence nearly idendically to the amplicons derived from native human CYP1A1 mRNA. For potassium determination after cryopreservation, two slices instead of one were used.

RESULTS

Fresh slices

Potassium content as a measure of slice viability did not decrease during 24 hr of incubation (Fig. 1). Albumin secretion differed considerably among individual livers ranging from 0.4 to 2.5 $\mu g \times hr^{-1} \times mg$ protein^{-1} after 2 hr incubation. After 24 hr it was maintained or decreased in two

Table 1. Concentration of specific CYP1A1-mRNA in fresh liver slices from six human livers after 6 or 24 hr of incubation in BNF containing medium as determined by competitive RT-PCR. Age of patients is given in parentheses: f = female, m = male, MTS = metastases, CA = carcinoma. In addition to drugs mentioned below, anaesthesia was performed with etomidate or propofol, isoflurane, fentanyl and atracurium

	Patient		CYP 1A1-mRNA (mol/μg total RNA)	
	Disease	Administered drugs	6 hr BNF exposure	24 hr BNF exposure
P1 f (67)	MTS of sigma CA	NAC, metamizol	9.71×10^{-18}	1.11×10^{-17}
P2 f (47)	hepatocell. CA	metronidazole, glibenclamide, metoprolol, hydrochlorothiazide	3.47×10^{-19}	6.07×10^{-19}
P3 m (49)	MTS of sigma CA	ranitidine, atenolol	3.47×10^{-18}	1.93×10^{-18}
P4 f (52)	portocaval fistula	digitoxin, verapamil, isosorbide-5-mononitrate, losartan	2.71×10^{-20}	not determined
P5 m (58)	Klatskin-tumour	none	5.42×10^{-20}	not determined
P6 f (57)	MTS of colon CA	pirenzepine, lactulose, verapamil	3.47×10^{-19}	not determined

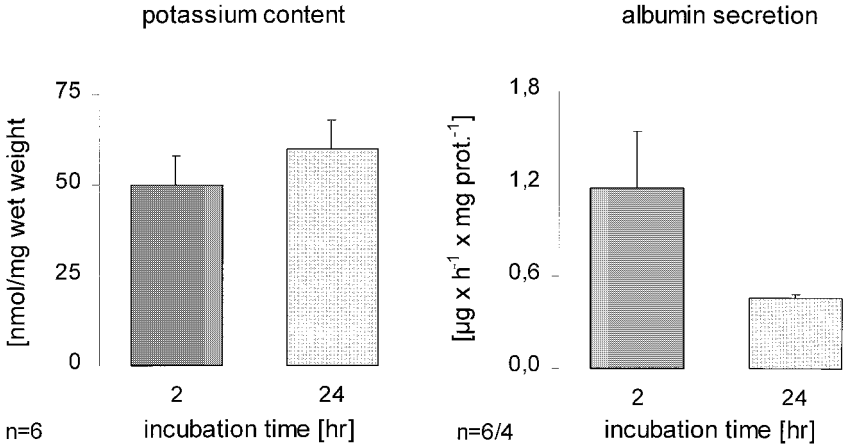

Fig. 1. Potassium content and albumin secretion of fresh human liver slices after 2 or 24 hr of incubation. Arithmetic means and SEM are shown. No significant differences between groups (paired *t*-test, $P \leqslant 0.05$).

samples each; the absolute values were very similar in all four samples (from two samples we did not have enough material to determine 24 hr values).

In untreated slices, CYP1A1-mRNA was detected neither after 6 nor after 24 hr of incubation, but in all cases distinct expression of this mRNA could be induced by 6 and 24 hr of exposure to BNF with no substantial differences between both times. One example of RT-PCR results is given in Plate 1. Considerable interindividual differences were found by competitive RT-PCR (Table 1), the mean concentration being $2.33 \pm 1.57 \times 10^{-18}$ mol CYP1A1-mRNA/μg total RNA after 6 hr of BNF exposure.

Cryopreserved slices

After 2 hr of incubation, potassium content was slightly lower than in fresh slices and did not decrease up to 6 hr of incubation (Fig. 2). Albumin secretion was measurable in all three samples investigated so far. In two of these three cases it reached the same range as the corresponding (relatively low) values before the freezing of slices, in one case (the highest fresh value at all) it decreased considerably after thawing.

RT-PCR was performed with all six specimens incubated in the absence or presence of BNF for 6 hr. A higher number of PCR cycles than with fresh slices was necessary to show the PCR products corresponding to CYP1A1-mRNA. In all but one case this mRNA was not detected in untreated cryopreserved slices. In three cases, which corresponded to the fresh samples with quite high concentrations of induced mRNA (cf. Table 1: P1, P2, P3), expression was clearly detectable after BNF exposure. In one case with traces of CYP1A1-mRNA even before BNF exposure (P3), its expression was distinctly enhanced by the inducer, thus amounting to 3.47×10^{-19} mol/μg total mRNA. This example of competitive RT-PCR is shown in Plate 2. In the other two cases with visible BNF effect the competitive PCR was not performed.

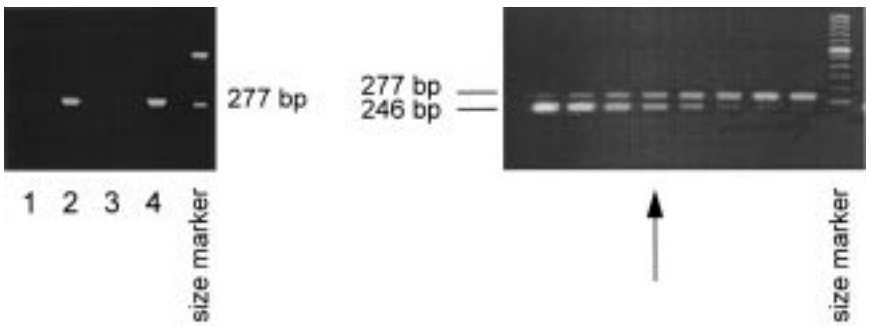

Plate 1. Electrophoresis of 277 bp RT-PCR products, corresponding to CYP1A1-mRNA (left) and of 277 bp as well as 246 bp RT-PCR products, the latter corresponding to internal standard RNA (right, competitive RT-PCR). RNA was isolated from fresh human liver slices after incubation for 6 or 24 hr (lanes 1 and 3: untreated slices after 6 and 24 hr of incubation, respectively; lanes 2 and 4: slices after BNF exposure for 6 and 24 hr, respectively. For competitive RT-PCR only a sample after BNF exposure for 6 hr is demonstrated. The arrow indicates approximately equal concentrations of both RT-PCR products.

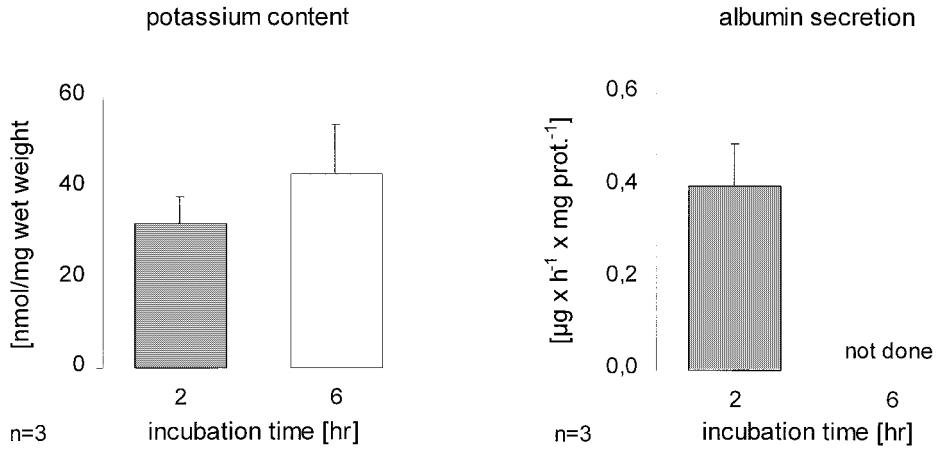

Fig. 2. Potassium content and albumin secretion of cryopreserved human liver slices after 2 or 6 hr of incubation. Arithmetic means and SEM are shown. No significant differences between groups (paired *t*-test, $P \leqslant 0.05$).

DISCUSSION

In principle, our standard protocol developed for the use of fresh rat liver slices (Müller *et al.*, 1998) is also suitable for human liver tissue, resulting in slices with acceptable viability and function. Potassium content in human slices proved to be lower than in rat liver slices and was in the range of other investigations (Fisher *et al.*, 1991, 1993) or even higher (Olinga *et al.*, 1998a,b). Albumin secretion by human liver slices as a liver specific differentiated function has not been published so far. In most of our six samples it was lower than in rat liver slices (Müller *et al.*, 1998). Interindividual differences have to be expected as for other parameters determined in human tissue. Our most important result—the proof of *in vitro* inducibility of CYP1A1 at the level of mRNA expression—encourages hope that this relatively simple and rapid procedure might be suitable to test xenobiotics for inducing effects on other mRNAs also. The proof of inducibility by measurement of enhanced CYP concentrations or increased metabolic rates in human liver slices, as descibed in the literature (Lake *et al.*, 1996, 1997), demands longer incubation with the risk of decreasing basic functions. In all six specimens used in this series of experiments, CYP1A1-mRNA was not detectable in untreated slices. However, we cannot exclude that other human livers might be induced *in vivo* before the preparation of slices. Further investigations are necessary to characterize *in vitro* inducibility after preceding *in vivo* induction. It is remarkable that a 6-hr exposure to BNF is enough for a maximal increase in CYP1A1-mRNA. After 24 hr no further increase in mRNA concentrations was detectable.

Cryopreserved human liver slices have been scarcely used so far. The potassium content of our cryopreserved slices was in the range of literature data (Fisher *et al.*, 1991, 1993) being 15–35% lower than in corresponding fresh slices. Other parameters of viability have also been described to decrease by 15–35% (de Kanter *et al.*, 1998). The maintenance of albumin secretion seems to depend on the indi-

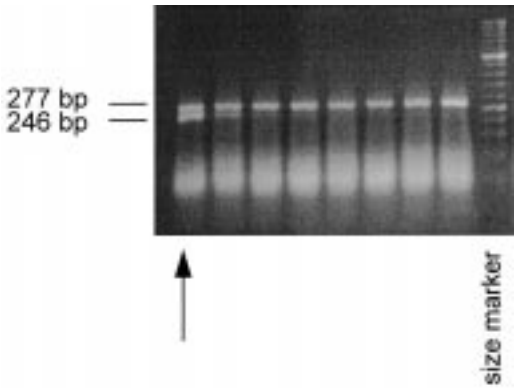

Plate 2. Electrophoresis of 277 bp and 246 bp RT-PCR products, corresponding to CYP1A1-mRNA and internal standard RNA, respectively, following competitive RT-PCR. RNA was isolated from cryopreserved human liver slices after BNF exposure for 6 hr. The arrow indicates approximately equal concentrations of both RT-PCR products.

vidual conditions of each liver taking into account the interindividual difference before freezing. In the livers investigated, there was no correlation between potassium concentration and albumin secretion. Perhaps this is due to differences in viability among cryopreserved slices from the same liver, which cannot be excluded. Nevertheless, a certain number of cells survived an incubation for up to 6 hr so that inducibility of CYP1A1-mRNA expression was possible *in vitro* at least in some samples. De Kanter *et al*. (1998) described that testosterone, lidocaine and 7-ethoxycoumarin metabolism was maintained at more than 80% after 1–3 hr of incubation of cryopreserved human liver slices. Inducibility *in vitro* after cryopreservation has not been reported in the literature so far. This can be proved only by methods detecting the first steps of changed gene expression because of the relatively short time of survival of cryopreserved tissue slices, and seems to depend additionally on sufficient quality of the slices before freezing. Further optimization of cryopreservation (including the subsequent incubation) has to be performed to enhance the amount of surviving cells.

In summary, fresh human liver slices can be used to detect the potency of xenobiotics of changing CYP1A1-mRNA expression *in vitro*. In principle, cryopreserved human slices are suitable, provided prerequisites are optimal.

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