Chapter 2

Phase I and phase II metabolic activities are retained in liver slices from mouse, rat, dog, monkey and human after cryopreservation

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Abstract

Precision-cut liver slices are described as valuable tool for in vitro metabolism studies of potential drug candidates. Recently, some papers reported successful cryopreservation conditions for liver slices, facilitating a broader and more efficient use of the tissue (particularly of human origin). The aim of this study is to evaluate the effect of cryopreservation on both phase I and phase II metabolism in liver slices prepared from mouse, rat, dog, monkey and human, using rapid freezing in the presence of 18% DMSO. Glucuronidation and sulfation activities (phase II) in both freshly prepared and cryopreserved liver slices were determined by rapid LC-MS/MS analyses using 7-hydroxycoumarin as a marker substrate. Testosterone was used as a marker substrate for cytochrome P450 mediated drug metabolism (phase I). Although the metabolic patterns and rates varied among the different species, the phase I and phase II metabolic capacities of the liver slices were well maintained after cryopreservation. Despite the good biotransformation capacity of cryopreserved slices a decrease in viability, expressed as ATP content and LDH leakage, was observed. MTT reduction was well maintained after cryopreservation. The possibility to cryopreserve liver slices will allow a more efficient utilisation of tissue, in particular from human, but also from dog and monkey. Finally, cryopreserved liver slices from mouse, rat, dog, monkey and human with good phase I and II metabolism activities are a useful in vitro tool to compare metabolite profiles of new chemical entities between species.

Introduction

In recent years much effort has been devoted to the development of in vitro systems and precision-cut liver slices are now a widely used tool for in vitro metabolic and toxicological studies [1]. The main advantage of this model over isolated hepatocytes is that slices can be easily prepared from rat, mouse, monkey, dog and man using the same method. The relative simple and straightforward preparation technique facilitates cross species comparison studies within drug discovery and development. During the past few years, different methods for slice cryopreservation have been described, such as slow [2,3], or rapid freezing [4-6] and vitrification [7], which differ mainly by the cooling rate and the concentration of cryoprotectant used. From a comparison between slow and rapid freezing, it was concluded that rapid freezing (by simply immersing into liquid nitrogen) was found optimal above slow freezing [8], which is normally used for the cryopreservation of isolated hepatocytes [9]. The possibility to cryopreserve liver slices made it possible to take advantage of the large number of slices that can be prepared from one liver (in particular from human liver). Cellular and drug metabolism activities in rat, monkey and human liver slices cryopreserved by fast freezing have been shown to be well maintained for up to 24 hours in culture [10-15] and was improved by raising the DMSO concentration to 18% (v/v) [16,17]. However, most studies were performed with rat or human liver slices and focus mainly on phase I mediated drug metabolism. As one of the most useful applications of cryopreserved liver slices is likely to be the comparison of drug metabolism profiles of new chemical entities in different laboratory species with that in man, we investigated the feasibility of the fast freezing method on liver slices from rat, mouse, dog, monkey and man.
For this, both phase I and phase II metabolism in fresh and cryopreserved liver slices prepared from mouse, rat, dog, monkey and human liver were studied. The phase II conjugating enzymes, UDP-glucuronosyltransferase and sulphotransferase, were assayed, using 7-hydroxycoumarin (7-HC) as a diagnostic substrate. Further, the pattern of hydroxylation of testosterone by several isoforms of cytochrome P450 (CYP) [18] was measured to characterize phase I metabolism. Besides metabolic capacity, also slice viability was assessed by measuring ATP-content, 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT) -reduction and lactate dehydrogenase (LDH) release.

Materials and Methods

Materials. The following compounds were obtained from the sources indicated: Williams’ Medium E, 7-hydroxycoumarin, 7-hydroxycoumarin glucuronide, dimethylsulfoxide (DMSO), d-glucose, insulin, corticosterone, androstene-3,17-dione and MTT were from Sigma-Aldrich (St. Louis, MO, USA); testosterone was from Fluka (Buchs, Switzerland); testosterone metabolites and 7-hydroxycoumarin sulphate were from Ultrafine Chemicals (Manchester, UK). University of Wisconsin organ preservation solution was from DuPont (Waukegan, IL, USA).

Animals. Male Sprague-Dawley rats (n=2) and male CD-1 mice (n=7, slices from these animals were pooled together) were maintained under a 12-h light/dark cycle in standard cages and bedding with free access to commercial food pellets and tap water. After anaesthesia with sodium thiopental (rat) or ketamine/xylazine/acepromazine (10/2.5/1) (mouse), the liver was removed and stored in cold Williams’ Medium E until use (max. 0.5 h). Without sacrificing the animals, small liver samples of male Beagle dog (n=1) and male Cynomolgus monkeys (n=2) were removed by surgery under ketamine (dog) and thiopental (monkey) anaesthesia and stored in ice-cold Williams’ Medium E until use (max. 0.5 h).

Organs. Human liver tissue was obtained from patients (n=4) subjected to surgery for carcinoma. Donors had given informed consent and were both male and female (aged 35-79 yr). Tumour-free tissue that was considered as surgical waste was used and stored in ice-cold University of Wisconsin preservation solution until use. Storage time was max 0.5 h.

Liver slice preparation. Liver slices (diameter 8 mm) were prepared in ice-cold Williams’ Medium E that was oxygenated with 95% O2 / 5% CO2 and supplemented with extra glucose (final conc. 25 mM), using a Krumdieck tissue slicer [1]. Cutting speed and slice thickness were properly regulated during the preparation. The slices obtained were subsequently stored in ice-cold Williams’ Medium E until use (within 1 h after the preparation).

Cryopreservation. The method of cryopreservation used is based on the so-called fast freezing method, as described earlier [6,16]. Shortly, the cryopreservation medium consisted of Williams’ Medium E containing 18% DMSO (v/v) as cryoprotectant. Five slices were incubated in an Erlenmeyer flask containing 5 ml of this cryopreservation medium. The flasks were shaken horizontally either at 54 times min-1 (mouse, rat, dog and monkey slices) or at 90-100 times min-1 (human slices) for 30 min at 4°C under 95% O2 / 5% CO2 atmosphere. After this incubation, the slices were transferred directly into a freezing vial containing 0.5 ml of ice-cold cryopreservation medium and immediately immersed and stored in liquid nitrogen (-196°C).

Thawing and pre-incubation of liver slices. Cryopreserved liver slices were thawed by transferring the freeze vials directly from liquid nitrogen into a water bath at 37°C. After thawing, slices were washed with ice-cold Williams’ Medium E to remove DMSO, and pre-incubated for 1 h at 37°C as described below. Fresh liver slices were pre-incubated under the same conditions.

Incubations. Slices were individually incubated in 6 well culture plates (Falcon, France) in 2 ml Williams’ Medium E under 95% O2 / 5% CO2 atmosphere (one slice per well) at 37°C while shaking horizontally at 90-100 times min-1. Both fresh and thawed slices from each liver were incubated in triplicate.

Testosterone metabolism by liver slices. Fresh and cryopreserved liver slices were incubated with 250 µM testosterone for 90 minutes. Prior to the immersion of the slices, testosterone was administered to the incubation medium as a 200-times concentrated solution in
methanol. Reactions were terminated by snap-freezing slices and medium together in vials using liquid nitrogen and stored at -20°C. After thawing, the internal standard corticosterone was added and samples were disrupted using a MSE Ultrasonic disintegrator (Fisons, Loughborough, UK). After centrifugation at 10000 g for 10 min, the pellet was discarded and the supernatant was evaporated under a stream of N₂ gas at 37°C for 1 hour. The dried-residues were reconstituted in water: MeOH (50:50 v/v) and analysed by reverse-phase HPLC.

The samples were injected at room temperature onto a C₁₈ Symmetry Column (5 µm, 4.6 x 150 mm, Waters) with a Symmetry guard column (5 µm). The mobile phases employed were solvent A (water) and solvent B (methanol: acetonitrile 9:1 v/v). During the 20-min run, the proportion of B in the mobile phase was increased from 45 to 70%. Flow rate was maintained at 1 ml/min.

The absorption of the eluent at 254 nm was monitored continuously using a UV/VIS detector. The elution order of standards after UV detection was: 6β-, 7α-, 16α-, 16β-, 2α-, 2β-hydroxytestosterone (OHT), corticosterone (internal standard), androstenedione and testosterone.

The quantification of testosterone metabolites present in the samples was performed by integration of the peaks detected, after normalisation with the internal standard corticosterone and comparing with a standard curve of authentic metabolites. The results were expressed as pmol/min/slice.

**Conjugation of 7-hydroxycoumarin by liver slices.** Fresh and cryopreserved liver slices were incubated with 50 µM 7-hydroxycoumarin for 90 minutes. Prior to the immersion of the slices, 7-hydroxycoumarin was administered to the incubation medium as a 1000-times concentrated solution in methanol. Reactions were terminated and samples were stored as described for testosterone. After thawing, samples were disrupted using a MSE Ultrasonic disintegrator (Fisons, Loughborough, UK). After centrifugation the samples at 10000 g for 10 min, the pellet was discarded. The supernatant was analysed by LC-MS/MS, using a Turbo Ion Spray source in positive mode and a HP1100 binary pump coupled to a Triple Quadrupole API 2000 (PE-Sciex). A SB-C₈ 5-µm, 4.6 x 12.5 mm column (Zorbax - Agilent Technologies) was applied using a mobile phase containing 10 mM ammonium formate, pH 4.0, and acetonitrile increasing from 5% to 95% within 0.4 min and then back to 5% in 1.4 min. The flow rate was 1 ml/min for the first 0.2 min to equilibrate the column quickly and after 0.2 min after injection the flow rate was reduced to 0.6 ml/min. The eluent from the column was led into the MS system only between 0.3 min and 1.05 min after injection of the sample. The positive ion/mass spectra were acquired by injecting 10 µl of the samples and quantification was performed by comparing the peak areas with authentic standards of each metabolite.

**ATP content.** The ATP content was determined using the ATPLite-M kit from Packard (Groningen, the Netherlands). Directly after incubation, slices were homogenised using a MSE Ultrasonic disintegrator (Fisons, Loughborough, UK) in 3 ml of incubation medium. 100 µl of slice homogenate was then added to a 96 well plate containing 50 µl of cell lysis solution from the ATPLite-M kit and gently mixed for 5 minutes at room temperature. Then, 50 µl of substrate solution (luciferase/luciferin) from the ATPLite-M kit was added to the plate and mixed for another 5 minutes. The plate was left in the dark for 10 minutes and the luminescence was read using a TopCount NXT Luminescence Instrument from Packard (Groningen, the Netherlands). The amount of ATP was calculated from a standard curve prepared and assayed under identical conditions.

**LDH release.** Directly after incubation, aliquots of liver slice culture medium and slice homogenate (from the ATP determination) were frozen into liquid nitrogen and stored at -80°C. After thawing, the aliquots were diluted ten folds with PBS containing 1% of BSA and 50 µl was measured for LDH activity using the Gr780 Promega kit (Madison, WI, USA). Results are expressed as percent of total liver slice LDH released into the culture media (LDH media + LDH slice = total LDH; LDH media/total LDH × 100 = % of total LDH released).

**Reduction of MTT.** Slices were tested for their activity towards the reduction of tetrazolium salt MTT. Fresh and cryopreserved liver slices were incubated individually with 1.2 mM MTT in 2 ml Williams’ Medium E at 37°C for 90 minutes and subsequently transferred into 1 ml acidified isopropanol. After extraction for 30 minutes, the absorbance of the organic phase was determined at 690 nm.
Results

Liver slices were prepared with a wet weight of 20-25 mg. After storage in liquid nitrogen (1 week – 3 months), cryopreserved liver slices from rat, mouse, monkey, dog and human were compared with fresh liver slices for testosterone metabolism, 7-hydroxycoumarin metabolism, ATP content, LDH release and MTT content.

**Testosterone metabolism by liver slices.** Incubation of liver slices with testosterone resulted in the formation of a variety of hydroxylated products that found to be linear with time for at least 90 min (not shown). Testosterone hydroxylation varied significantly between the different species tested, but did not change qualitatively after cryopreservation (figure 1).

The rate of formation of 16α-, 16β-, and 2α- (in rat) of 2β- (in dog) hydroxytestosterone and androstenedione (rat and mouse) significantly increased after cryopreservation, while no differences were observed in cryopreserved slices from monkey and human. Figure 2 shows a representative testosterone HPLC chromatogram from a fresh and a cryopreserved human liver slice, showing the testosterone metabolites detected.

![Figure 1](image.png)

**Figure 1.** Formation of testosterone metabolites (6β-, 7α-, 16α-, 16β-, 2α-, 2β-hydroxytestosterone and androstenedione) in fresh and cryopreserved liver slices after 90 min incubation. Values are means of independent experiments (rat n=2; mouse n=7, slices from these animals were pooled together; dog n=1; monkey n=2; human n=4), for each experiment three slices were used.
Figure 2. Representative testosterone metabolism chromatograms of fresh (A) and cryopreserved (B) human liver slices after 90 min incubation, showing the metabolites of testosterone, 6β-, 16α-, 16β-, 2β-hydroxytestosterone, androstenedione and the internal standard corticosterone. X is an unknown metabolite.
Conjugation of 7-hydroxycoumarin by liver slices. Incubation of liver slices with 7-hydroxycoumarin gave rise to both sulphate and glucuronic acid conjugates. The formation of conjugates was linear with time for at least 90 min (not shown). Figure 3 illustrates the metabolite formation from 7-HC in both fresh and cryopreserved liver slices. 7-HC was preferentially conjugated with glucuronide by both fresh and cryopreserved human liver slices, whereas the conjugation with sulphate was minimal. In both fresh and cryopreserved liver slices from mouse, rat, dog and monkey, both a significant amount of 7-hydroxycoumarin sulphate and glucuronic conjugates were formed, illustrating the maintained glucuronidation and sulphation capacity after cryopreservation. There were no significant differences observed in the formation of 7-hydroxycoumarin conjugates by fresh and cryopreserved liver slices from all species studied.

Figure 3. Formation of 7-hydroxycoumarin glucuronide and 7-hydroxycoumarin sulphate in fresh and cryopreserved liver slices after 90 min incubation. Values are means of independent experiments (rat n=2; mouse n=7; slices from these animals were pooled together; dog n=1; monkey n=2; human n=4), for each experiment three slices were used.
**Liver slices cryopreservation**

**ATP content.** After cryopreserved slices were thawed and subsequently cultured for 2 hours, ATP content was determined. The cryopreserved liver slices revealed markedly lower ATP level in rat (-70%), mouse (-40%), monkey (-50%) and human (-40%) in comparison to fresh prepared liver slices (Table 1).

<table>
<thead>
<tr>
<th>Species</th>
<th>Fresh</th>
<th>Cryopreserved</th>
<th>Ratio thawed/fresh</th>
</tr>
</thead>
<tbody>
<tr>
<td>rat</td>
<td>8.5 ± 3.8</td>
<td>3.2 ± 0.4</td>
<td>0.3</td>
</tr>
<tr>
<td>mouse</td>
<td>6.6 ± 0.7</td>
<td>3.7 ± 0.5</td>
<td>0.6</td>
</tr>
<tr>
<td>dog</td>
<td>n.d.</td>
<td>1.2 ± 0.4</td>
<td>-</td>
</tr>
<tr>
<td>monkey</td>
<td>1.9</td>
<td>0.9</td>
<td>0.5</td>
</tr>
<tr>
<td>human</td>
<td>6.8 ± 0.4</td>
<td>3.9 ± 0.7</td>
<td>0.6</td>
</tr>
</tbody>
</table>

Data are means ± SD of independent experiments (rat n=2; mouse n=7, slices from these animals were pooled together; dog n=1; monkey n=2; human n=4), for each experiment three slices were used, except for monkey where only one slice was used.

**LDH leakage.** Cryopreserved liver slices from rat and mouse showed a 69-85% leakage of total LDH content, which was considerable more than the leakage from fresh slices from all studied species, except from monkey that showed only a low loss of intracellular LDH. The LDH release from cryopreserved monkey and human liver slices did not differ significantly from that of the corresponding fresh liver slices (Table 2). The total LDH content from cryopreserved liver slices of all species did not differ significantly from that of the corresponding fresh liver slices (Table 2).

<table>
<thead>
<tr>
<th>Species</th>
<th>Fresh</th>
<th>Cryopreserved</th>
<th>Total medium</th>
<th>Percentage leakage</th>
</tr>
</thead>
<tbody>
<tr>
<td>rat</td>
<td>1337</td>
<td>44 (3 %)</td>
<td>1030</td>
<td>706 (69%)</td>
</tr>
<tr>
<td>mouse</td>
<td>521</td>
<td>165 (32%)</td>
<td>479</td>
<td>409 (85%)</td>
</tr>
<tr>
<td>dog</td>
<td>n.d.</td>
<td>n.d.</td>
<td>251</td>
<td>73 (20%)</td>
</tr>
<tr>
<td>monkey</td>
<td>204</td>
<td>16 (8%)</td>
<td>214</td>
<td>12 (6%)</td>
</tr>
<tr>
<td>human</td>
<td>99</td>
<td>16 (16%)</td>
<td>118</td>
<td>36 (31%)</td>
</tr>
</tbody>
</table>

Data are means ± SD of independent experiments (rat n=2; mouse n=7, slices from these animals were pooled together; dog n=1; monkey n=2; human n=4), for each experiment three slices were used, except for monkey where only one slice was used.

**Reduction of MTT.** In contrast to ATP and LDH, there were no significant differences in the level of MTT reduction observed between fresh and cryopreserved liver slices of all species (Table 3).
Table 3. MTT reduction by fresh and cryopreserved liver slices

<table>
<thead>
<tr>
<th>Species</th>
<th>Fresh (OD$_{690}$/slice)</th>
<th>Cryopreserved</th>
<th>Ratio thawed/fresh</th>
</tr>
</thead>
<tbody>
<tr>
<td>rat</td>
<td>0.67 ± 0.05</td>
<td>0.66 ± 0.11</td>
<td>1.0</td>
</tr>
<tr>
<td>mouse</td>
<td>0.56 ± 0.05</td>
<td>0.54</td>
<td>1.0</td>
</tr>
<tr>
<td>dog</td>
<td>0.50 ± 0.07</td>
<td>0.36 ± 0.02</td>
<td>0.7</td>
</tr>
<tr>
<td>monkey</td>
<td>0.50 ± 0.10</td>
<td>0.57 ± 0.05</td>
<td>1.0</td>
</tr>
<tr>
<td>human</td>
<td>0.37 ± 0.07</td>
<td>0.27 ± 0.04</td>
<td>0.7</td>
</tr>
</tbody>
</table>

Data are means ± SD of independent experiments (rat n=2; mouse n=7, slices from these animals were pooled together; dog n=1; monkey n=2; human n=4), for each experiment three slices were used, except for mouse (cryopreserved liver slices) where only one slice was used.

**Discussion**

Previous studies have demonstrated the usefulness of precision-cut liver slices as an *in vitro* model system for investigating drug metabolism and toxicity [1]. Most of these studies focus on rat and/or human liver slices and address only phase I metabolism. In the current study, five species (including human) were compared for both phase I and II metabolism. In addition, some parameters indicative for slice viability were investigated. Using the same cryopreservation method for all species, no significant loss of both phase I and phase II mediated drug metabolism was observed after cryopreservation. The results obtained in liver slices are comparable with previous findings as reviewed before [19]. As expected, the different species demonstrated marked differences in metabolite patterns of testosterone and 7-hydroxycoumarin and these qualitative differences between the species were maintained after cryopreservation. The main testosterone metabolites, observed in fresh and cryopreserved liver slices in each species, were 6β-OHT and androstenedione. 7α-OHT, a CYP2A mediated metabolite, was only detected in fresh and cryopreserved slices from mouse and rat, while 2α-OHT, mediated by CYP2C, was only detected in fresh and cryopreserved rat liver slices. 2β-OHT was detected in all species studied. 16α-OHT and 16β-OHT were formed only in liver slices from rat, monkey and human.

When slices were incubated with 7-hydroxycoumarin, phase II metabolism took place and gave rise to the formation of both glucuronide and sulphate conjugates in each species. After cryopreservation, the rates of conjugation of 7-hydroxycoumarin to its glucuronide and sulphate derivates were unaffected. Although less pronounced as observed with testosterone, the formation of 7-hydroxycoumarin glucuronide and 7-hydroxycoumarin sulphate was different among the species as well. Remarkable was the observation that in human liver slices, the amount of 7-hydroxycoumarin sulphate was minimal.

The results of the viability parameters measured, demonstrated that some degree of tissue damage was found in cryopreserved liver slices, in particular ATP and LDH release were affected due to cryopreservation. ATP content was decreased and LDH release increased (except for monkey and human), whereas the total LDH content was unaffected during the cryopreservation. On the other hand, the mitochondrial reduction...
of MTT was not changed after cryopreservation. The use of various viability parameters allows one to investigate cellular injury at different sites. These cellular sites include energy status, membrane integrity, and the ability of cells to carry out various biochemical functions. Together, these viability parameters provide a better picture of the overall state of the tissue slice than when only one parameter was investigated. According to the current results, the sensitivities of these viability parameters to detect cellular dysfunction can be ranked as follows: ATP > LDH > MTT. This is in accordance with other results, as reviewed before [19]. Although it seems that the decrease in ATP content and the increase in LDH leakage does not affect phase I and II metabolism, it might limit the application of cryopreserved liver slices in toxicological studies [1,20].

The current results confirm that cryopreservation of liver slices is an useful method for characterizing both phase I and phase II drug metabolism and that the method is suitable for the cryopreservation of liver slices from various species. In contrast, previously developed methods for preserving liver slices had to be adapted for each species [2,21]. Another advantage of the present procedure is its simplicity without the need of special equipment as is needed for computer-controlled freezing or total vitrification. Also, cryopreserved liver slices from man and animal species in drug metabolism research can reduce the use of experimental animals [20].

In conclusion, the method described here permits the formation of a liver slice bank from rat, mouse, monkey, dog and human, allowing the easy comparison of qualitative differences in metabolic profiles (both phase I and phase II mediated) of potential drug candidates among different species, including human.
Liver slices cryopreservation

References

Liver slices cryopreservation
