Human and rat organ slices

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A simple method for cryopreservation of liver slices from man and other species

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Abstract

The cryopreservation of liver slices permits optimal use of large livers and provides for the availability of the slices at any desired time. Moreover, it allows one to study liver slices from various species in the same experiment. We studied the effect of cryopreservation on the viability and metabolic activity of liver slices from rat, monkey, dog, hamster, minipig, rabbit and human. Precision-cut liver slices were prepared using a Krumdieck tissue slicer. Human donor liver tissue was obtained and studied at the Dept. Pharmakinetics and Drug Delivery at the University of Groningen. Animal livers were from control animals in toxicity studies at Solvay. Slices were cryopreserved by incubation in 12% dimethylsulphoxide (DMSO) in Williams’ medium E for 30 min on ice, followed by fast freezing into liquid nitrogen. Viability was determined by measuring alanine aminotransferase activity retention in the slices, and by measuring urea synthesis, and phase I and II metabolic activity by testosterone metabolism. The viability of rat, monkey, dog and human liver slices after thawing was retained close to fresh values during 3 h of incubation (80-100% of fresh values). In addition, no significant differences were detected in metabolite patterns of the three drugs tested between fresh and cryopreserved liver slices. These results show that cryopreservation of liver slices from various species by the same simple cryopreservation procedure can maintain viability and metabolic competence as measured in fresh slices. Therefore, cryopreserved liver slices from various species can be used to compare the metabolite patterns of xenobiotics such as candidate drugs. Efficient use of tissue can be accomplished by cryopreserving liver slices, thus reducing the use of experimental animals. In addition, optimization of the choice of animal species for toxicity testing of drugs in development allows reduction of experimental animal use and facilitates a safer first administration to man.

Introduction

Precision-cut liver slices have been proven to be a valuable tool in drug development and many other research areas (for a review, see [109]). However, the availability of livers from larger animals and man is limited, whereas at the same time - when available - the amount of tissue exceeds the actual experimental capacity. Cryopreservation of liver slices could therefore facilitate more efficient utilization of the tissue and permit their use at any desired time. Moreover, cryopreservation of human liver slices would allow comparison of liver characteristics of individual human subjects, and between man and other species, in one experiment. In drug development, cryopreserved liver slices from various species, may be used to select, for toxicity studies, the animal species that opti-
nally mimics human metabolism of the drug under investigation. This will prevent toxicity studies in inadequate animal models, reduce animal use, and result in a safer first administration to humans.

Previously, it was shown that a simple cryopreservation procedure, which was optimized for cooling rate, DMSO (dimethylsulphoxide) concentration and pre-freezing conditions, retained urea synthesis capacity and testosterone metabolism in rat and monkey liver slices [62]. This method consists of a 30-minute pre-freezing period in the presence of 12% DMSO on ice, fast cooling by direct immersion into liquid nitrogen and quick thawing [62].

Here, we describe the application of this cryopreservation method to liver slices from rat, monkey, dog, hamster, minipig, rabbit and man. We compared freshly prepared and cryopreserved liver slices according to various functional parameters. Urea synthesis from ammonia and ornithine was studied to obtain information about the integrity of various steps in the urea cycle and energy status. Cytochrome P450 activity was studied by determination of the metabolism of testosterone, covering various cytochrome P450 iso-enzymes activities and availability of co-factors.

Materials and Methods

Livers. Human liver tissue was obtained from livers taken from multi-organ donors. Consent from the legal authorities and from the families concerned was obtained for the explantation of organs for transplantation purposes. The donor livers were reduced in order to perform reduced size or split liver transplantation. The donor livers were perfused in situ with cold University of Wisconsin solution, before explantation. The livers were stored in cold UW solution on ice until reduction of the liver. The liver tissue remaining after bipartition, which was considered as surgical waste, was stored again in cold UW solution until the start of the slicing procedure (total cold storage period: 10-16h).

Male Wistar rats, male and female Beagle dogs were obtained from Harlan CPB (Zeist, the Netherlands); a male New Zealand White rabbit from Broekman Instituut (Someran, the Netherlands); a female rhesus monkey from the Biomedical Primate Research Center (Rijswijk, the Netherlands) was sacrificed because of intestinal infection; Cynomolgus monkeys (male and female) from Inveresk Research International (Edinburgh, UK); the male Yucatam minipig from NOTOX (ʼs-Hertogenbosch, the Netherlands) and the hamster was from the University of Utrecht (Utrecht, the Netherlands). Monkeys and dogs were sacrificed as controls in toxicity studies for new drugs. All the animals had free access to food and water until sacrifice.

Preparation of liver slices. Precision-cut liver slices (8 mm) were prepared in ice-cold Krebs-Henseleit buffer, by using a Krumdieck tissue slicer, as described earlier [18]. The wet weight of the slices was 16-20 mg.

Incubation. Slices were individually incubated for 3 h in 25 ml Erlenmeyer flasks, containing 5 ml Williams medium E (Gibco BRL, Breda, the Netherlands) under carbogen atmosphere (95% O2/5% CO2). The Erlenmeyer flasks were shaken back and forth (110 times/min) in a 37°C water bath (GFL, Hannover, Germany). Incubations were supplemented with NH4Cl (10 mM) and ornithine (1 mM) (Sigma-Aldrich Chemie, Zwijndrecht, the Netherlands) for urea synthesis or testosterone (0.25 mM, dissolved in methanol; Fluka, Buchs, Switzerland). After various time points, medium samples were taken, then the slices were homogenized on ice in the used incubation medium by sonication.
(human slices) (Vibra cell, Sonics materials, Dandurry, Connecticut, USA) at level 4 for 10 s, or (animal slices) by using a Potter-Elvehjem homogenizer (B. Braun, Melsungen, Germany). Aliquots of medium and homogenate were stored at -80°C until required for analysis.

Freezing and thawing conditions. Slices were cryopreserved and thawed as described earlier [62]. Briefly, five slices were pre-incubated in 5 ml Williams’ medium E, containing 12% (v/v) DMSO, on ice for 30 min. The five slices and 1 ml medium were then quickly transferred into 2-ml freeze vials (Greiner, Alphen a/d Rijn, the Netherlands) and immersed directly in liquid nitrogen. Thawing was performed quickly in a 37°C water bath. Immediately after thawing, slices were transferred to 10 ml fresh, oxygenated, ice-cold Williams’ medium E, to wash out the DMSO for 1 min. Slices were then transferred to Erlenmeyer’s flasks and incubated exactly as for freshly prepared slices.

Biochemical determinations. Urea synthesis during a 3 h incubation period was determined colorimetrically in medium samples [62]. The formation of hydroxylated testosterone metabolites from testosterone was determined by HPLC in homogenate extracts after incubation for 3 h [313]. Slice protein content was determined in the homogenate using Coomassie Blue as described elsewhere [62].

Results

Urea synthesis in fresh and cryopreserved liver slices from various species is shown in figure 1. Urea synthesis, which involves various energy-demanding steps and transport across mitochondrial membranes, is maintained after cryopreservation. Also, inter-species differences in urea synthesis were maintained after cryopreservation.

In figure 2 the biotransformation of testosterone to the main metabolite (of 3-10 that were detected) by fresh and cryopreserved liver slices is shown in figure 2. We conclude that cryopreserved liver slices maintained their capacity for testosterone metabolism in the species studied. From the HPLC chromatograms, it was also concluded that metabolite profiles (which differ between species) are fully maintained after cryopreservation (data not shown).

An important application of liver slices is species comparison of metabolite profiles of drugs in experimental animals and humans in drug development.

Figure 1. Urea synthesis in fresh and cryopreserved animal liver slices. Values are means of data for five slices ± SEM.
Therefore, liver slices from 3 human individuals were investigated for urea synthesizing capacity (table 1) and metabolism of testosterone (table 2). Because of the inter-individual differences in human subjects, results are individually presented. It is shown that, in cryopreserved liver slices, $6\beta$-hydroxytestosterone formation is close to the amount formed in fresh liver slices. Not surprisingly, there are considerable inter-individual differences in testosterone biotransformation rates.

**Discussion**

From the urea and testosterone results presented for animal and human liver slices, we conclude that the same freezing method can maintain specific liver functions in liver slices, irrespective of species of origin. This was confirmed by determination of the amount of alanine aminotransferase (ALT) activity retained in slices and of metabolism of lidocaine and 7-ethoxycoumarin (data not shown). We found that thickness and storage conditions appeared to be critical; liver slices weighing less than 16 mg wet weight lost viability after cryopreservation, and storage in liquid nitrogen is imperative [62].

**Table 1. Urea synthesis in human liver slices (nmol/min/mg slice protein)**

<table>
<thead>
<tr>
<th></th>
<th>freshly prepared</th>
<th>cryopreserved</th>
<th>% of fresh values</th>
</tr>
</thead>
<tbody>
<tr>
<td>liver 1</td>
<td>3.0 ± 0.2</td>
<td>3.1 ± 0.4</td>
<td>104 ± 10</td>
</tr>
<tr>
<td>liver 2</td>
<td>11.0 ± 2.4</td>
<td>9.2 ± 0.7</td>
<td>83 ± 16</td>
</tr>
<tr>
<td>liver 3</td>
<td>7.2 ± 0.8</td>
<td>6.5 ± 0.9</td>
<td>89 ± 26</td>
</tr>
</tbody>
</table>

Values are means of data for five slices ± SEM.
An advantage of this method is its simplicity. No special equipment is necessary, allowing the method to be applied in any laboratory, which has the facility to store precision-cut slices in liquid nitrogen.

The results presented suggest the possibility of a significant reduction in experimental animal use by comparing metabolite profiles of new drugs from different species, including man, in one single experiment. In this way, a more rational species choice of the animal species that optimally mimics drug metabolism in man is possible. Also, it is now possible to set up a liver slice bank in liquid nitrogen. Moreover, liver material can be exchanged between laboratories wishing to study diseased liver material or liver from rare donor types.

### Table 2. Testosterone metabolism in human liver slices (pmol 6β hydroxytestosterone/min/mg slice protein)

<table>
<thead>
<tr>
<th></th>
<th>freshly prepared</th>
<th>cryopreserved</th>
<th>% of fresh values</th>
</tr>
</thead>
<tbody>
<tr>
<td>liver 1</td>
<td>194 ± 12</td>
<td>184 ± 36</td>
<td>95 ± 21</td>
</tr>
<tr>
<td>liver 2</td>
<td>143 ± 23</td>
<td>114 ± 27</td>
<td>80 ± 28</td>
</tr>
<tr>
<td>liver 3</td>
<td>117 ± 9</td>
<td>124 ± 9</td>
<td>106 ± 11</td>
</tr>
</tbody>
</table>

Values are means of data for five slices ± SEM.