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ScienceDirect
Cryobiology 54 (2007) 1–12
CRYOBIOLOGY
www.elsevier.com/locate/ycryo

Cryopreservation of rat precision-cut liver and kidney slices by rapid freezing and vitrification ☆

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Received 3 January 2006; accepted 27 September 2006
Available online 12 December 2006

Abstract

Precision-cut tissue slices of both hepatic and extra-hepatic origin are extensively used as an *in vitro* model to predict *in vivo* drug metabolism and toxicity. Cryopreservation would greatly facilitate their use. In the present study, we aimed to improve (1) rapid freezing and warming (200 °C/min) using 18% Me_2SO as cryoprotectant and (2) vitrification with high molarity mixtures of cryoprotectants, VM3 and VS4, as methods to cryopreserve precision-cut rat liver and kidney slices. Viability after cryopreservation and subsequent 3–4 h of incubation at 37 °C was determined by measuring ATP content and by microscopical evaluation of histological integrity. Confirming earlier studies, viability of rat liver slices was maintained at high levels by rapid freezing and thawing with 18% Me_2SO. However, vitrification of liver slices with VS4 resulted in cryopreservation damage despite the fact that cryoprotectant toxicity was low, no ice was formed during cooling and devitrification was prevented. Viability of liver slices was not improved by using VM3 for vitrification. Kidney slices were found not to survive cryopreservation by rapid freezing. In contrast, viability of renal medullary slices was almost completely maintained after vitrification with VS4, however vitrification of renal cortex slices with VS4 was not successful, partly due to cryoprotectant toxicity. Both kidney cortex and medullary slices were vitrified successfully with VM3 (maintaining viability at 50–80% of fresh slice levels), using an optimised pre-incubation protocol and cooling and warming rates that prevented both visible ice-formation and cracking of the formed glass. In conclusion, vitrification is a promising approach to cryopreserve precision-cut (kidney) slices.

Keywords: Precision-cut liver slices; Precision-cut kidney slices; VS4; VM3; Vitrification; Rapid freezing; Tissue slice bank

Precision-cut liver slices are frequently used as an *in vitro* model for *in vivo* drug metabolism and toxicity [9,11,29,35,37]. Cryopreservation would offer a possibility to store slices, forming a tissue slice bank from which slices can be used for species-comparison studies on drug metabolism and toxicity at any desired time-point. Particularly the use of human tissue, of which the supply is irregular and unreliable, can be highly facilitated in this manner. A number of cryopreservation methods for liver slices that have shown to be successful to some extent, have been developed, amongst others by our laboratory [3,5,7,12,13,23–25,27,28,31,46]. There is an increasing awareness that extra-hepatic organs contribute substantially to drug metabolism and that using liver slices alone may give an incomplete view of *in vivo* metabolism [6,8,26,36,41,44]. However, the cryopreservation of precision-cut slices of extra-hepatic origin has not received much attention, with only a method for dog and human kidney slices reported [25].

In the present study we aimed to improve cryopreservation of rat liver slices and to develop new cryopreservation protocols for kidney slices. Two approaches were used for

☆ The research described in this article is funded by grants of the Dutch Platform Alternatives to Animal Testing (ZonMW) and by the Technology Foundation STW, Applied Science Division of NWO and the Technology Programme of the Ministry of Economic Affairs.
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doi:10.1016/j.cryobiol.2006.09.002

this purpose. The first approach was rapid freezing. For liver slices, we have developed a simple, rapid (200 °C/min) freezing method [5,7,10] using 18% Me_2SO[1] and showed that post-thaw viability and phase I and II biotransformation activity of cryopreserved rat liver slices were maintained at least during 4 h after thawing [5]. This method was now also applied to kidney slices. The second approach used was vitrification. Essentially, vitrification is the solidification of a liquid not induced by crystallization but by an extreme elevation in viscosity during cooling, so that the solution becomes a glass [19] and cell damage by ice crystal formation can be prevented. A liquid can be successfully vitrified, when cooling rates used are higher than the so-called critical cooling rate (v_{crc}), above which the chance of ice-crystal formation is small. Likewise, devitrification (ice-growth out of ice nuclei that are formed during cooling) should be prevented by warming with rates above the critical warming rate of the liquid (v_{crw}).

Traditionally, the major obstacle to the successful use of vitrification as a means of cryopreservation has been the toxicity of the high concentrations of cryoprotectant agents (CPAs) that must be used to prevent ice crystallization with cooling and warming rates that are achievable with tissue [15]. Toxicity can be a direct result of osmotic damage which arises because the cell membrane is less permeable to most CPAs than to water. In the present study we tended to limit osmotic damage by introducing and removing CPA stepwise, allowing time at each step for the cells to reverse the better part of their induced volume changes before moving on to the next step. Moreover, we attempted to prevent extreme cell swelling during removal of the CPAs by the use of impermeable sugars in CPA washout media by balancing in this manner osmoles of intracellular CPA against osmoles of extracellular sugar, thus making the extracellular osmolality closer to the intracellular osmolality [34,38]. Approaches to reduce chemical toxicity of a CPA solution without diminishing its glass-forming tendency include the use of a combination of CPAs with different mechanisms of toxicity, that even possibly antagonize each other's toxicity. For this reason, in the present study highly concentrated cryoprotectant (CPA) mixtures, VS4 and VM3, were used for vitrification of tissue slices. VS4 consists of 1,2 propanediol, Me_2SO and formamide in buffered saline with glucose, with a total molarity of 7.5 [23,24] and Table 1. VM3 is a more recently developed commercially available (http://www.21cm.com) low toxicity, high molarity (8.44 M) mixture of three permeating CPAs (ethylene glycol, Me_2SO and formamide) and three extracellular specific "ice-blocking" compounds, that further increase its glass-forming tendency without increasing toxicity [21,22]. Another approach that was used in the present study to decrease chemical toxicity was to shorten the time-period and the temperature at which slices were exposed to the CPA mixtures.

Table 1
Composition of 'full strength' VS4 and VM3

		VS4[a]	VM3[b]
Buffer components	NaCl (mM)	10	—
	$NaHCO_3$ (mM)	—	10
	KCl (mM)	28.3	28.3
	$MgCl_2$ (mM)	0.40	0.40
	$CaCl_2$ (mM)	0.05	0.05
	Dextrose (mM)	180	90
	Mannitol (mM)	—	45
	Lactose (mM)	—	45
	Reduced glutathion (mM)	5	5
	Adenine HCl (mM)	1	1
	K_2HPO4 (mM)	7.2	7.2
CPAs	Me_2SO (g/L)	215.7	223.1
	Formamide (g/L)	124.3	128.6
	1,2-Propanediol (g/L)	150.0	—
	Ethyleneglycol (g/L)	—	168.4
	Supercool x, iceblocker (g/L)	—	10
	Supercool y, iceblocker (g/L)	—	10
	PVP K12/5000 kDa (g/L)	—	70
pH		7.4	7.4
Molarity CPAs		7.5	8.4

[a] From [16].
[b] From [21].

Previously we found that the cooling rate required to permit vitrification of VS4 in liver slices is ca. 10 °C/min until the glass transition point (approximately −125 °C) [4], which is easy to achieve. However, the warming rate required to prevent devitrification of liver slices varied between 20 and 1600 °C/min in slices of different rat livers [4]. These rates are high and technically difficult to achieve. The v_{crc} and v_{crw} of VM3 were shown to be lower than for VS4 and are, respectively, <1 and ~3 °C/min [22], allowing cooling and warming rates to be optimized in the present study so that apart from visible ice formation also cracking of the formed glass (induced by uneven cooling and warming) could be prevented.

The viability of liver and kidney slices was tested by measuring their ATP content and by evaluating the histomorphology. Both ATP content and slice histomorphology have been shown to be sensitive to cryopreservation damage [30] and to correlate with slice metabolic capacity (unpublished data).

Materials and methods

Animals

All procedures on vertebrate animals were done under DEC (committee of animal ethics) approval. Male rats (Wistar, obtained from Harlan CPB, Horst, The Netherlands) were used for all experiments and were housed under standard vivarium conditions. All animals had free access to food and water until being anaesthetized for surgery.

[1] *Abbreviations used:* WME; Williams medium E, Me_2SO; dimethylsulfoxide, v_{crc}; critical cooling rate, v_{crw}; critical warming rate, FCS; fetal calf serum, CPA; cryoprotectant, PVP; polyvinylpyrrolidone.

Chemicals

Formamide, 1,2-propanediol, Krebs Henseleit buffer, Hepes buffer, insulin, gentamicin, adenine, trehalose and glutathione were obtained from Sigma, Axel, The Netherlands; William's medium E (+glutamax) (WME), phosphate buffered saline and foetal calf serum (FCS) were from Gibco BRL, Breda, The Netherlands; Premixed VM3 and VMP solutes were a gift from 21st Century Medicine, Rancho Cucamonga, California, USA. University of Wisconsin solution (UW) was purchased from DuPont Pharmaceuticals, Waukegan, IL, USA. The Celsis Biomass ATP assay kit was derived from Omnilabo, Breda, The Netherlands. Me_2SO (>99.9% pure) and all other chemicals were from Baker, Deventer, The Netherlands.

Preparation of liver and kidney slices

Due to the fact that experiments were done at different laboratories, small deviations in the preparation and incubation protocol described further on have occurred for some experiments (mainly experiments with kidney slices and VM3 and 18% DMSO). Viability of control slices did not differ between the two laboratories (data not shown). Procedures for which these apparently immaterial variations existed are noted with an * below.

Animals were anaesthetised with 65% CO_2, 35% O_2 before the liver and/or kidneys were removed. *For some experiments rats were anaesthetised with isoflurane and N_2O/O_2. From the liver, cores (8 mm in diameter) were produced using a 9 mm tissue-coring tool (Alabama R&D) attached to a mechanical drill (Metabo, The Netherlands) that was placed perpendicular to the liver lobe surface. Kidney cores were produced likewise, except that 5 mm diameter cores were produced using a 6 mm coring tool which allowed for visual separation of medulla and cortex slices. Subsequently, these cores were sliced using a Krumdieck tissue slicer filled with ice-cold oxygenated Krebs Henseleit buffer. All slices were stored in ice-cold oxygenated WME with 10% FCS until further use (usually 60–90 min). *In some experiments, slices were stored in University of Wisconsin solution.

Pre-incubation with the CPA

Slices were pre-incubated in 25 or 100 ml Erlenmeyer flasks (six slices per 25 ml flask, 18 slices per 100 ml flask) placed in a gently shaking water bath filled with ice. Each Erlenmeyer flask contained 5 ml (25 ml flask) or 15 ml (100 ml flask) CPA mixed in various proportions with (oxygenated) carrier solution. The CPAs used were 18% v/v Me_2SO, VS4, and VM3. VS4 consists of Me_2SO, 1,2-propanediol and formamide (weight ratio 21.5:15:12.4) (Table 1) in a carrier solution containing buffered saline, glucose, adenine and glutathione (see Table 1 for the final composition of 'full strength' VS4). VS4 has a total concentration of 7.5 M. The cryoprotectants of VM3 have been described in [21]. VM3 has a total concentration of 8.4 M and contains in addition to Me_2SO, formamide, and ethylene glycol, two commercially available anti-nucleating agents or "ice blockers" [47,48] and 7% low molecular weight polyvinylpyrrolidone (PVP). VM3 was prepared in a carrier solution called LM5 [21], for the final composition of 'full strength' VM3, see Table 1. Because both VS4 and VM3 are highly concentrated CPA solutions, pre-incubation was executed stepwise with the intention to limit osmotic damage. For this purpose, VS4 was mixed to various concentrations with VS4 buffer (see Table 2; VS4 buffer is VS4 without CPAs). For VM3 introduction, a special pre-incubation solution, VMP (which consisted of VM3 minus PVP; [21]) was mixed in various proportions with VS4 buffer (the exact pre-incubation schedule is given in Table 2). In some experiments, as indicated in the results section, the last step of pre-incubation (with 'full strength' VM3), was done at −20 °C, in an attempt to further decrease VM3 toxicity. 18% Me_2SO (v/v), used for rapid freezing, was applied to liver slices in one single step of 30 min on ice. For kidney slices a two-step protocol was used, in order to limit osmotic damage. These slices were incubated for 15 min in 10% Me_2SO followed by 15 min in 18% Me_2SO.

Table 2
Stepwise CPA introduction and removal

VS4 addition steps[a]				VS4 washout steps[a]		
25%VS4	50% VS4	75% VS4	100% VS4	75% VS4	50% VS4	25% VS4
15 min	15 min	15 min	15/30 min	10 min	10 min	10 min
VM3 addition steps[b]				VM3 washout steps[b,c]		
8% VMP[b]	15% VMP	26% VMP	VM3	26% VMP	15% VMP	8% VMP
15 min	15 min	15 min	15 min	10 min	10 min	10 min

Note. Values in the table represent the time span of each step, given in minutes with each row representing consecutive pre-incubation or washing steps.

[a] Steps designate % v/v of undiluted VS4 in VS4 buffer. 100% v/v VS4 = 49% w/v cryoprotectants (7.49 M).

[b] Steps designate % w/v VMP or VM3 solutes in LM5 buffer as indicated. VM3 = 61% w/v cryoprotectants (8.44 M) and VMP = 54% w/v cryoprotectants (~8.44 M).

[c] Trehalose was present at a concentration of 300 mM in all of these washout steps as well as in the 0% VMP 10 min step immediately following the 8% VMP washout step.

Cryopreservation

For rapid freezing with 18% Me_2SO, 3–6 slices were placed in 2 ml cryovials (Greiner, Alphen a/d Rijn, The Netherlands) with 0.5 ml 18% Me_2SO. Hereafter, the cryovials were directly submersed in liquid nitrogen [5,7].

To prevent ice-crystal formation during cooling and warming with VS4, higher cooling and warming rates were required than could be reached with the cryovials [4]. Therefore, slices were pre-incubated with VS4, then rapidly transferred to aluminium foil pads placed on melting ice. The foil was kept double-folded until the slices were actually placed between the two sides of the foil to prevent water condensation on the foil. Thereafter, the slices were cryopreserved by direct immersion of the double-folded foil in liquid nitrogen. Cooling rates were ca. 800 °C/min [4]. VM3 has a lower v_{crc} and v_{crw} than VS4, i.e. <1 and <3 °C/min respectively, so cooling rates of slices were varied to obtain a protocol that prevented both ice formation upon cooling and warming and cracking of the formed glass. For this purpose a computer-controlled cooling/warming device (Sylab Ice-cube, Hoekloos cryoservice, Hedel, The Netherlands) was used.

Rewarming and CPA removal

Slices cryopreserved with 18% Me_2SO were re-warmed by placing the cryovials in a 37 °C water bath until ice was no longer visible (ca. 1.5 min), quickly washed in WME + 10% FCS (10 s) and then incubated at 37 °C. For kidney, a two-step washing protocol was used, with the intention to limit osmotic damage. These slices were incubated for 10 min in 10% Me_2SO at 0 °C before incubation at 37 °C. Slices that were vitrified in cryovials with VM3 were re-warmed within the computer-controlled cooling/warming device (the warming rate used was varied within the experiments as indicated in the result section). VS4 pre-incubated slices that were vitrified after being sandwiched between aluminium foil, were re-warmed by placing the foil in VS4 on melting ice. The warming rates achieved by this method are discussed below. After rewarming, VS4 and VM3 were removed according to the stepwise protocols given in Table 2. For removal of VM3, 300 mM trehalose was mixed with the washing solutions.

To determine possible adverse effects of pre-incubation with the CPAs on the slices, slices underwent the procedure of CPA addition and washout without being cryopreserved.

Incubation and viability testing

Slices were incubated in a 25 ml Erlenmeyer flask (one slice/flask for 8 mm liver slices or two slices per flask for 5 mm kidney slices) in a shaking water bath (110 shakes/min), under humid carbogen (95% O_2, 5%CO_2) in 5 ml WME, supplemented with FCS (5%), 0.1 μM insulin, 50 mg/l gentamicine and D-glucose (to a medium concentration of 25 mM). *For some experiments, slices were incubated in 6-wells plates (one slice per well) in WME containing gentamycin and glucose (for details see [9]). After incubation for 3–4 h at 37 °C viability was determined by measuring ATP content and by microscopical evaluation of histological integrity.

The ATP content of liver and kidney slices was determined as follows. Liver slices were cut in two equal parts. One part was used for histomorphological examination (see below). The other part was immersed in 70% ethanol in HPLC water, containing 2 mM EDTA (pH 10.9). One of the two kidney slices from an Erlenmeyer flask was used for the determination of ATP content and the other for histomorphology examination. For ATP determination, slices were homogenized using a Branford sonifier (50% duty cycle, 5 s). An aliquot of the homogenate was taken and mixed 1/1 with Nucleotide Releasing Medium from the assay kit. Thereafter, the solution was diluted 1/10 with 0.025 M Hepes buffer to decrease the ethanol concentration. Subsequently, to 100 μl of the solution, 50 μl luciferin/luciferase solution from the assay kit was added and the amount of luminescence as a measure of ATP content was measured using a Lumac Biocounter M500 (Lumac, The Netherlands). *For some experiments, ATP was determined in a slightly different matter (see [9]). ATP content was determined relative to the protein content of the slices.

Slice protein content was determined in the slice homogenate as described previously [30], except that now an I-lab 600 automatic spectrophotometer (Instrumentation Laboratory, Lexington, USA) was used for the measurements.

For histomorphological examination slices were fixed in 70% ethanol and further processed as described by de Graaf et al. [5]. Slice integrity was determined in the haematoxylin and eosin stained cross sections by estimating the percentage of viable cells. Viability was scored in general by one observer. For some experiments, scoring was done by two (independent) observers, of which one had no prior knowledge of the experimental set-up, but no significant difference between the scoring of the two observers appeared then; For each of the three slices of one treatment, one section was made of its cross section. Within this cross section, all cells were scored (in this way, very roughly, 5000 cells were scored for kidney and 8000 cells for liver). Living and death cells were not actually counted, but their percentage was estimated. Cells were judged as 'dead', when they exhibited irreversible injury indicated by eosinofilic cytoplasm, and nuclear changes like pyknosis (condensed nucleus), karyorrhexis (fragmented nucleus) or karyolysis (loss of nucleus).

Differential scanning calorimeter measurements

A differential scanning calorimeter (DSC7, Perkin Elmer, Oosterhout, The Netherlands) was used for determination of v_{crw} of slices pre-incubated with VS4 for two

purposes (a) to monitor whether or not optimal equilibration with a CPA had taken place (in case of optimal equilibration the v_{crw} of the slices do not further decrease with increasing pre-incubation time) and (b) to determine whether or not the feasible (or maximally achievable) warming rate of 800 °C/min was higher than the v_{crw} of the VS4 pre-incubated slices, so that devitrification was prevented. Detailed description of DSC measurements on slices was published by us previously [4].

Statistical analysis

Statistical significance of decrease of viability after treatment of slices by the various protocols was determined by a one tailed, paired, student-*t* test.

Results

Tissue slice viability after pre-incubation with 18% Me_2SO and rapid freezing

Previously, we have reported that viability and functionality of rat liver slices cryopreserved by rapid freezing in the presence of 18% Me_2SO were maintained close to fresh slices levels for at least 4 h after thawing. Results in the present study are consistent with this observation (Fig. 1). Both the ATP content and the amount of viable cells in cryopreserved slices, 4 h after thawing, were approximately 70% of fresh slice levels. Fig. 2B shows a virtually intact slice that was cryopreserved with 18% Me_2SO and cultured for 3 h: few pycnotic cells can be seen only at the edges of the slice. Also in accordance with previous studies [5], viability of fresh liver slices was not affected by pre-incubation with 18% Me_2SO.

Pre-incubation of kidney slices with 18% Me_2SO significantly decreased viability (data not shown). Because this may be due to osmotic shock, a protocol was developed to minimize Me_2SO toxicity by pre-incubating stepwise (15 min in 10% Me_2SO, 15 min in 18% Me_2SO). Moreover, while liver slices were washed in WME/10% FCS directly after thawing, kidney slices were first placed in 10% Me_2SO for 10 min before washing. Treated in this manner, both non-frozen cortical and medullary slices were unaffected by 18% Me_2SO (Fig. 1). Nevertheless, ATP levels of kidney slices that were treated in this way and then cryopreserved by rapid freezing were very low, <20% of fresh levels, while few unaffected cells were found upon histomorphological examination in either cortical or medullary slices (Figs. 1, 3B and 4B).

Tissue slice viability after pre-incubation with VS4 and vitrification

Initially, to vitrify kidney slices with VS4, a method was used that was found to maintain viability of rat liver slices at acceptable levels [4], incubating 30 min with full strength VS4 before vitrifying the slices. However, pilot experiments with kidney slices demonstrated a significant loss of viability of especially cortical slices using this method. Therefore, the length of the pre-incubation step with 'full strength' VS4 was varied and it appeared that viability was increased with shorter pre-incubation time (data not shown). To

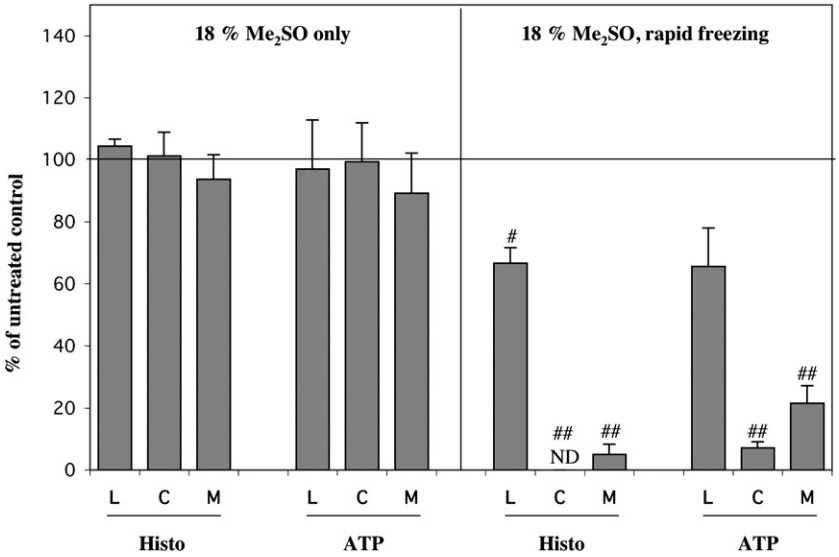

Fig. 1. Viability of liver (L), renal cortex (C) and renal medulla (M) slices after pre-incubation with 18% Me_2SO (v/v) and rapid freezing, relative to levels of untreated slices (not pre-incubated with 18% Me_2SO or cryopreserved). Mean ATP content of untreated slices was 9.6 ± 1.5 (liver slices), 5.4 ± 1.1 (renal cortex slices) and 9.7 ± 2.2 (renal medullary slices) nmol/mg protein. Histomorphologic appearance of slices was quantified as the percentage of intact (not necrotic) cells in the slice cross section. Mean % of intact cells of untreated slices was 72.2 ± 4.5 (liver slices), 71.3 ± 3 (renal cortex slices) and 77.7 ± 1.8 (renal medullary slices) due to the unavoidable damage caused by tissue slicing. All slices were incubated at 37 °C for 3–4 h prior to viability testing. Bars give the means of 2–5 experiments (three slices per experiment) + SEM. ND = intact cells not detected. $^{\#}$Values are significantly lower than those of slices that were only treated with 18% Me_2SO with $p < 0.05$ (*t*-test) ($^{\#\#}p < 0.01$).

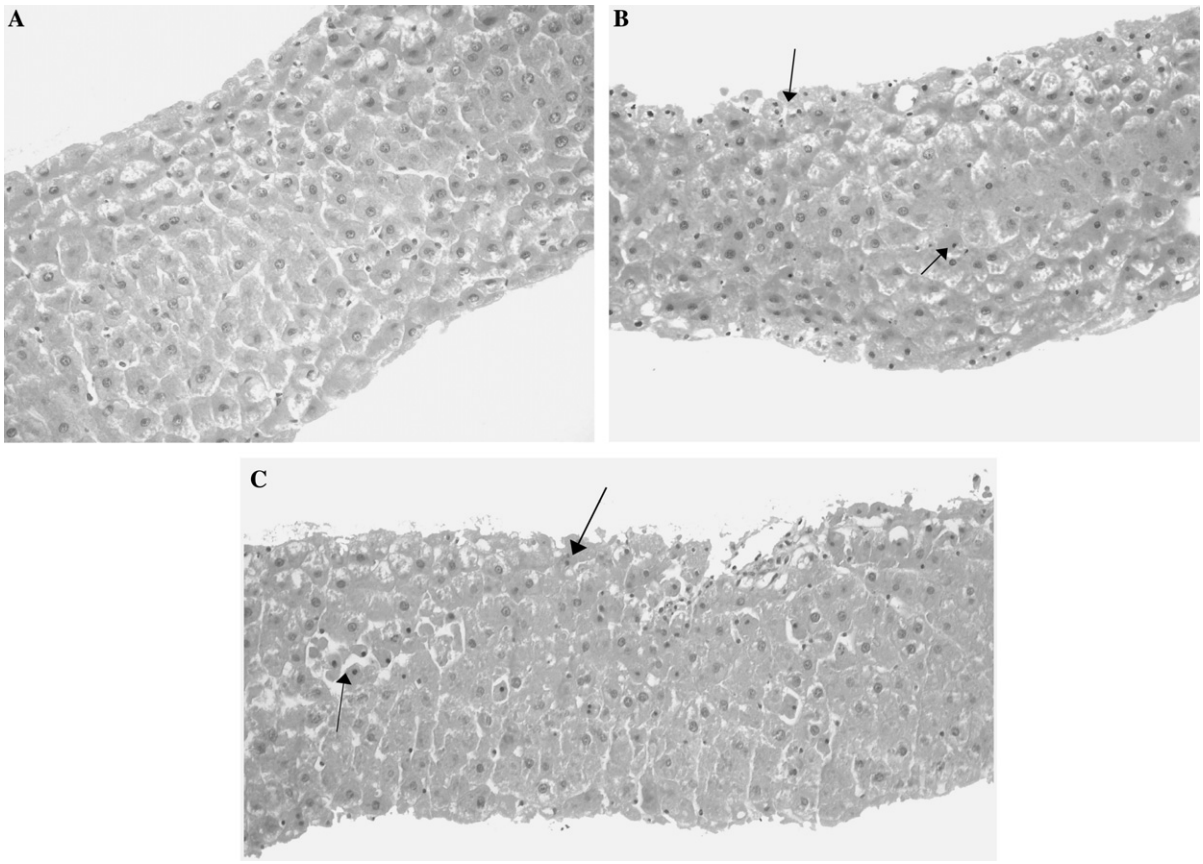

Fig. 2. Histomorphology of liver slices: (A) untreated slice (B) slice cryopreserved by rapid freezing after pre-incubation with 18% Me_2SO (C) slice vitrified with VS4. All slices were incubated at 37 °C for 3–4 h. Examples of necrotic cells in (B and C) are indicated with an arrow.

ensure that shortening of the pre-incubation period did not cause incomplete penetration of VS4, kidney slices were pre-incubated for 10, 20 or 30 min with full strength VS4 and v_{crw} determined. We have previously shown that the critical warming rate (i.e. the warming rate required to prevent devitrification, v_{crw}) of vitrified liver slices is usually close to the maximal warming rate that can be reached when slices are warmed between aluminium foil sheets (800 °C/min, from now on called the feasible warming rate) [4]. When equilibration with VS4 is incomplete, the v_{crw} is expected to be above this level. With kidney slices, v_{crw} was not changed by shortening pre-incubation to 10 min, indicating that VS4 penetration was complete (data not shown).

After optimisation of the pre-incubation protocol for VS4 (final protocol in Table 2), liver and kidney slices were vitrified. The quantitative results are shown in Fig. 5 and the histomorphological appearance of liver and kidney cortical and medullary slices treated and vitrified with VS4 is shown in Figs. 2C, 3C and 4C. Some diffuse necrosis can be observed in liver slices and kidney medulla slices, while the kidney cortex appears to be much more affected. For each individual experiment, one slice per organ was used to assess the v_{crw} by DSC. Measured v_{crw} varied between 350 and 3500 °C/min for liver slices and were 298 and 194 °C/min for kidney cortical and 2578 and 498 °C/min for medullary slices. For some of the livers and kidney medulla, the v_{crw} of slices (bars labelled L^* and M^* in Fig. 5) was higher than the feasible warming rates of 800 °C/min and to some extend crystallization may have occurred upon warming. VS4 vitrified liver slices showed a viability only 40–60% of that of untreated slices, despite the fact that VS4 toxicity was low (though statistically significant, a decrease of only 10%–20% of viability was observed after VS4 treatment and subsequent incubation) and no ice was formed during either cooling or warming. Lower recovery was obtained when devitrification was expected (L^* bars), but the difference was substantial only for histomorphological integrity and not for ATP recovery and did not reach the level of statistical significance in either case. For kidney cortical slices, viability of slices treated with VS4 ranged from about 50% (histomorphology) to 80% (ATP content). Vitrification further reduced viability substantially despite the fact that v_{crw} was always well below the feasible warming rate: recovery was about 10% based on histomorphology or <40% based on ATP content, in comparison to untreated slices. Renal medullary slices, however, were well preserved by vitrification with VS4, and there was no indication that devitrification was lethal to this tissue (although the number of observations was too limited to draw firm conclusions about the latter observation).

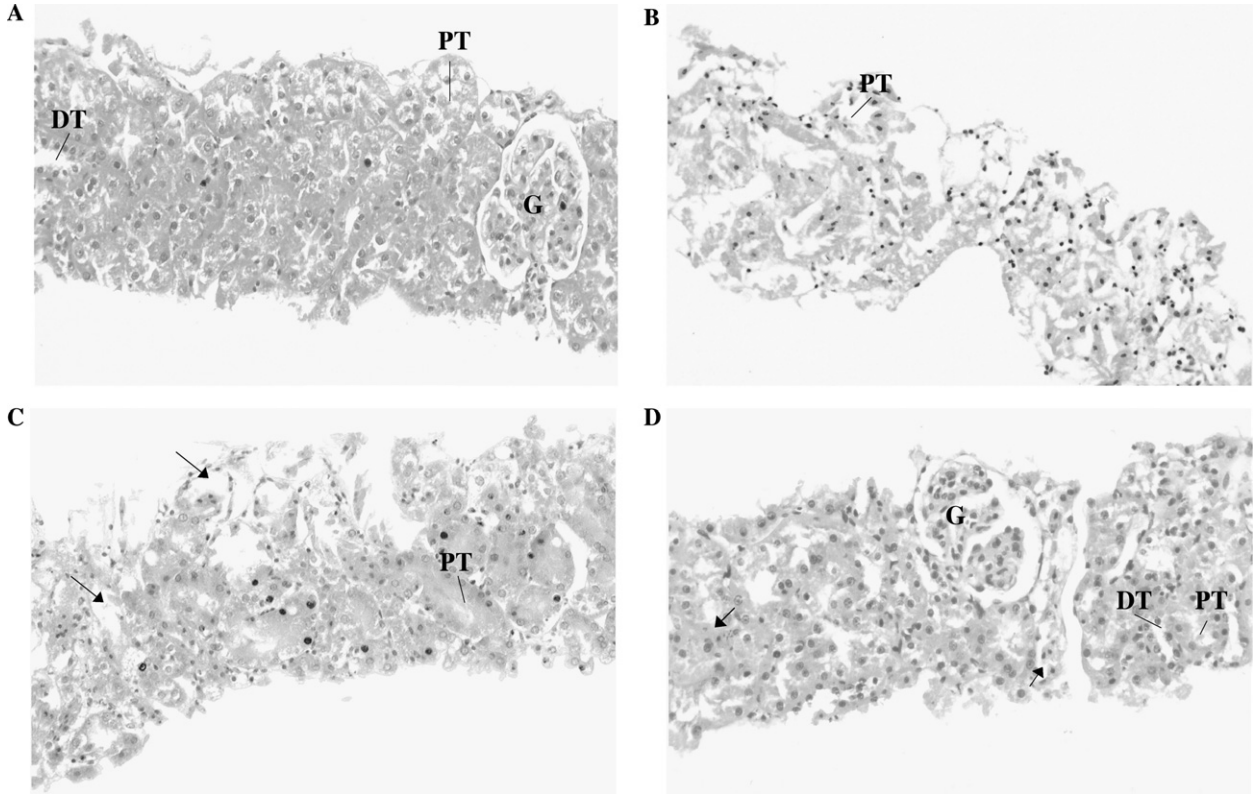

Fig. 3. Histomorphology of kidney cortical slices (A) untreated slice (B) slice cryopreserved by rapid freezing after pre-incubation with 18% Me_2SO (C) slice vitrified with VS4 (D) slice vitrified with VM3. All slices were incubated at 37 °C for 3–4 h. Examples of necrotic cells in (C and D) are indicated with an arrow. DT; distal tubulus, PT; proximal tubulus, G; glomerulus.

Vitrification of liver and kidney slices with VM3

Initially, to pre-incubate kidney slices with VM3, a stepwise protocol was used with a 30 min pre-incubation period with VM3 as final step. As with VS4 however, the viability of kidney cortical slices was decreased by VM3 treatment while kidney medullary slices were somewhat less affected. To increase viability of VM3 treated kidney slices, several variations were made in the pre-incubation protocol. Pilot experiments demonstrated that kidney slices incubated with VM3 for 15 min instead of 30 min and washed with 300 mM trehalose added to the washing medium with the intention to decrease osmotic effects, showed the highest viability (data not shown). Another small (but not statistically significant) improvement was achieved by pre-incubating the kidney slices at −20 °C instead of at 0 °C with VM3 (bars marked with C^{**} or M^{**} in Fig. 6).

Because the critical cooling rate (v_{crc}) and the v_{crw} (respectively <1 and ≈3 °C/min) of VM3 are much lower than of VS4, it was possible to vary cooling rates. In preliminary experiments it was noticed that the glass that was formed from VM3 in the cryovials was susceptible to cracking (Fig. 7A) when the vials were rapidly cooled (100 °C/min), leading to fragmentation of the slices and viability loss (data not shown). It is known that cracking in a glass at low temperatures can be avoided by lowering the cooling rate around the glass transition point [1,20]. Optimisation of the vitrification technique for VM3 has led to the following protocol: Cryovials containing VM3 and slices were initially cooled to −120 °C at 60 °C/min and then slowly (5 °C/min) to −150 °C (the lowest temperature that could be reached with the computer controlled freezing device). Upon re-warming, the vials were slowly warmed at 5 °C/min until −120 °C and then at 60 °C/min until 0 °C. In this manner, the obtained glass was virtually unfractured and the slices were not fragmented after thawing (Fig. 7B).

Fig. 6 shows the viability of renal cortical and medullary slices after treatment and vitrification with VM3. The decrease of viability of cortical slices caused by treatment with VM3 was limited and reached 65% (histomorphology) to 75% (ATP content) of fresh slice values. When slices were also vitrified, viability decreased only very slightly and generally non-significantly, to approximately 55% to 70% of fresh slice values. Cortical slices that were pre-incubated at −20 °C with VM3 had a somewhat higher viability with and without vitrification than slices that were treated at 0 °C, though these differences were not statistically significant. A photograph of the relatively well preserved microscopic appearance of a cortical slice treated and vitrified with VM3 and cultured 3 h after re-warming is shown in Fig. 3D. ATP content of medullary slices was decreased to 60% of that of fresh (untreated) slices after treatment with VM3. Vitrification decreased ATP content only

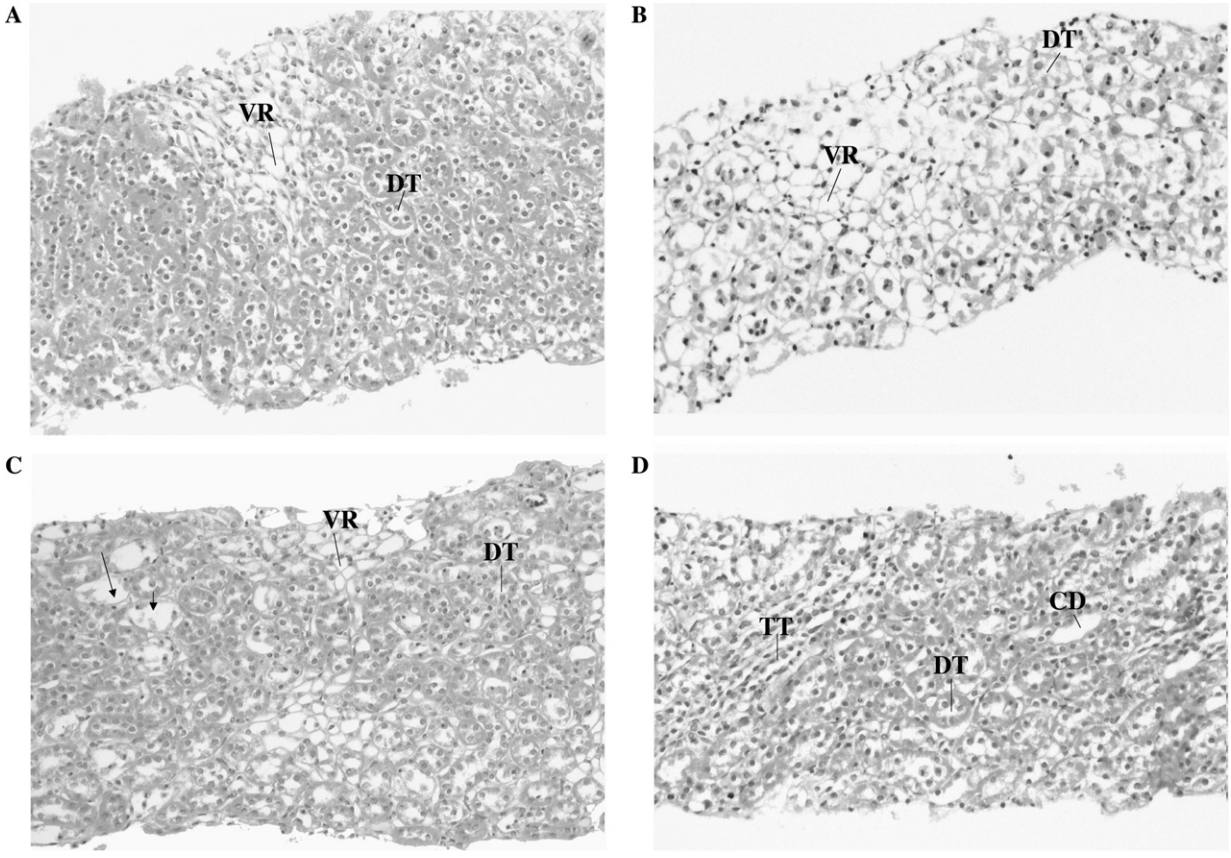

Fig. 4. Histomorphology of kidney medullary slices (A) untreated slice (B) slice cryopreserved by rapid freezing after pre-incubation with 18% Me_2SO (C) slice vitrified with VS4 (D) slice vitrified with VM3. All slices were incubated at 37 °C for 3–4 h. Examples of necrotic cells in (A and B) are indicated with an arrow. DT; distal tubulus, VR; vasa recta, TT; thin tubulus, CD; collecting duct.

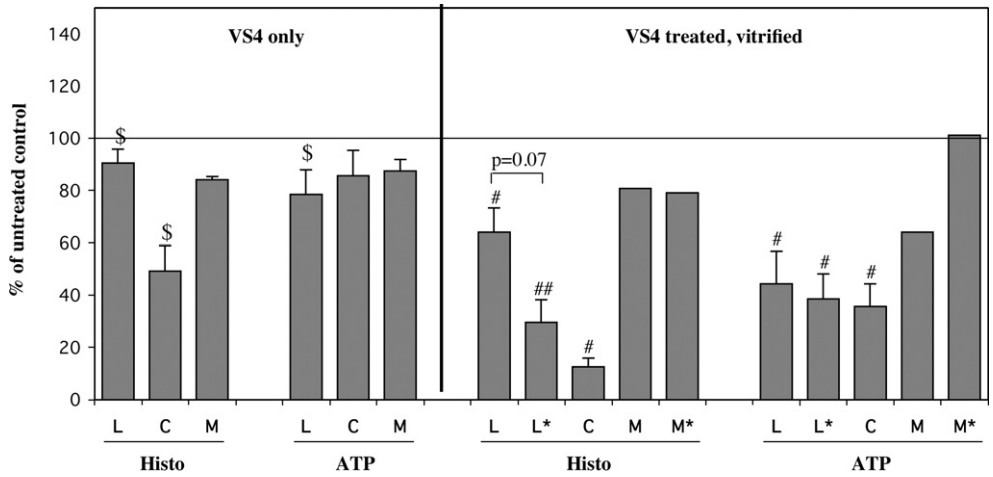

Fig. 5. Viability of liver (L), renal cortex (C) and medulla (M) slices after pre-incubation with VS4 and vitrification, relative to levels of untreated slices (not pre-incubated with VS4 or vitrified). Mean ATP content of untreated slices was 17.6 ± 1.6 (liver slices), 5.2 ± 0.01 (renal cortex slices) and 6.2 ± 0.5 (renal medullary slices) nmol/mg protein. Histomorphologic appearance of slices was quantified as the percentage of intact (not necrotic) cells in the slice cross section. Mean % of intact cells of untreated slices was 71.6 ± 1.9 (liver slices), 61.3 ± 6.3 (renal cortex slices) and 79.2 ± 0.9 (renal medullary slices). All slices were incubated for 4 h at 37 °C prior to viability testing. L* and M*: of these slices v_{crw}s were higher than feasible warming rates. The final pre-incubation step with 'full strength' VS4 was 30 min with liver slices and 10–15 min with kidney slices. Bars give the means of 2–5 experiments (three slices per experiment), bar represents one experiment (three slices) in the case of vitrified medulla slices + SEM. $^{\$}$Values are significantly lower than those of untreated slices with $p < 0.05$ (t-test). $^{\#}$Values are significantly lower than those of slices that were only treated with VS4 with $p < 0.05$ (t-test) ($^{\#\#}p < 0.01$).

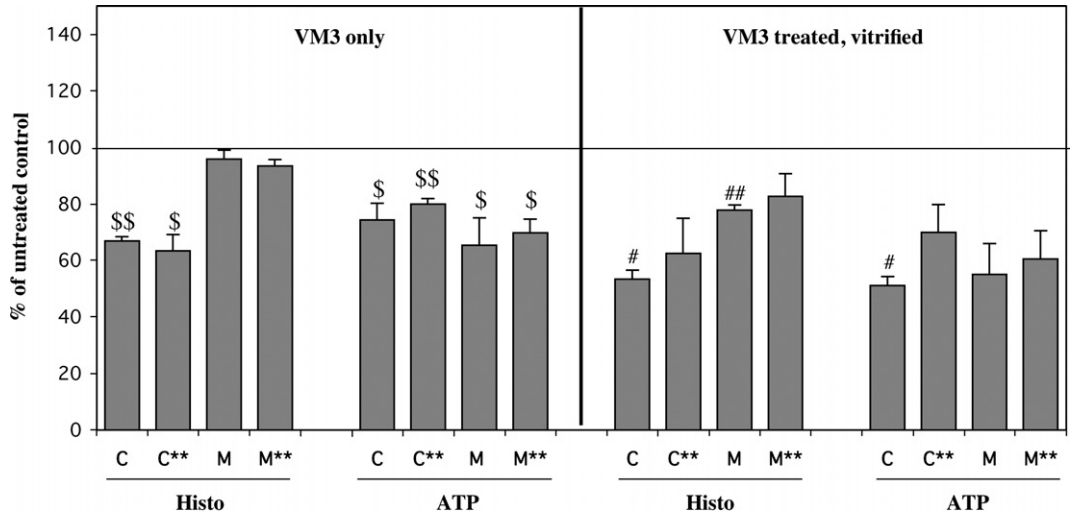

Fig. 6. Viability of liver (L), renal cortex (C) and medulla (M) slices after pre-incubation with VM3 and vitrification, relative to levels of untreated slices (not pre-incubated with VM3 or vitrified). Mean ATP content of untreated slices was 5.9 ± 0.8 (renal cortex slices) and 13.1 ± 2.2 (renal medullary slices) nmol/mg protein. Histomorphologic appearance of slices was quantified as the percentage of intact (not necrotic) cells in the slice cross section. Mean % of intact cells of untreated slices was 78.9 ± 5.9 (renal cortex slices) and 80.0 ± 0.0 (renal medullary slices). **C and **M: slices were pre-incubated at −20 °C with VM3. All slices were incubated for 3 h at 37 °C prior to viability testing. Bars give the means of three experiments (three slices per experiment) + SEM, however for **C one experiment was not taken into account because ice crystal formation due to devitrification was observed during re-warming of these slices. ND = intact cells Not Detected. $Values are significantly lower than those of untreated slices with $p < 0.05$ (t-test) ($^{\$\$}p < 0.01$). #Values are significantly lower than those of slices that were only treated with VM3 with $p < 0.05$ (t-test) ($^{\#\#}p < 0.01$).

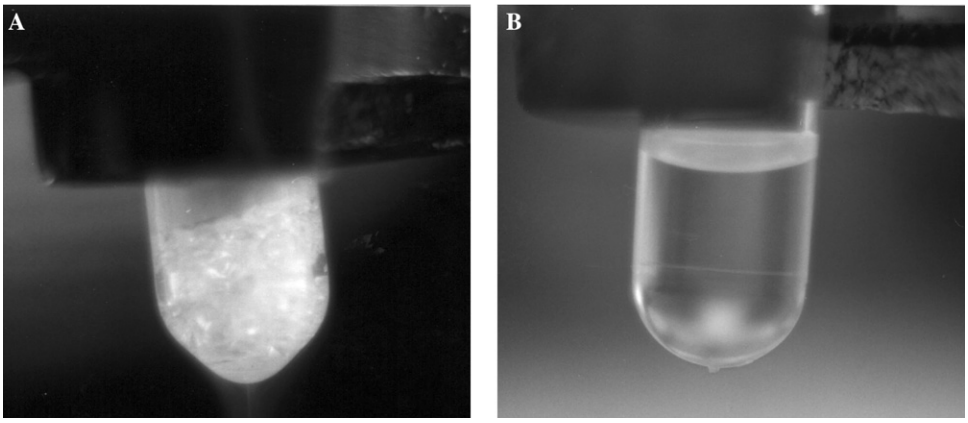

Fig. 7. Influence of cooling rate on stability of the formed VM3 glass. (A) Vial was directly submerged in liquid nitrogen (cooling rate −100 °C/min) (B) vial was cooled at 1.5 °C/min near the glass transition point of VM3 (∼−125 °C).

slightly. The number of viable cells in the slice cross section, as observed by studying the histomorphology of medulla slices was barely affected by VM3 treatment (Fig. 6) and decreased slightly to 80%–85% of fresh slice values after vitrification. A photomicrograph of the histomorphology of an almost completely normal-appearing renal medullary slice after vitrification and rewarming with VM3 is shown in Fig. 4D.

Pilot experiments with liver slices showed no improvement of viability of slices vitrified with VM3 in comparison with those vitrified with VS4 (data not shown).

Discussion

Rapid freezing with moderately concentrated cryoprotectant solutions (18% Me_2SO) has been proposed by our laboratory as a simple and adequate way to cryopreserve rat liver slices, maintaining viability and phase I and II biotransformation activity of cryopreserved rat liver slices for at least 4 h after thawing. Others who have used rapid freezing for the cryopreservation of liver slices (using 18% Me_2SO or more) have found similar results [27,42]. In the present study, liver slices again remained viable after cryopreservation by rapid freezing, however kidney slices were severely damaged despite the fact that cryoprotectant toxicity was prevented by stepwise introduction and removal of Me_2SO.

Within the present study, viability of slices was determined by examining slice histomorphology and by measuring their ATP content. Both parameters have appeared to be very sensitive to cryopreservation damage in previous

studies [30,43]. Both these studies found that ATP content of cryopreserved slices decreased rapidly after thawing, while phase I metabolism appeared to more stable. Also for kidney slices it was found that mitochondria were target of cryopreservation damage resulting in a decrease in ATP content of slices, while other functional parameters were retained [45]. Moreover, recently, at our laboratory we also found a significant positive correlation between CYP3A mediated metabolism of testosterone in human (fresh) liver slices during the first 3 h after slice preparation and ATP content at $t = 0$ (directly after cutting the slices) (unpublished data). For these reasons, we feel that these parameters are good (first) indicators of slice viability and predictive for slice functionality i.e. metabolic capacity, and thus suitable for method development as done in the present study.

The mechanism by which rapid freezing maintains viability of liver slices is not completely clear. de Kanter and Koster [7] have proposed that vitrification within the slice, promoted by compartmentalization of water within the tissue, could possibly explain the success of rapid freezing. Recent studies by DSC measurements, however, have pointed out that with 18% Me_2SO and rapid freezing, ice was formed in liver slices in the same amount as in the cryopreservation solution, with ice crystals probably formed both within the liver cells and in the intercellular space [4]. A number of authors have reported examples of cell types that survive intracellular ice formation caused by freezing rapidly [32,33,49], provided that these cells were rapidly thawed as well. It was proposed that small intracellular ice crystals would re-arrange during slow warming, growing to a size deleterious to the cells [33]. With liver slices, slow warming after rapid freezing also results in loss of viability (unpublished data), consistent with the hypothesis that intracellular ice re-crystallization is responsible for cryopreservation damage by rapid freezing with 18% Me_2SO. The question arises why rat kidney slices do not survive rapid freezing. Possibly, they are more susceptible to injury from small intracellular ice crystals. Studies to compare the exact location and size of ice crystals in rapidly frozen liver and kidney slices would be helpful for explaining the differences between the responses of these different tissues.

In principle, vitrification can overcome the problems due to both intracellular and extra-cellular ice formation since it allows ice-free cooling to liquid nitrogen temperatures. Traditionally, the major obstacle to the successful use of vitrification as a means of cryopreservation has been the toxicity of the high concentrations of CPAs that must be used to prevent ice crystallization [15,19,18]. In the present study we noticed that different tissues were affected differently by CPA toxicity. Renal cortex slices were particularly sensitive to VS4 exposure. Injury caused by CPA exposure can arise from either direct biochemical changes in the tissue or from osmotic forces if the latter are not adequately controlled. Approaches to reduce chemical toxicity of a CPA solution without diminishing its glass-forming tendency include using a combination of CPAs with different mechanisms of toxicity, using agents that antagonize each other's toxicity and using reduced temperatures of exposure [2,17,19] and this is the scientific basis of the development of traditional high molarity mixtures of CPAs like VS4. More recently, two new approaches have been developed that are beginning to overcome the obstacle of CPA toxicity. The first includes the use of mixtures of dimethylsulfoxide, an amide (like formamide), and ethylene glycol that are thought to preserve hydration of cellular biomolecules even when used at extremely high concentrations [21]. The second is the use of specific "ice-blocking" compounds that include molecules able to prevent the nucleation of ice [47,48]. VM3 is a new-generation vitrification solution that combines both of these new approaches. In the present studies, for kidney slices, the 8.4 M VM3 solution appeared to be generally less damaging than the 7.5 M VS4 solution.

Another approach to reducing chemical toxicity is to decrease the exposure time to the CPA. When decreasing the exposure time, one should confirm that the tissue is still adequately equilibrated with the CPA. The use of a DSC can be of help for this purpose, monitoring possible elevation of critical cooling and warming rates when equilibration is not complete upon reduction of exposure time. In the present study, we indeed found that reducing the exposure time to VS4 and VM3 reduced toxicity without increasing critical warming rates.

In the present study we limited osmotic damage by introducing and removing CPA stepwise, allowing time at each step for the cells to reverse the better part of their induced volume changes before moving on to the next step. However, the exact protocol could be further optimised. In addition, impermeable sugars were added to the CPA washout media because this is known to reduce or prevent cell swelling during CPA removal by balancing osmoles of intracellular CPA against osmoles of extracellular sugar, thus making the extracellular osmolality closer to the intracellular osmolality [34,38]. The use of 300 mM trehalose in the washing medium increased viability of VM3-treated renal cortical slices.

Vitrification at readily attainable cooling rates was originally proposed by Fahy [14] and Fahy and Hirsh [15] as a method for avoiding all damaging events associated with freezing, provided only that CPA toxicity could be prevented. A major attraction of vitrification according to Fahy and Hirsh is that in principle there is no need to worry about finding optimal cooling and warming rates for different cell types in an organ as long as cooling and warming rates are rapid enough to avoid ice crystal formation during freezing and thawing, a prediction confirmed by experimental results [40]. The cooling rate needed to permit vitrification of 10 mg samples of VS4 in VS4 buffer according to DSC measurements (approximately 10 °C/min) is easily achievable with slices. The warming rate required to prevent devitrification of these samples has been found to be much higher (1500–6990 °C/min) [4].

However, we have found previously that liver slices have less tendency to devitrify than bulk solutions [4], in agreement with the results of Peyridieu et al. for kidney and cardiac tissue [39], resulting in v_{crw} for slices that are much lower than for VS4 itself. In most tissue samples devitrification is prevented using warming rates of 800 °C/min. However in the present study, in tissue slices that had a v_{crw} higher than the feasible warming rate and in which devitrification was anticipated, viability was not different from that of slices with a v_{crw} lower than the feasible warming rates. This suggests that devitrification may be tolerable if insufficient time is allowed for recrystallization on rewarming.

According to the proposition of Fahy and Hirsh [15], in slices with a v_{crw} lower than the feasible warming rate, vitrification should not further decrease viability. In the present study this was indeed the case when VM3 pre-incubated kidney slices were vitrified: most of the viability loss was caused by treatment with VM3, and very little injury was caused by vitrification and rewarming per se. In contrast, liver slices that were vitrified with VM3 and both liver and kidney cortical slices that were vitrified with VS4 at 800 °C/min had a clearly lower viability than slices that were treated with the mixtures but not vitrified, despite the fact that no ice formation was observed (or measured). There are several possible explanations for this injury. One possibility is that the decrease of viability is due to ''chilling injury,'' which is injury caused by cooling per se that is not cooling rate dependent, and another is that the injury observed is caused by ''thermal shock,'' which is injury induced by high cooling rates such as those used with VS4. Support for the existence of thermal shock in kidney slices comes from studies showing that rabbit kidney cortical slices cooled slowly to −30 °C (a temperature greater than their freezing point in the studies performed) recovered better than slices cooled rapidly to the same temperature [17]. The present experiments suggest that both these types of injury can be avoided during the vitrification of VM3 treated rat renal cortex slices with low cooling rates.

In conclusion, rapid freezing after pre-incubation with 18% Me_2SO maintains viability of liver slices as determined by measuring ATP content and examining slice histomorphology, but is deleterious for rat kidney slices for reasons that remain to be understood. Vitrification with relatively non-toxic, high molarity mixtures as VS4 and particularly VM3 is a promising alternative approach for cryopreservation of particularly kidney slices, maintaining viability of these slices at acceptable levels.

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