Chapter 4

The Effect of Hypothermic in Situ Liver Perfusion with TransSend W on the Toxicity of Cryoprotectant Agents in Precision-Cut Liver Slice

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Abstract

Precision cut liver slices (PCLS) are widely used as ex vivo model in studying pathology, and drug metabolism and toxicity because they contain all liver cell types in their original tissue architecture. Successful cryopreservation of PCLS would greatly facilitate their use and commercial standardization. However, toxicity of the cryoprotectants (CPA), especially for vitrification (vitrification solution (VS)), needed to prevent ice crystal formation, appeared to be a major obstacle especially for vitrification-based cryopreservation. Although hypothermic in-situ organ perfusion is beneficial for organ and tissue cold storage before transplantation, its application in cryopreservation of PCLS has not yet been studied. In the present study, we examined if hypothermic liver perfusion with TransSend W, a tissue preservation solution, could improve the condition of rat PCLS and reduce the vulnerability of not only the hepatocytes but also the non-parenchymal cells (NPC) (Kupffer cells, stellate cells and endothelial cells) to the CPA toxicity. Three CPAs (Sol Y, Sol Z and M22) were used. Perfusion appeared to improve the viability of slices exposed to CPA as indicated by ATP and LDH content relative to control slices although morphology did not show a clear difference. M22 appeared the most toxic, whereas Sol Y and Sol Z showed low toxicity (ATP= 90% of control). Immunohistochemical staining of cell specific markers for the different NPC cell types showed that perfusion reduced the loss of marker expression that was induced by exposure to the CPA solutions Sol Z and M22 but not Sol Y, in Kupffer cells and endothelial cells but the beneficial effect was not observed in stellate cells. Perfusion also protected against the reduction of phase I and phase II metabolism in slices exposed to CPA and even restored it to control values for slices exposed to Sol Y and Sol Z. The protective effect of hypothermic perfusion could not be ascribed to an increased glutathione content but was possibly due to a down-regulation of stress-related genes. The differential response of NPC and hepatocytes to CPA toxicity highlights the heterogeneous properties of the various cell types in the liver slices and emphasizes the need for further investigations in the responses of the NPC during cryopreservation. In conclusion, in-situ hypothermic liver perfusion with TransSend W proved to be beneficial toward CPA-induced injury in PCLS, which might be an important step for successful cryopreservation of PCLS.

Keywords: cryoprotective agents; cryoprotectant toxicity; tissue banking; cryopreservation; vitrification; hypothermic perfusion, precision-cut liver slices
Introduction

Precision-cut liver slices (PCLS) are increasingly used as an in vitro model to study drug metabolism and toxicity [1-3]. However, the availability of PCLS especially of human liver is limited and hampered by the irregular and low availability of human liver tissue for research. Cryopreservation of PCLS would strongly improve their application possibilities in ADME-tox studies. However, intracellular ice formation (IIF) which occurs during cryopreservation is strongly correlated to cell death and should be avoided to increase the survival rate of the slices after cryopreservation [4]. Vitrification-based cryopreservation (glass formation without ice formation), appeared to improve the post-cryopreservation viability by preventing mechanical damage from ice crystal formation [5].

To achieve successful vitrification, high concentrations of cryoprotectants (CPA) also called vitrification solution (VSs) containing both permeating CPAs and non-permeating CPAs are needed to prevent ice formation [6, 7]. In general, these CPAs are quite toxic to the tissues, organs and cells at the concentration used for vitrification. Therefore strategies such as reducing the toxicity by combining CPAs with different mechanisms of toxicity (cryoprotectant toxicity neutralization [8]), by loading the tissue with CPA at lower temperature [6] and by reducing the exposure time to the maximal concentration of CPA during CPA loading [9]. By applying these strategies, the CPAs M22 and VM3 were introduced and their toxicity appeared to be low in kidney slices with more than 90% and 80% recovery of ion transport function, respectively [10]. However, our previous data indicated that these CPAs were still toxic in rat PCLS and decreased the ATP content by ca. 40% even when all strategies mentioned above were applied [11].

In-situ hypothermic perfusion is the initial step for organ transplantation to flush out blood cells and was successful to prolong the time for hypothermic preservation and to improve post-operation viability and functions of the transplanted organs [12, 13]. In general, the function of such an hypothermic organ preservation solution is to minimize the damage due to formation of free radicals, maintain ionic balance, minimize cell and tissue swelling, and stimulate recovery upon rewarming [14, 15]. Hypothermic perfusion with TransSend B containing among others the two nonpermeable components, polyglycerol and lactose and the anti-oxidants glutathione and chlorpromazine was shown to be effective as cold flush/cold storage solution for preservation of kidney tissue and protection from damage due to ischemia [16]. After hypothermic kidney perfusion with TransSend B M22 was found to be barely toxic in kidney slices [7, 10] whereas M22 appeared to be toxic in our rat PCLS experiments in which in-situ hypothermic perfusion was absent [11]. So far, in-situ liver perfusion with polyglycerol and lactose containing perfusion solutions as a strategy to protect against CPA toxicity in rat PCLS has not been investigated.

Based on the hypothesis that in situ perfusion, by improving the liver condition prior to CPA exposure, could protect against CPA toxicity, in the present study, we investigated the effect of hypothermic in-situ perfusion with a modified version of the TransSend
solution that was designed for liver preservation TransSend W (Table 1). We investigated the effect of perfusion on the viability and functionality of precision-cut liver slices (PCLS), and on the expression of several genes known to be involved in inflammation and injury of cells. Moreover, using the same parameters, we determined the effect of perfusion with TransSend W on the susceptibility of PCLS to the toxicity of cryoprotectant agents. Three different CPAs (M22, Sol Y and Sol Z) (table 2) were used, which differ in composition with respect to the content of polyvinylpyrrolidone (PVP and N-methylformamide. In addition to the overall response of the PCLS with respect to viability (morphology, LDH and ATP) and redox state (glutathione and reduced glutathione) we paid special attention to the response of the different cell types in the liver slices (hepatocytes, Kupffer cells, stellate cells and endothelial cells), by analyzing the metabolism of 7-ethoxycoumarin (by hepatocytes), the NO production as a reaction to LPS (mediated by Kupffer cells) and the immunohistochemical expression of ED2 (Kupffer cells), RECA-1 (endothelial cells) and desmin (stellate cells).

Materials and Methods

Chemicals and Reagents

D-glucose monohydrate and NaHCO$_3$ were obtained from Sigma-Aldrich (St Louis, MO, USA). Gentamicin and William’s medium E (WME) supplemented with Glutamax I were purchased from Gibco (Paisley, UK). TransSend W solution (Table 1), and premixed concentrated CPAs (M22, Sol Y and Sol Z) and LM5 (Table 2) were a gift from 21st Century Medicine, Fontana, California, USA and are commercially available. SYBR Green and Taq Master Mixes were purchased from Applied Biosystems, Warrington, UK and Eurogentech, respectively. All primers were purchased from Sigma Genosys. University of Wisconsin preservation solution (UW) was from Du Pont Critical Care, Waukegab II, USA. All other chemicals were of the highest purity and are commercially available.

Animals

Male Wistar rats (HsdCpb:WU) (300–350 g) were purchased from Charles River (Maastricht, The Netherlands). The rats were housed under a 12-h light/dark cycle in a temperature- and humidity-controlled room with food (Harlan chow no. 2018, Horst, The Netherlands) and tap water ad libitum. The animal experimental protocol was approved by the Animal Ethical Committee of the University of Groningen.

Preparation of rat PCLS

Rats were anesthetized with isoflurane/O$_2$. For the perfusion experiments the liver was perfused with 30 mL ice-cold TransSend W perfusion medium (Table 1) in situ via the vena cava before the liver was removed. The liver was stored in ice-cold TransSend W until slicing. For the control experiments the livers were removed without perfusion and stored in ice-cold UW solution until slicing. PCLS were prepared as described previously [1]. In
brief, cylindrical cores of 5 mm diameter were made by drilling a hollow drill bit into the liver. These cores were sliced with a Krumdieck tissue slicer (Alabama R&D, Munford, AL, USA) in ice-cold, oxygenated Krebs-Henseleit buffer. PCLS (5 mm diameter, 200-300 μm thick and 5 mg wet weight) from the perfused livers were stored in ice-cold TransSend W solution until use and those from the non-perfused liver were stored in ice cold UW.

### Table 1 Composition of TransSend W preservation solution

<table>
<thead>
<tr>
<th></th>
<th>mM</th>
<th>g/liter</th>
</tr>
</thead>
<tbody>
<tr>
<td>Polyglycerol</td>
<td>49.40</td>
<td>13.00</td>
</tr>
<tr>
<td>GSH</td>
<td>3.25</td>
<td>1.00</td>
</tr>
<tr>
<td>glycerine</td>
<td>3.25</td>
<td>0.24</td>
</tr>
<tr>
<td>Chlorpromazine HCl</td>
<td>N/A</td>
<td>0.0032</td>
</tr>
<tr>
<td>Glucose</td>
<td>63.05</td>
<td>11.36</td>
</tr>
<tr>
<td>α Lactose*H₂O</td>
<td>29.25</td>
<td>10.54</td>
</tr>
<tr>
<td>Na Acetate</td>
<td>1.30</td>
<td>0.18</td>
</tr>
<tr>
<td>AdenineHCl</td>
<td>0.65</td>
<td>0.11</td>
</tr>
<tr>
<td>NaCl</td>
<td>26.00</td>
<td>1.52</td>
</tr>
<tr>
<td>K₂HPO₄*3H₂O</td>
<td>4.55</td>
<td>1.04</td>
</tr>
<tr>
<td>CaCl₂*2H₂O</td>
<td>0.65</td>
<td>0.10</td>
</tr>
<tr>
<td>MgCl₂*6H₂O</td>
<td>0.65</td>
<td>0.13</td>
</tr>
<tr>
<td>Waymouth MB 752/1</td>
<td>N/A</td>
<td>35% of usual</td>
</tr>
</tbody>
</table>

Osmolarity: 293 mOsm; pH: 7.4

### Step-wise method for CPA loading and unloading

For CPA loading/unloading, 100% CPA working solutions (Table 2) were diluted using LM5 to create the loading step solutions and unloading step solutions of increasing respectively decreasing concentrations while sucrose was included in all the unloading solutions at a final concentration of 300 mM [11]. Eight concentration steps were used to load and unload the slices. To load the slices with the CPAs, about 10-20 slices were placed in a beaker and exposed in 4 steps of 15-min to the CPA solutions with increasing concentration: 25%, 50%, 75% and 100% of the working solution. After that, slices were immersed in the CPAs at decreasing concentrations of 75%, 50% and 25% working solution with 300 mM sucrose followed by solely LM5 with sucrose in 4 steps of 10 min on ice for unloading. During all steps, the beakers were placed on melting ice and gently shaken [7].
Incubation and viability testing

After the different treatments, slices were incubated in 12-well plates (Greiner Bio-One, the Netherlands) under humidified carbogen (95% O₂, 5% CO₂) at 37°C with gentle shaking at 90 rpm/min. Each well contained 1 slice in 1.3 mL WME with 25 mM D-glucose and 50 µg/mL gentamicin. After incubation for 3 or 24 hours, slices were collected for the determination of viability and functionality. Incubation of control slices (not exposed to CPAs) was started directly after slice preparation.

Table 2 Composition of 100% working solutions of CPA solutions

<table>
<thead>
<tr>
<th>Cryoprotectant Solution</th>
<th>Sol Y</th>
<th>Sol Z</th>
<th>M22</th>
</tr>
</thead>
<tbody>
<tr>
<td>Me₂SO (g/L)</td>
<td>223.1</td>
<td>223.1</td>
<td>223.1</td>
</tr>
<tr>
<td>Formamide (g/L)</td>
<td>128.6</td>
<td>128.6</td>
<td>128.6</td>
</tr>
<tr>
<td>Ethylene glycol (g/L)</td>
<td>168.4</td>
<td>168.4</td>
<td>168.4</td>
</tr>
<tr>
<td>N-Methylformamide (g/L)</td>
<td>—</td>
<td>—</td>
<td>30</td>
</tr>
<tr>
<td>3-Methoxy,1,2-propanediol (g/L)</td>
<td>40</td>
<td>40</td>
<td>40</td>
</tr>
<tr>
<td>PVP K12 (g/L)</td>
<td>—</td>
<td>—</td>
<td>28</td>
</tr>
<tr>
<td>Sucrose (g/L)</td>
<td>—</td>
<td>20</td>
<td>—</td>
</tr>
<tr>
<td>Supercool X-1000* (g/L)</td>
<td>10</td>
<td>10</td>
<td>10</td>
</tr>
<tr>
<td>Supercool Z-1000* (g/L)</td>
<td>20</td>
<td>20</td>
<td>20</td>
</tr>
<tr>
<td>5X LM5-- (mL/L)#</td>
<td>200</td>
<td>200</td>
<td>200</td>
</tr>
<tr>
<td>Total CPA Molarity</td>
<td>8.83</td>
<td>8.89</td>
<td>9.35</td>
</tr>
</tbody>
</table>

*Ice blocker
# The 1X LM5 carrier solution contained 90 mM glucose, 45 mM mannitol, 45 mM lactose, 28.2 mM KCl, 7.2 mM K₂HPO₄, 5 mM reduced glutathione, 1 mM adenine HCl, 10 mM NaHCO₃.

Determination of functionality and viability of PCLS

ATP Determination

The ATP content of PCLS was determined as a viability parameter in PCLS as described before [11]. For ATP determination, slices were collected individually in 1 mL 70% ethanol with 2 mM EDTA (pH 10.9), snap-frozen in liquid nitrogen and stored at -80°C until analysis. After homogenization for 45 s using a Mini-BeadBeater-8 (BioSpec, Bartlesville, OK, USA), the slices were centrifuged at 16,100 g for 5 min and the ATP level in the supernatant was determined using the ATP Bioluminescence assay kit CLS II (Roche diagnostics, Mannheim, Germany) in a black 96-well plate Lucy1 luminometer (Anthos, Durham, NC, USA) according to the manufacturer’s protocol. The amount of luminescence as a measure of ATP content was measured using a Packard LumiCount® Luminescence Microplate Reader (Packard, United Kingdom).
Histomorphology

Slices were prepared for the examination of histological integrity as follows: after fixation in 4% buffered formaldehyde in phosphate-buffered saline solution for at least 12 hours at 4°C, slices were dehydrated in a graded alcohol series and Histosolve (Thermo Scientific, Pittsburgh, USA). Subsequently, slices were vertically embedded in paraffin and cross-sectioned at 4-µm thickness. The sections were stained with hematoxyline and eosin (H&E).

LDH leakage

The leakage of the enzyme lactate dehydrogenase (LDH), from liver slices in the culture medium was measured on a Hitachi Automatic Analyzer (Roche Diagnostics, Mannheim, Germany), according to a routine laboratory procedure. Samples of the medium were collected after 3 h of slice incubation, snap-frozen and stored at -80°C until analysis. To measure the total LDH content in fresh liver slices, three liver slices were collected after 1 hour incubation and homogenized in 1.3 mL culture medium. The homogenate was centrifuged and stored at -80°C until analysis.

Nitrate/nitrite (NOx) formation

The functionality of the Kupffer cells was determined after incubation of PCLS with LPS (final concentration 40 µg/ml) for 8 and 24 hours. The NOx production was assessed by determining NO$_2^-$ and NO$_3^-$ levels in the culture medium using Griess reagent according to Moshage et al. [17]. In brief, the nitrate in the sample was first reduced to nitrite in presence of NADPH (Roche, Basel, Switzerland), nitrate reductase (Roche, Basel, Switzerland), and flavin adenine dinucleotide (FAD) (Roche, Basel, Switzerland) at 37°C for 30 min. Then 2.2 µl 0.5 M Na-pyruvate (Sigma) and 0.55 µl lactate dehydrogenase (5 mg/ml) Roche, Basel, Switzerland) in serum-free WME were added to degrade NADPH. After incubation at 37°C for 10min, 5 µl 30% ZnSO$_4$ was added to precipitate the enzymes, and samples were centrifuged at 2000 g for 5 min. 100 µL of the supernatant was then transferred to a new plate and the total amount of nitrite was measured at 550 nm after formation of the red-violet diazo dye after adding 50 µL 1 % sulfanylamide in 5% H$_3$PO$_4$ (Sigma) and 50 µL 0.1% naphthylethylenediamine (Sigma) at room temperature for 15 min using a linear standard curve of potassium nitrite. Assays were performed in duplicate.

Immunohistochemical staining of non-parenchymal liver cells

The presence of non-parenchymal liver cells in the slices was determined by immunohistochemical staining after 3 h of incubation. For this purpose, slices were embedded in Tissue-Tek after incubation and frozen in isopentane (-80°C). The frozen sample blocks were kept at - 80°C until cryosectioning. Four µm thick tissue sections were cut using a cryostat (Leica CM 3050) at -20°C, placed on Super-Frost plus slides (Menzel-
Glaser, Braunchweig, Germany) and air-dried. The sections were stored at -20°C until further use.

For immunohistochemical staining of Kupffer cells (ED2), endothelial cells (RECA-1) and stellate cells (desmin), sections were fixed in acetone for 10 min at room temperature and then rehydrated in PBS for 5 min. Then, sections were incubated with primary mouse antibodies against ED2 (1:100; Serotec, Oxford, UK), RECA-1 (1:1000; Serotec, Oxford, UK) and desmin (1:400; sigma, Saint Louis, USA) for 1 h at room temperature. After washing with PBS, the sections were incubated with 0.75% H$_2$O$_2$ solution in PBS for 30 min to block endogenous peroxidase. Then the sections were incubated with the secondary antibody, RAMPO (rabbit anti-mouse immunoglobulin labeled with horseradish peroxidase 1: 100) (Dako Cytomation, Denmark) and the tertiary antibody GARPO 1:100) (Goat anti-rabbit immunoglobulin labeled with horseradish peroxidase) (Dako Cytomation, Denmark) for 30 min sequentially with intermittent washings in PBS. Thereafter the sections were stained with AEC (3-amino-9-ethylcarbazole) solution and counterstained with haematoxylin for 1 min. The sections were embedded in Kaiser’s glycerin gelatin (Merck, Darmstadt, Germany) after washing under tap water for 5 min. The stained area in the digital photomicrographs was quantified using Cell^D imaging software (Olympus) according to the standard procedure and is presented as % positive area per field.

**Metabolism of 7-ethoxycoumarin**

The phase I and phase II metabolic activity of PCLS were evaluated by measuring metabolite formation from 7-ethoxycoumarin (7-EC) (Fluka Chemie, Buchs, Switzerland). Liver slices were incubated with 500 µM 7-EC at 37°C for 3 hours, and the medium samples were stored at -20°C until analysis. The hydrolysis of 7-EC to 7-hydroxycoumarine (7-HC) by Cytochrome P450 mediated phase I reaction, the conjugation of 7-HC to 7-HC-glucuronide (7-HC-Gluc) and 7-HC-sulfate (7-HC-Sulf) as products of phase II metabolism were measured in the culture medium using an HPLC method as described before [18].

**Glutathione content (GSH+GSSG) of PCLS**

After 3 hour incubation, the liver slices were washed in 0.9% NaCl solution, collected individually into Eppendorf tubes, immediately frozen in liquid nitrogen and stored at -80°C until analysis. The slices were homogenized in 400 µL ice cold 50 mM Tris-HCl/ 1 mM EDTA buffer (pH 7.4) using a mini bead beater for 45 s and then centrifuged for 3 min at 16,100 g at 4°C and the supernatant was used to determine the levels of reduced and total glutathione. To determine the content of reduced glutathione 150 µl of the supernatant was mixed with 15 µL 50% TCA and kept on ice until the next step. Then the mixture was centrifuged at 161,00 g for 5 min and 50 µl of the supernatant was pipetted on a clear 96-well plate. To assess the level of total glutathione, first oxidized glutathione (GSSG) in another 150 µL aliquot of the supernatant of the slice homogenate was reduced by glutathione reductase in the presence of 6 µM of NADPH and incubated at 37°C for 15
The Effect of Hypothermic in Situ Perfusion on Cryoprotectant Toxicity

Thereafter 15 µl 50% TCA of 0 °C was added. Subsequently the sample was centrifuged at 2,000 g for 5 min and 50 µl of the supernatant was transferred on clear 96-well plate. To all samples, 200 µL 5,5’-dithio-bis(2-nitrobenzoic acid) was added and absorbance was measured at 405 nm using a spectrophotometer. A linear standard curve of reduced glutathione (Sigma-Aldrich) was used.

Gene expression

Three slices from the same experiment were collected after incubation and total RNA was isolated with the RNAeasy mini kit (Qiagen, Hilden, Germany) according to manufacturer’s instructions. RNA concentration and quality were measured using a Nanodrop ND100 spectrometer (Wilmington, DE USA). The ratio of absorbance at 260/280 was above 1.8 for all samples. cDNA was synthesized from RNA samples using a Promega Reverse Transcription kit (Promega, Madison, USA) as published before [19]. RT-PCR was conducted using SensiMix SYBR Green (Quantace, London, UK). The primers used for RT-PCR are listed in Table 3. The PCR reaction was performed as follows: 10 min at 95°C and then 40 cycles of amplification at 95°C for 15 s, at 56°C for 15 s and at 72°C for 40 s, followed by a dissociation stage (at 95°C for 15 s, at 60°C for 15 s and at 95°C for 15 s). All assays were performed at least in duplicate. The comparative threshold cycle (C_T) was used for quantification of the mRNA. The relative expression of genes 2^\Delta C_T was calculated by the difference of the C_T of the target gene and the C_T of the reference gene (GAPDH) from the same sample.

Table 3 Sequences of the primers for quantitative Real-Time PCR rat genes

<table>
<thead>
<tr>
<th>Gene</th>
<th>Forward primer(5’ – 3’)</th>
<th>Reverse primer(5’ – 3’)</th>
</tr>
</thead>
<tbody>
<tr>
<td>GAPDH</td>
<td>CTGTGGTCATGAGCCCCCTCC</td>
<td>CGCTGGTGCTGAGTATGTCG</td>
</tr>
<tr>
<td>MDR-1a</td>
<td>GATGGAATTGATGAGCTGGAC</td>
<td>AAGGATCAGGAAACAAAGA</td>
</tr>
<tr>
<td>MDR-1b</td>
<td>GAAATAATGCTTATGAAATCCCAAAG</td>
<td>GGTTCATGTCATGTCCTCCTTTGA</td>
</tr>
<tr>
<td>IL-1α</td>
<td>TCGGGAGGAGAGCAGCCTCTAA</td>
<td>GAAAGCTCGGATGGAATGGAAT</td>
</tr>
<tr>
<td>IL-1β</td>
<td>AGAGCTCAGGAGGAGCAGT</td>
<td>GCAGTGCAGCTCCTAATGG</td>
</tr>
<tr>
<td>iNOS</td>
<td>CGTCGATGTTCCAAGCAA</td>
<td>CCCTGAGACTTCCTACCTGT</td>
</tr>
<tr>
<td>eNOS</td>
<td>TACCCCTACCCGATAACAA</td>
<td>TCTGGCCCTTCTGCTATTT</td>
</tr>
<tr>
<td>IL-6</td>
<td>ATGTTTGTGACAGCCACTGC</td>
<td>ACAGTGCACTCAGTGCTGTC</td>
</tr>
<tr>
<td>IL-10</td>
<td>GAGGACGACGCAGAAAGG</td>
<td>GCAGAACAAGTACACCAAGTA</td>
</tr>
<tr>
<td>TNF1α</td>
<td>GGCCACGCTTCTCTCTCTT</td>
<td>TTTGCTACGAGGTGGCTAC</td>
</tr>
<tr>
<td>HO-1</td>
<td>CTGCCATGAAACACTCTGGAGAT</td>
<td>GCAGGAAGGCGGCTCTTG</td>
</tr>
<tr>
<td>HSP70</td>
<td>GGTGCATGATGCTTCTTGGTTTA</td>
<td>GGTTGGCCATGCTGAGGTTGTT</td>
</tr>
<tr>
<td>MHC-II</td>
<td>CGTCGGGTATCAGAGGAGG</td>
<td>ATAGCCAATGACACCTGAG</td>
</tr>
</tbody>
</table>
Statistical Analysis

All experiments were performed with a minimum of 3 livers and at least 3 slices from each liver. Results were compared using two-tailed unpaired Student’s t-test. The criterion for significance is a p value <0.05. Statistical differences for ATP content were determined using values normalized to controls in each experiment. For gene expression data, the significance of the differences was determined using the ΔCt values.

Results

Influence of perfusion with TransSend W on the viability of PCLS and on their susceptibility to the toxicity of CPAs

The viability of PCLS was determined by the ATP content, histomorphological appearance and the LDH content and leakage in the medium after 3 hour of incubation.

As shown in Fig 1A, the in-situ perfusion significantly improved the recovery of ATP content of PCLS compared to the control after loading/unloading CPAs from 70% to 95% for Sol Y, from 69% to 89% for Sol Z and from 54% to 78% for M22 respectively. M22 was more toxic than Sol Y and Sol Z with or without perfusion. As shown in Fig. 1B, the ATP content of control slices without perfusion (18 nmol/mg protein) after 3 h incubation was significantly higher than that of control slices with perfusion (12.2 nmol/mg protein).

Leakage of LDH is a well-known indicator of cell membrane integrity and cell viability [20]. The initial content of LDH in fresh slices after the perfusion with TransSend W was higher than that in the non-perfused slices (Fig. 4). In general, the LDH leakage into the medium was similar in all samples (both the control slices and the slices treated with CPA) for both perfusion and non-perfusion. As a result the percentage of LDH leakage was lower in slices from perfused livers. The only exception was the non-perfused slices treated with M22, where LDH leakage was significantly higher than that from the non-perfused control. Moreover, perfusion reduced the absolute amount of LDH leakage from the M22-treated slices into the medium.

Histomorphological appearances of slices (Fig.3) from perfusion and non-perfusion closely resembled each other both for the control and for each of the CPA treatments. In general, after CPA treatment more pyknotic and condensed nuclei were observed in the hepatocytes. Moreover the sinusoidal cells became more round, and the number of non-parenchymal cells seemed reduced. Therefore we decided to perform immunohistochemical staining for markers of the Kupffer, endothelial and hepatic stellate cells.
Figure 1. ATP content of PCLS after treatment with different CPA and 3 hours of additional incubation at 37°C. (A) ATP content is expressed as relative values to the control; (B) ATP content is expressed as nmol/mg per protein. 3 CPA solutions were tested within one experiment with 11 rats for no perfusion and 9 rats for perfusion with TransSend W, with four slices for each rat. Data are expressed as mean + SEM. **p<0.01 and ***p<0.001 when comparing perfusion with no perfusion; #p<0.05 when compared with the corresponding control; $ p<0.05 and $$ p<0.01 when compared with M22 in the same group: perfusion or no perfusion.

Figure 2. LDH leakage after 3 h incubation of PCLS. 3 CPA solutions were tested within one experiment with 2 rats for no perfusion and 3 rats for perfusion with TransSend W, with 3 PCLS per experiment. *p<0.05 when comparing perfusion and no perfusion; #p<0.05 when compared with the corresponding control.
The presence of the different non-parenchymal cells (Kupffer cells, endothelial cells and stellate cells) in slices was determined by immunohistochemical staining for ED2, RECA-1 and desmin respectively (Fig. 4). The staining intensities for the non-parenchymal cells in control slices were not affected by the perfusion. The staining for all three cell types in slices of non-perfused livers was decreased after CPA treatment compared to non-CPA treated controls, suggesting toxicity of the CPAs to the NPC. Interestingly, the perfusion of the liver with TransSend W significantly reduced the loss of Kupffer cell and endothelial cells staining in slices after Sol Z and M22 treatment. The staining for ED2 and RECA-1 in slices treated with Sol Z and M22 was not significantly different from their corresponding non CPA-treated controls.

The effect of perfusion and CPA treatment on the functionality of Kupffer cells was assessed by measuring LPS-stimulated NOx production in the medium after 24 hours of incubation with LPS (Fig.5). LPS treatment resulted in higher NO production in slices of perfused livers than in slices of non-perfused livers (205 vs 160 μM, p<0.05). CPA treatment significantly decreased the LPS-stimulated NOX production compared to the control to the same extent in slices of both non-perfused and perfused livers.
Figure 4. Immunohistochemical staining for ED2 (KC marker), desmin (HSC marker), and RECA-1 (EC marker) in PCLS treated with different CPA solution. Representative photomicrographs and quantitative analysis of ED-2, RECA-1 and desmin. Scale bars: 100 µm. Perf = perfusion. No Perf = no perfusion. **p<0.01 and ***p<0.001 when compared with no perfusion; #p<0.05 when compared with the corresponding control.

The Effect of Hypothermic in Situ Perfusion on Cryoprotectant Toxicity
Influence of perfusion with TransSend W on 7-EC metabolism of PCLS treated with CPA

Besides investigation of the functionality of Kupffer cells, we also investigated whether in situ perfusion of the liver with TransSend W could prevent decrease of the metabolic rate towards the model compound 7-EC due to CPA treatment. Phase I metabolism of 7-EC (500 µM) towards 7-HC by cytochrome P450 and Phase II metabolism of 7-HC to 7-HC–Gluc and 7-HC–Sulf by conjugation with UDP-glucuronyltransferase and sulfotransferase (Fig 6A) were examined. The total formation of 7-HC as indicated in Fig 6B is calculated as the sum of all phase I and phase II metabolites: 7-HC, 7-HC–Gluc and 7-HC–Sulf. The results in Fig 6B-D indicate that perfusion did not change phase I or phase II metabolic capacity of the control slices, the slight differences in 7-HC–Gluc and 7-HC–Sulf between control slices of perfused and non-perfused livers were not significant. Exposure to the CPAs reduced the formation of all metabolites in slices from non-perfused livers, although the decrease was not significant for 7-HC–Sulf formation after exposure to Sol Y and Sol Z. In contrast in slices prepared from perfused livers exposure to Sol Y and Sol Z did not cause any reduction in metabolite formation. Exposure to M22 reduced phase I metabolism and glucuronidation but to a lesser extent than in slices from non-perfused livers (Fig. 6B and 6C). In general, compared to glucuronidation, the sulfation rate (Fig. 6D) in slices was less affected by CPA treatment regardless of perfusion or non-perfusion. Our results indicated that in-situ perfusion of a rat liver with TransSend W protected both phase I and phase II metabolic capacity of hepatocytes from the toxicity of CPA.

Figure 5. LPS-stimulated NOx production in the culture medium by PCLS in 24 h. Data are expressed as mean + SEM. #p<0.05 when compared with the corresponding control. NC are control slices that were not stimulated with LPS. Control with LPS from non-perfused livers vs perfused livers: 160 µM vs 205 µM. #p<0.05 when compared with the corresponding control;
The Effect of Hypothermic in Situ Perfusion on Cryoprotectant Toxicity

Figure 6. (A) Metabolic pathway of 7-ethoxycoumarin (7-EC), and metabolic conversion of 500 µM 7-EC into (B) 7-HC, (C) 7-HC-Gluc and (D) 7-HC-Sulf. Data are expressed as Mean ± SEM (n=3). *p<0.05 when compared with and without perfusion; #p<0.05 when compared with the corresponding control.

Influence of perfusion with TransSend on GSH content of PCLS treated with CPA

The effect of perfusion with TransSend W on the GSH, GSSG and total glutathione in PCLS is shown in Fig. 7. The perfusion with TransSend W only decreased the GSH content in the control slices, but not in the slices treated with CPA. While the GSH content in slices of non-perfused livers was decreased after CPA treatment (although only significantly after M22 treatment), the GSH content of perfused slices was not affected by CPA treatment. The total glutathione levels were not affected by perfusion or CPA treatment.

Influence of perfusion with TransSend on gene expression of PCLS

Gene expression of a set of genes including inflammation-related cytokines (IL6 (interleukin 6), TNF-α (Tumor Necrosis Factor α), IL10 (interleukin 10), IL α (interleukin α) and IL β (interleukin β)), general markers for cellular stress (eNOS (endothelial nitric oxide synthase), iNOS (inducible nitric oxide synthase), HO-1 (heme-oxygenase 1), Hsp70 (heat shock protein 70)), MDR-1a (muti-drug resistance-1a), MDR-1b (muti-drug resistance-1b) and MHC-II (major histocompatibility complex II) were measured in both
perfused and non-perfused control slices after 3 hours incubation. IL-6, TNF-α, iNOS, and eNOS were significantly down-regulated due to perfusion, suggesting a reduced inflammatory response in slices (Fig. 8). Also MDR 1a and MDR 1b were significantly down-regulated. The gene expression of IL10, ILα, ILβ, Hsp70 and HO-1 known for their contribution to protect against ROS and of MHC-II was not affected by perfusion.

Figure 7. GSH and total glutathione content of rat PCLS after CPA treatment with or without perfusion and 3 hours of additional incubation at 37°C. Data are expressed as mean ± SEM (n=3), using 3 PCLS for each group in every experiment. *P<0.05 when compared with the group without perfusion. #p<0.05 when compared with the corresponding control.

Figure 8. The expression of selected genes in PCLS after TransSend W in situ perfusion and 3 hours additional incubation at 37°C. Expression levels were normalized with GAPDH and expressed as fold change compared to slices of non-perfused livers. Data are from 6 rats for perfusion and 6 rats for no perfusion, using 4 PCLS per experiment. *P<0.05, **p<0.01 and ***p<0.001 when compared with slices without TransSend W perfusion.
Discussion

PCLS retain a high degree of integrity of the liver architecture, cell-cell interactions and cellular heterogeneity. To successfully cryopreserve PCLS, it is important that all the different liver cell types remain viable both after CPA loading/unloading and after vitrification. The basic principle of hypothermic perfusion with a preservation solution is to minimize the deleterious effect of ischemia during harvesting of the organ and subsequent cold storage by removing blood cells, providing an appropriate osmotic condition to avoid cell swelling, providing some substrates for cell protection and reducing temperature at the same time. Hypothermic perfusion is a standard practice in order to preserve organs for tissue transplantation [15]. TransSend W is an organ preservation solution designed specifically for liver perfusion [21]. Besides the two impermeants, polyglycerol and lactose to prevent cell swelling under hypothermic condition, other additives such as adenine (nucleotide precursors)[22], glutathione[12] and chlorpromazine [16] (antioxidant) and appropriate buffer capacity [12] are also included in TransSend W. The aim of the present study was to investigate whether in-situ perfusion with TransSend W is able to improve the conditions (viability and functionality) of PCLS before and after CPA treatment which is important for the successful cryopreservation of PCLS.

ATP content, reflecting the energy status and overall metabolic capacity, is considered as a sensitive marker for viability [1]. Our results indicated that in-situ perfusion significantly improved the recovery of the viability of PCLS treated with CPA compared to the control slices. Interestingly, the initial ATP content of the control slices of the perfused livers were significantly reduced compared to that of the non-perfused livers. We (unpublished data) and other scientists [9, 23, 24] have observed that it takes 1-6 h for ATP to recover to the maximal values for non-perfused slices and ATP levels remain stable at least 48-72 h. The observed difference for ATP content in perfused vs non-perfused slices may be explained by assuming that the time for recovery may vary between the different experimental systems (perfusion vs non-perfusion). In contrast to the ATP content, the initial total LDH content of slices from perfused livers was significantly higher than from non-perfused livers, which may indicate that LDH leakage during slicing was reduced in these perfused livers. During slicing, cell damage can result from the cold ischemia injury due to perturbations in osmoregulation, energetics, and aerobic metabolism [25] or result from free radicals [26-29]. Perfusion also protected against leakage of LDH caused by M22 treatment, and M22 appeared to be the most toxic among the CPAs tested based on the ATP content. For the other two CPAs, leakage of LDH was similar to that of control slices and a significant protective effect of perfusion could not be observed. The beneficial effect on preserving the LDH total content and on reducing leakage from M22 toxicity might be due to the inhibition of the formation of free radicals or reduced metabolic activity before and during slicing by in-situ perfusion with TransSend. Chlorpromazine, one of the components in TransSend, is capable of a one-electron transfer to Fe3+ to form a cationic free radical which therefore can act as an antioxidant to terminate a free radical cascade [30].
Chapter 4

Moreover, chlorpromazine has shown protective effects in rat liver [31, 32] and kidney [33] against ischemia injury.

The beneficial effect of in-situ hypothermic perfusion was further confirmed by the immunohistochemical staining for Kupffer cells and endothelial cells in PCLS after Sol Z and M22 treatment. In-situ perfusion with TransSend clearly protects non-parenchymal cells from the damage of the toxicity of certain CPAs although the protective effect was not universal for all the cell types. Moreover, perfusion increased the reaction to LPS in control slices but all CPAs reduced the reaction to LPS and perfusion did not protect against this decreased reaction. Kupffer cells are activated by LPS to increase iNOS expression, NO production and cytokines excretion, which in turn activate the hepatocytes to increased expression of iNOS and formation of NO. Therefore, whether the observed difference in NO production was due to reduced Kupffer cell activity or reduced response of the hepatocytes or both still has to be determined.

Interestingly, the in-situ perfusion with TransSend W protected the biotransformation function of the hepatocytes from the toxicity of all CPA treatments as indicated by the phase I metabolism of 7-HC. It appeared that the protection was not due to an induction of the metabolic enzymes by the TransSend W since perfusion did not increase the phase I metabolism in the control slices. The increased phase II glucuronide formation by CPA-treated slices after perfusion is most likely the result of the increased formation of phase I metabolites and does not represent an improved capacity for phase II glucuronidation since the increased amount of phase II glucuronide (7-HC-Gluc) was similar to the increase in phase I metabolite (7-HC). The relative amount of the two phase II metabolites: glucuronide and sulfate was the same as previously reported by our lab [2, 34].

The results of the effect of exposure to CPAs on phase I metabolism in slices from perfused livers were in line with the corresponding ATP results. Interestingly, the results of IHC staining for the markers of the different NPC cells were not necessarily correlated with the general viability markers (ATP or LDH leakage). In slices from perfused livers M22 was more toxic than Sol Y, which seemed to be nontoxic as indicated by ATP levels and leakage of LDH, but the IHC staining for NPC was generally lower after Sol Y than that after M22 exposure in slices from perfused livers. Hepatocytes account for more than 85% of the liver volume and for 60% of the total number of liver cells while NPC cells including Kupffer cells, endothelial cells and stellate cells constitute 40% of the cell number [1]. The responses by the hepatocytes therefore contribute more to the overall changes in viability parameters than the individual NPC cell types. Our results indicated the importance of studying the effect of the CPAs on each of the different cell types in the liver slices.

GSH is an important endogenous antioxidant for the detoxification of oxygen-derived free radicals and therefore protects cells against oxidative stress and reactive metabolites [35, 36]. TransSend W contains GSH and chlorpromazine which can act as free-radical scavengers [37]. We speculated that perfusion with TransSend W could increase the GSH
content, which might contribute to the beneficial effect of in-situ perfusion before tissue slicing and CPA load/unloading. Interestingly, the GSH content in the control slices was significantly lower after perfusion while total glutathione remained the same.

Instead, the beneficial effect could be explained by the fact the perfusion suppressed the inflammatory responses as indicated by the down-regulation of gene expression of IL-6, TNF-α, MHC-II, eNOS and iNOS. An up-regulation of MDR genes has previously been observed in ischemic organs [38] or due to hypoxia [39], and our results show that the expression of both MDR 1a and MDR 1B were lower after perfusion. Taken together, these data indicate that in-situ perfusion exhibits protective effects against ischemia injury during preparation and subsequent reperfusion damage during incubation of the PCLS.

**Conclusion**

We found that hypothermic in-situ perfusion with TransSend W was protective to liver slices in particular when exposed subsequently to CPAs as indicated by both the general viability markers (ATP and LDH leakage) and the presence and functionality markers for different cells types including hepatocytes, Kupffer cells, stellate cells and endothelial cells (IHC staining, NOx and metabolism). Although the exact mechanism of protecting tissue by perfusion with TransSend W against toxicity of CPA is still unknown, the protective effects observed might be due to the better tissue condition before CPA loading (LDH content and lower expression of cell stress-related genes). In conclusion, the in-situ perfusion with TransSend W is protective to PCLS exposed subsequently to CPA, which indicates that this perfusion may be a first step to successful development of improved vitrification protocols for PCLS.

**References**


