Cryopreservation of Rat Precision-cut Liver Slices is Associated with Major Metabolic Stress and Ionic Perturbations

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Abstract

Background/Aims: Cryopreserved precision-cut liver slices (PCLS) in culture exhibit a rapid decline in ATP level and anabolic processes by unknown mechanisms. The aim of this study is to elucidate the key events explaining the alterations occurring in cryopreserved PCLS. *Methods:* Glucose metabolism, mitochondrial activities, ionic homeostasis and caspase-3 activity were assessed in fresh or cryopreserved (rapid freezing-thawing conditions) rat PCLS within the first hour of incubation in Williams' medium E at 37°C. *Results:* Despite a similar glycolytic activity under both conditions, only fresh PCLS were able to gradually recover their ATP and potassium content. Glycogen content dropped more rapidly in cryopreserved than in control PCLS. Only cryopreserved PCLS exhibited a decline in O_2 consumption and a lower ATP/ADP ratio from 30 min of incubation with a loss of coupling of oxygen consumption to ATP synthesis and mitochondrial calcium transport after 1 hour of incubation. Caspase-3 activation was already present in cryopreserved PCLS after a few minutes of incubation. *Conclusions:* The lack of restoration of potassium/sodium exchange might be a primary event in the metabolic alterations occurring in cryopreserved PCLS after thawing, that contributes to further mitochondrial alterations, caspase activation and compromises ATP-dependent anabolic pathways.

Introduction

Precision-cut liver slices are widely used as in vitro tool to study the putative toxicity and metabolism of xenobiotics [1, 2]. PCLS offer various advantages over isolated hepatocytes such as the preservation of tissue architecture, the maintenance of cell polarity, cell heterogeneity and cell-cell interactions and the lack of use of hydrolytic enzymes. Freshly prepared PCLS are able to maintain, at least for a few hours, efficient phase I (cytochrome P450 dependent oxidation) and phase II

(conjugation reactions) metabolism of xenobiotics, as well as important liver metabolic functions (β-oxidation, gluconeogenesis, glycogen storage, protein synthesis…) [2-4]. Cryopreservation of PCLS, that would allow to store precious tissues such as human tissue samples i.e., is much less successful than cryopreservation of isolated cells. Several PCLS cryopreservation methods, including slow or fast freezing and vitrification methods, have been described in recent years (for an overview see [2]). Most of the data concern the use of cryopreserved PCLS in xenobiotics metabolism: the maintenance of cytochrome P450-catalysed activities upon thawing and incubation has been largely described in rat and human PCLS cryopreserved by different methods, whereas several studies demonstrate that phase II enzyme activities (sulfation, glucuronidation…), which are more dependent on the energy status of hepatocytes, are more susceptible to cryopreservation [3, 5-8]. De Graaf *et* al [9] observed an improvement of phase II enzyme activities and functionality in PCLS cryopreserved in 18% DMSO as compared to usual concentrations of 10-12% or 30% DMSO. Therefore we performed a comparison of different DMSO concentrations on cryopreserved PCLS viability in the present study. We have recently shown that PCLS cryopreserved in 10% DMSO by ultrarapid freezing and incubated at 37°C in Williams' medium E are able, after thawing, as compared to controls (non cryopreserved PCLS), to maintain CYP3A2 activity and content and CYP2E1 content during 24 hours of incubation, whereas paracetamol glucuronidation and sulfation dropped to 24 and 21 % of the control value, respectively, 1 hour after thawing [3]. We have also observed a significant decrease in ATP and glycogen content, and an increase in LDH released in the medium after a few hours of incubation. Others authors report, despite the maintenance of drug metabolism that the absolute values of several viability and functionality parameters are considerably altered in cryopreserved PCLS within 24 hours of incubation [7-10]. It seemed essential to analyse the biochemical mechanisms underlying the global loss of functionality and integrity of cryopreserved PCLS observed upon incubation after thawing in order to propose scientifically based modifications of culture conditions prone to improve cell viability after thawing.

Materials and Methods

Materials

Williams' medium E and fetal calf serum (FCS) were purchased from Gibco BRL (Middlesex, UK). Dimethyl sulfoxide (DMSO), ouabain and enzymes (the purest grade available) were obtained from Sigma Chemicals (St Louis, USA). L-Glutamine was purchased from ICN Biomedicals Inc (Ohio, USA). Insulin (Actrapid HM®, Novo Nordisk, Bagsvaerd, Denmark) and dexamethasone phosphate (Decadron®, MSD, New Jersey, USA) were commercially available. Radio-labelled chemicals were purchased from Amersham (Buckinghamshire,UK). All other chemicals used were of the purest grade commercially available.

Preparation of PCLS

Perfusion *in situ* of the liver of an adult fed male Wistar rats (Iffa Credo, Brussels, Belgium) weighing 280 to 320 g was performed with ice-cold Krebs-Ringer solution prior to liver removal. Tissue cores (10mm) were prepared from the freshly excised liver, and PCLS (about 250 μm thickness) were rapidly prepared in oxygenated ice-cold Krebs-Ringer buffer using a Krumdieck slicer according to a procedure previously described [3]. Fresh PCLS were stored during at least 30 min in ice-cold Williams' medium E supplemented with L-glutamine (2 mM), insulin (100 nM), dexamethasone (10 nM) (hereafter referred to as "preincubation medium") and 10% FCS (v/v) under an atmosphere of 95% O_2 / 5% CO_2 until incubation at 37°C.

PCLS cryopreservation and thawing

PCLS cryopreservation and thawing were performed as previously described [3]. Briefly, prior to freezing, PCLS were stored in ice-cold oxygenated "preincubation medium" supplemented with 10% DMSO (v/v) for 30 min for most experiments. One experiment was performed with 18% DMSO (v/v). They were then rapidly frozen by immersion in liquid nitrogen and kept in liquid nitrogen until use. A rapid thawing and a washing step in "preincubation medium" containing 10% FCS (v/v) for 5 min were performed before incubation as the fresh ones.

PCLS incubation

PCLS were placed into glass vials containing "preincubation medium" supplemented with 25 mM glucose (2 ml medium per slice). Vials were continuously oxygenated (95% O_2 / 5% CO_2) all along the incubation and placed in a shaking water bath at 37°C. No pH change was observed during the incubation. This incubation procedure allowed to maintain the functionality of PCLS during at least 24 hours [3]. At various time points, incubation medium samples and PCLS were carefully taken for analysis.

PCLS membrane integrity

The loss of membrane integrity was estimated by measuring the activity of lactate dehydrogenase (LDH) in the culture medium, according to the procedure of Wroblesky and Ladue [11]. The results are expressed as international units (IU) of LDH per milligram of proteins.

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Adenine nucleotides analysis

PCLS were taken, washed twice in saline and sonicated in 2% perchloric acid. The intracellular AMP, ADP and ATP contents were quantified on neutralised perchloric acid extracts by HPLC on a 110 X 4.7-mm PartiSphere 5 SAX anion-exchange column (Whatman, Maidstone, Kent, UK) by the method of Hartwick and Brown [12] modified according to Vincent *et al* [13].

Assessment of glucose metabolism

Glycolysis was estimated by determining the lactate concentration in the medium according to the procedure described by Hohorst *et al* [14] and by measuring the rate of detritiation of [3-^{3}H]glucose. After 1 hour incubation in Williams'medium E containing 11 mM glucose, PCLS were further incubated in medium supplemented with [3-^{3}H]glucose (0.2 µCi/ml). The kinetics of release of ^{3}H$_2$O in the medium was measured, during 8 min, after separation from [3-^{3}H]glucose [15]. Glucose uptake was measured, during the first 5 min of incubation, by following the incorporation of 2-deoxy-D-[1-^{3}H]glucose into PCLS (9 µCi/ mmol in Williams'medium E 11 mM glucose). The radioactivity inside the tissue was quantified using a liquid scintillation counter (Pharmacia, Wallac liquid scintillation counter, type 1410). Glycogen content of PCLS was quantified by a procedure as previously described [3].

PCLS mitochondria

Mitochondria were isolated from fresh or cryopreserved PCLS incubated for 15 or 60 min at 37°C [16] and used for the measurement of oxygen consumption and F0F1-ATPase activity [17].

Oxygen consumption of PCLS and isolated mitochondria

Oxygen consumption of PCLS was measured by using a Clark-type oxygen electrode (Yellow Springs Inst., OH, USA) in a chamber containing the Krebs-Ringer solution supplemented with 10 mM glucose at 30°C. The medium was air-equilibrated at 0.22 mM oxygen [18]. Oxygen consumption rate of freshly isolated mitochondria was measured at 30°C in medium containing 120 mM KCl, 2.5 mM KH$_2$PO$_4$, 10 mM Hepes, 5 mM MgCl$_2$ and 1 mM EDTA, pH 7.2.

Ionic homeostasis

PCLS were rinsed in distilled water and sonicated in 3.5% perchloric acid. After centrifugation, the supernatant fraction was assayed for K$^+$ by flame photometry. For Na$^+$/K$^+$-ATPase activity measurement, liver plasma membrane isolation was performed on PCLS as described by Sennoune *et al* [19]. Briefly, PCLS were taken, rinsed and homogenised in 10 vol ice-cold buffer (8% saccharose, 0.1 mM phenylmethanesulfonyl fluoride, 1mM EDTA and 30 mM imidazol/HCl pH 7.4). After sequential differential centrifugations (120 g for 5 min; 6800 g for 15 min; 48380 g for 30 min), the final pellet was resuspended in 8% saccharose and 30 mM imidazol/HCl pH 7.4. Na$^+$/K$^+$-ATPase activity was determinated using slight modifications of a method previously described by measuring the rate of release of inorganic phosphate (Pi) from ATP at 37°C [20]. Briefly, the incubation mixture (500 µl) consisted of 50 mM Tris-HCl (pH 7.4), 100 mM NaCl, 25

mM KCl, 3 mM MgCl$_2$, 1 mM EGTA and 5 mM NaN$_3$. The reaction was started by the addition of 2 mM ATP and stopped after 20 min of incubation at 37°C by adding 5 ml of HCl 0.1 M. Each ATPase assay was performed with 15 min preincubation in the presence and absence of 1 mM ouabain to assess the ouabain-inhibition component of the total Mg^{2+}-ATPase activity.

Protein and triglyceride synthesis

Protein synthesis by PCLS was assayed by measuring the incorporation of L-[U-^{14}C]leucine (175 µCi/mmol) into acid-precipitable proteins [21]. Triglyceride synthesis was estimated by measuring the incorporation of [1-^{14}C]acetate into intracellular triglycerides. PCLS were incubated in the medium containing 2 mM [1-^{14}C]acetate (0.2 mCi/mmol), rinsed and sonicated in 400 µl of NaCl 0.9%. After triglyceride extraction with isopropanol/heptane (4:1), radioactivity of organic phase was measured using a liquid scintillation counter (Pharmacia, Wallac type 1410) [22].

Measurement of caspase activity

Caspase-3-like activity was evaluated using the FluorAce™ apopain assay kit from Bio-Rad (California). The results are expressed as activity units of caspase 3 per milligram of proteins.

Protein determination

All results were expressed per mg of proteins. Protein measurement was performed in duplicate in PCLS for all conditions and at all time points after sonication. The amount of protein was assessed in PCLS by the method of Lowry *et al* [23] using bovine serum albumin as standard.
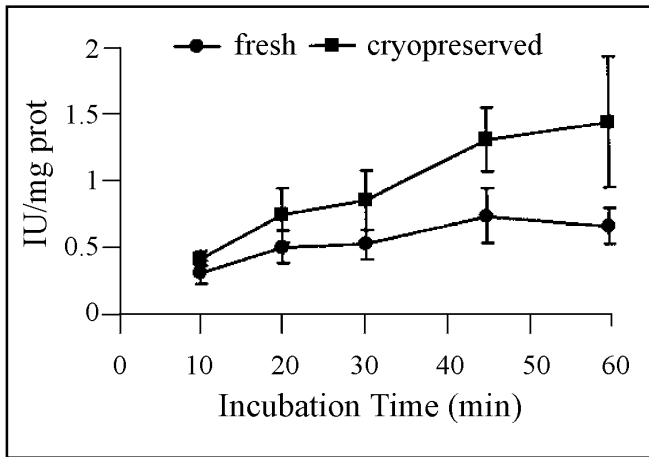
Statistical analysis

All the parameters were measured at least in duplicate on two slices obtained from the same rat (except for mitochondria isolation, which was performed on 20 slices). A mean value has been calculated for each experiment. The values presented in the tables and figures correspond to the mean ± SEM of at least 3 experiments (performed on 3 different rats). Statistical analysis was performed by an ANOVA by using SPSS™ 9.0 statistical software. Bonferroni correction of the P values was used for statistical comparison between results at a given time point. Student's t-test was used when an ANOVA test was not suitable. For all tests, P<0.05 was considered statistically significant.

Results

PCLS viability

Cellular integrity of fresh PCLS and PCLS cryopreserved in 10% DMSO was evaluated by measuring LDH leakage in the incubation medium (FIG. 1). LDH activity gradually increased at a higher extent in cryopreserved than in fresh PCLS. Intracellular potassium was initially low in both fresh PCLS and PCLS cryopreserved in 10% DMSO. After 10 min of incubation, the potassium content was progressively

Fig. 1. LDH leakage into the medium of fresh PCLS and PCLS cryopreserved in 10% DMSO at various incubation times. Data are expressed as the mean ± SEM (n=4). Statistical analysis: ANOVA; treatment interaction: $P<0.05$; time-treatment interaction: $P>0.05$; Bonferroni analysis: $P>0.05$.
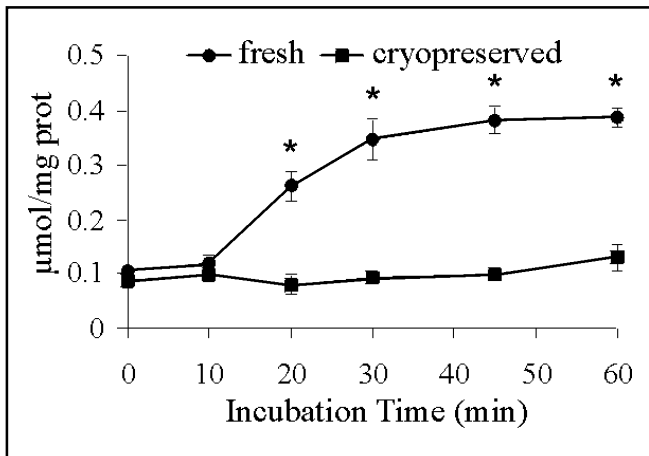
Fig. 2. Potassium content in fresh PCLS and PCLS cryopreserved in 10% DMSO at various incubation times. Data are expressed as the mean ± SEM (n=3). Statistical analysis: ANOVA; treatment interaction: $P<0.05$; time-treatment interaction: $P<0.05$; Bonferroni analysis: * $P<0.05$ compared with fresh PCLS after the same incubation period.

restored in fresh PCLS only to reach normal values after 30 min of incubation, whereas it remained low all along the incubation in cryopreserved PCLS (FIG. 2). Na$^+$/K$^+$-ATPase activity was 61.11 ± 14.70 and 59.08 ± 11.79 nmol Pi/min.mg proteins, respectively, in fresh and PCLS cryopreserved in 10% DMSO after 30 min of incubation (Student's paired t-test: $p>0.05$). The intracellular concentration of AMP, ADP and ATP in PCLS was measured under both conditions (Table 1). The total

amount of nucleotides dropped in both fresh and cryopreserved PCLS (10% DMSO) within the first 10 min of incubation, then increased in fresh PCLS and decreased from 30 to 60 min of incubation in cryopreserved PCLS. Only fresh PCLS were able to gradually increase their ATP content (and ATP/ADP ratio) upon incubation. The AMP and ADP contents of both fresh and cryopreserved PCLS were similar during the first hour of incubation. After 60 min of incubation, cryopreservation of PCLS in 18% DMSO improved cell lysis, evaluated by measuring LDH leakage in the medium, potassium and ATP content as compared to PCLS cryopreserved in 10% DMSO but those parameters were significantly altered as compared to fresh values (Table 2).

Assessment of glucose, protein and lipid metabolism

Lactate production in the medium (FIG. 3) was similar during the first hour of incubation in both fresh and PCLS cryopreserved in 10% DMSO. Glycolysis, estimated by measuring the rate of detritiation of [3-^{3}H]glucose, was similar under both conditions and corresponded to 132.3 ± 10.1 dpm/mg proteins.min and 113.3 ± 20.9 dpm/mg proteins.min in fresh and cryopreserved PCLS, respectively (n=4, Student's t-test: $p>0.05$) after 1 hour of incubation. Glycogen content was similar in fresh PCLS and PCLS cryopreserved in 10% DMSO at time 0 (± 450 μg/mg proteins) but decreased more rapidly in cryopreserved than in fresh PCLS (FIG 3). After 60 min of incubation, similar glycogen content was observed in PCLS cryopreserved in 10 or 18% DMSO (table 2). PCLS capacity to take up extracellular 2-deoxy-D-[1-^{3}H]glucose after the first 5 min of incubation was similar under both conditions and correponded to 1031 ±123 and 1286 ± 128 dpm/mg proteins in fresh and PCLS cryopreserved in 10% DMSO respectively (n=4, Student's t-test: $p>0.05$). The incorporation of L-[U-^{14}C]leucine into proteins and of [1-^{14}C]acetate into triglycerides (FIG. 4 and table 2) increased with time in fresh PCLS whereas neither protein nor lipid synthesis could be shown in PCLS previously cryopreserved in 10 or 18% DMSO.

Oxygen consumption of PCLS and mitochondria isolated from PCLS

In order to assess respiration capacity, oxygen consumption by intact fresh PCLS or PCLS cryopreserved in 10% DMSO, previously incubated for 0, 15, 30, 45 and 60 min at 37°C was measured using a Clark-type electrode (FIG. 5). At time 0, oxygen

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Table 1. Adenine nucleotides content in fresh PCLS and PCLS cryopreserved in 10% DMSO. Data are expressed as nmol/mg protein. Results are presented as the mean ± SEM (n=3). Total adenine nucleotide content was calculated by adding ATP, ADP, and AMP contents. Statistical analysis: ANOVA; treatment interaction: P<0.05 (ATP and ATP/ADP ratio) and P>0.05 (AMP and ADP); time-treatment interaction: P<0.05 (ATP/ADP ratio) and P>0.05 (AMP, ADP and ATP); Bonferroni analysis: * P<0.05 compared with fresh PCLS after the same incubation period.

Incubation Time (min)	AMP		ADP	
	fresh	cryopreserved	fresh	cryopreserved
0	1.07 ± 0.38	1.84 ± 0.30	4.30 ± 0.79	3.96 ± 0.39
10	0.67 ± 0.11	0.83 ± 0.06	1.96 ± 0.25	2.80 ± 0.13
20	0.64 ± 0.03	0.56 ± 0.10	1.89 ± 0.49	2.43 ± 0.24
30	0.67 ± 0.03	0.68 ± 0.18	1.42 ± 0.09	2.26 ± 0.33
60	0.55 ± 0.03	0.36 ± 0.03	1.35 ± 0.28	1.52 ± 0.58

Incubation Time (min)	ATP		ATP/ADP ratio		Total	
	fresh	cryopreserved	fresh	cryopreserved	fresh	cryopreserved
0	2.87 ± 0.68	1.74 ± 0.35	0.69 ± 0.18	0.43 ± 0.07	8.23	7.54
10	2.39 ± 0.64	1.86 ± 0.11	1.20 ± 0.22	0.67 ± 0.03	5.01	5.49
20	3.70 ± 0.48	2.10 ± 0.23	2.09 ± 0.26	$0.88 \pm 0.13*$	6.22	5.08
30	4.37 ± 0.37	2.57 ± 0.47	3.06 ± 0.08	$1.17 \pm 0.23*$	6.46	5.51
60	5.83 ± 0.95	$1.96 \pm 0.78*$	4.38 ± 0.22	$1.28 \pm 0.02*$	7.72	3.84

	fresh PCLS	cryopreserved PCLS	
		10% DMSO	18% DMSO
LDH leakage (IU/mg protein)	0.41 ± 0.06	$2.36 \pm 0.21*$	$1.47 \pm 0.19*^a$
K^+ content (μmol/mg protein)	0.67 ± 0.06	$0.14 \pm 0.01*$	$0.26 \pm 0.08*^a$
ATP content (nmol/mg protein)	5.55 ± 0.53	$0.56 \pm 0.08*$	$2.00 \pm 0.52*^a$
Glycogen content (μg/mg protein)	347.6 ± 67.8	$231.2 \pm 73.1*$	$221.2 \pm 69.8*$
Protein synthesis (dpm/mg protein)	1491.0 ± 234.5	$58.1 \pm 19.9*$	$127.8 \pm 48.4*$
Caspase 3 activity (unit/mg protein)	0.99 ± 0.17	$15.05 \pm 1.31*$	$9.59 \pm 2.00*^a$

Table 2. Functionality of fresh PCLS and PCLS cryopreserved in 10% or 18% DMSO after 60 min of incubation. Data are expressed as the mean ± SEM (n=3). Statistical analysis: ANOVA; treatment interaction: P<0.05; Bonferroni analysis: * P<0.05 compared with fresh PCLS; [a] P<0.05 compared with PCLS cryopreserved in 10% DMSO.

consumption was very low under both conditions (± 2 nmol O_2/min.mg proteins) and increased similarly to reach a maximum after 30 min. Thereafter, fresh PCLS maintained a constant O_2 consumption level whereas a decline in O_2 consumption was observed in cryopreserved PCLS, which appeared significant after 60 min of incubation. The effect of the addition of different substrates or inhibitors was assessed in mitochondria isolated from fresh and cryopreserved PCLS, which had been previously incubated for 15 min or 60 min at 37°C (FIG. 6). O_2 consumption in presence of glutamate/malate (1) as electron donor was similar in all conditions. The addition of amytal (2) significantly decreased O_2 consumption in fresh PCLS mitochondria only. The addition of succinate (3) (substrate for complex II) induced an increase in O_2 consumption at the same extent in all conditions. The addition of ADP (4) as a substrate
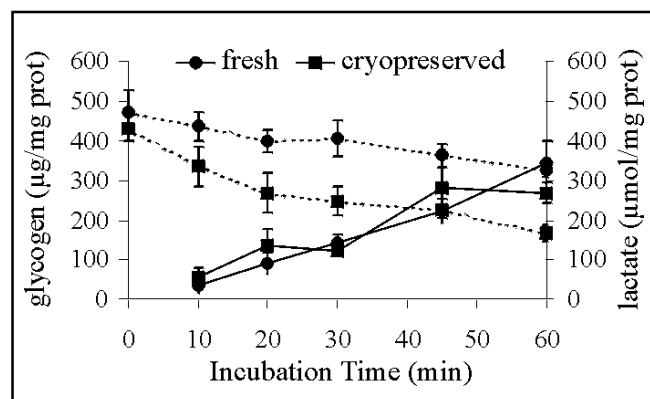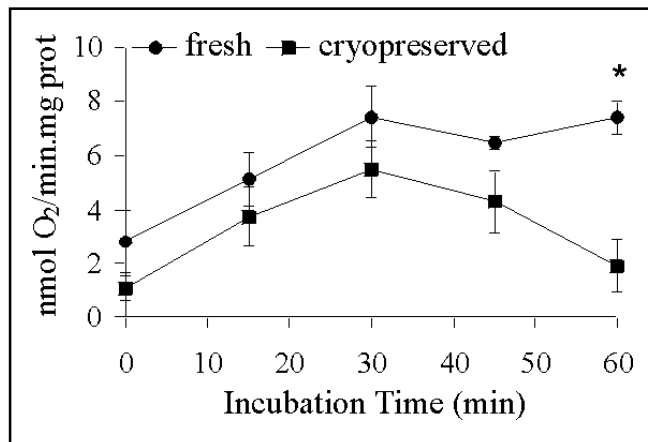

Fig. 3. Lactate concentration (—) in the medium and glycogen content (····) in fresh PCLS and PCLS cryopreserved in 10% DMSO at various incubation times. Data are expressed as the mean ± SEM (n=3). Statistical analysis: ANOVA; treatment interaction: P<0.05 (glycogen) and P>0.05 (lactate); time-treatment interaction: P>0.05 (lactate and glycogen); Bonferroni analysis: P>0.05.
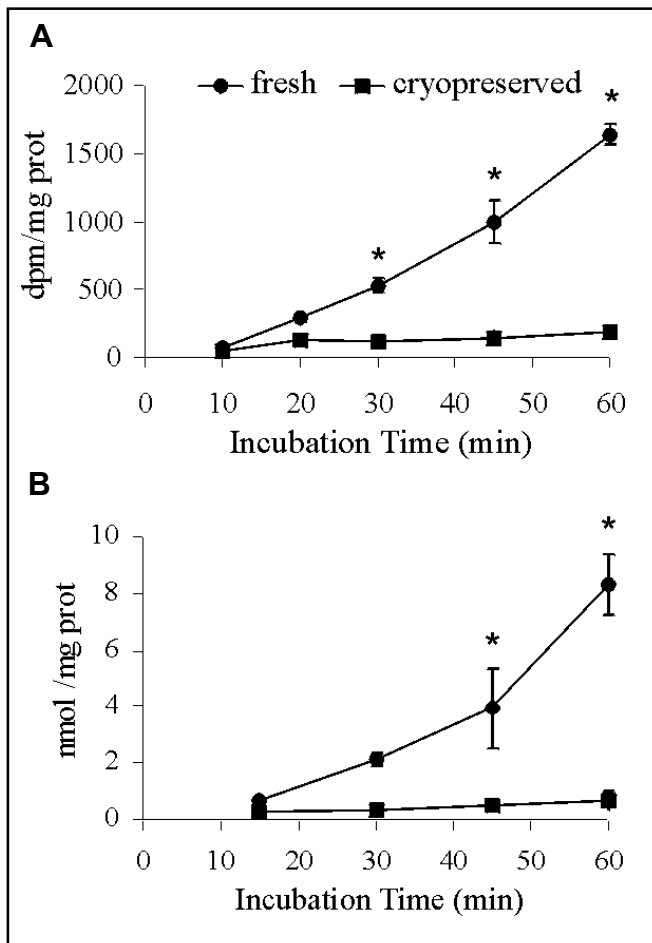
Fig. 4. Incorporation of L-[U-^{14}C]leucine into proteins (A) and [1-^{14}C]acetate into triglycerides (B) in fresh PCLS and PCLS cryopreserved in 10% DMSO at various incubation times. Data are expressed as the mean ± SEM (n=3). Statistical analysis: ANOVA; treatment interaction: P<0.05; time-treatment interaction: P<0.05; Bonferroni analysis: * P<0.05 compared with fresh PCLS after the same incubation period.

Fig. 5. Oxygen consumption in fresh PCLS and PCLS cryopreserved in 10% DMSO at various incubation times. Data are expressed as the mean ± SEM (n=5) . Statistical analysis: ANOVA; treatment interaction: P<0.05; time-treatment interaction: P>0.05; Bonferroni analysis: * P<0.05 compared with fresh PCLS after the same incubation period.

of F0F1-ATPase for ATP synthesis induced an increase in O$_2$ consumption in mitochondria of both fresh and cryopreserved PCLS at time 15 min. After 1 hour of incubation, no increase in O$_2$ consumption was observed in cryopreserved PCLS as compared to controls. The addition of oligomycine (5), a specific inhibitor of the F0F1-ATPase, led to a significant decrease in O$_2$ consumption only in fresh mitochondria after 15 min or 1 hour of incubation as compared to the previous condition. The addition of CaCl$_2$ (6) induced a similar increase in O$_2$ consumption in fresh and cryopreserved PCLS mitochondria after 15 min of incubation, whereas a further increase was observed only in fresh PCLS

mitochondria isolated after 1 hour of incubation but not in cryopreserved. Uncoupling by CCCP (7) provoked a significant rise in O$_2$ consumption in all conditions but at a lower extent in cryopreserved PCLS as compared to control after 60 min of incubation.

F0F1-ATPase activity

F0F1-ATPase activity, assayed after 60 min of incubation, was statistically increased in PCLS cryopreserved in 10% DMSO (94.34 ± 10.26 and 132.95 ± 5.09 nmol Pi/min.mg proteins in fresh and cryopreserved PCLS, respectively) (Student's paired t-test: p<0.05).

Caspase-3-like activity

Caspase-3-like activity remained low in fresh PCLS upon the first hour of incubation (± 1 unit/mg proteins) whereas, in PCLS cryopreserved in 10% DMSO, it increased by 2 fold after 30 min of incubation to reach values 11 times higher than control values after 1 hour of incubation (FIG. 7). A further increase was observed in cryopreserved PCLS thereafter (30.63 ± 3.94 unit/mg proteins after 3 hours of incubation). After 60 min of incubation, cryopreservation of PCLS in 18% DMSO improved caspase-3-like activity increase as compared to PCLS cryopreserved in 10% DMSO but values were higher than fresh PCLS (Table 2).

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Fig. 6. Oxygen consumption by mitochondria of fresh PCLS and PCLS cryopreserved in 10% DMSO at various incubation times. (**A**) Typical pattern of oxygen consumption by mitochondria isolated from fresh or cryopreserved PCLS previously incubated for 15 or 60 min at 37°C. The numbers correspond to addition of 5 mM glutamate and 1 mM malate (1), 1 mM amytal (2), 5 mM succinate (3), 1 mM ADP (4), 3.3 µg/ml oligomycine (5), 1.3 mM $CaCl_2$ (6) and 1.5 µM CCCP (7) in 3 ml medium containing mitochondrial suspension (± 1 mg prot). (**B**) Mean ± SEM of oxygen consumption under the different conditions (n = 3 to 6). Statistical analysis: Student's t-test; * P<0.05 vs fresh 60, • P<0.05 vs cryopreserved 15 for the same condition; [a] P<0.05 vs previous condition (2 versus 1, 3 versus 2, …) for the same treatment and at the same time of incubation.
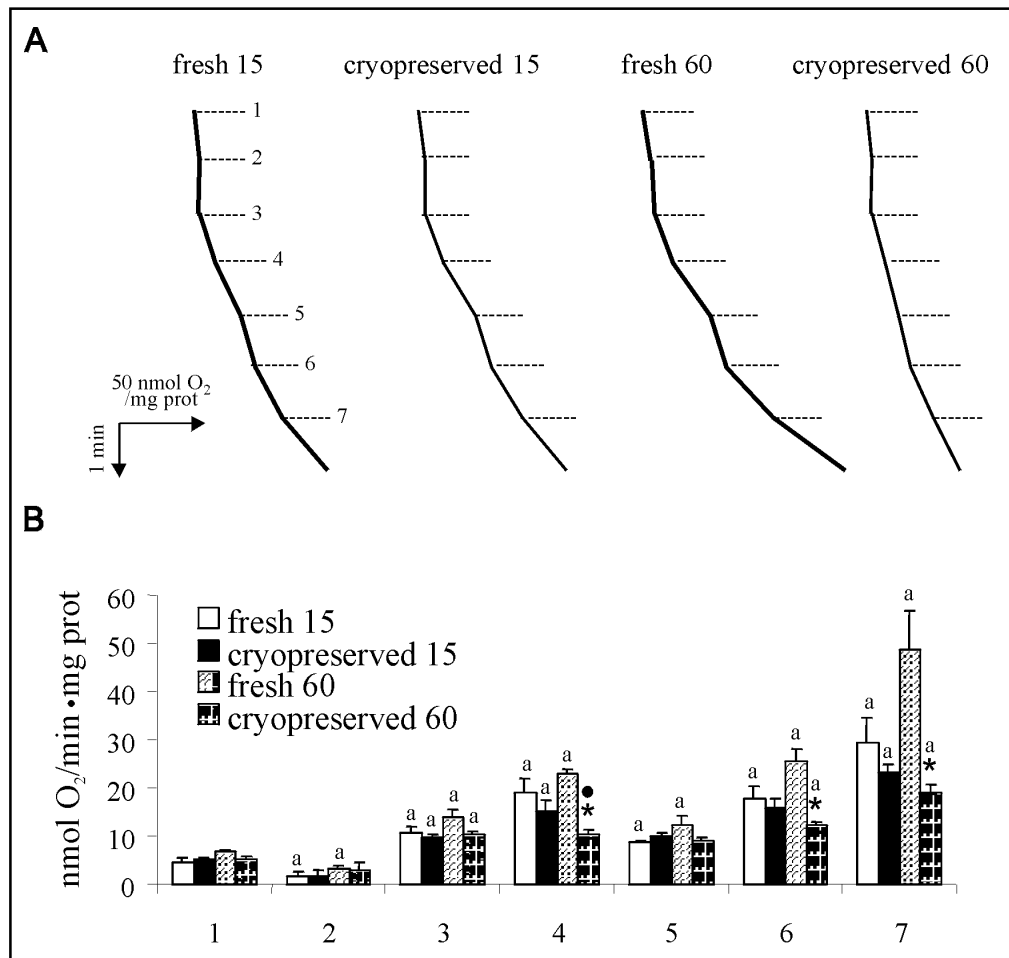
Discussion

Only few studies report successful cryopreservation of PCLS and most demonstrate that only few functions such as CYP-dependent oxidative metabolism are maintained for several hours upon incubation after thawing, whereas highly "energy" consuming processes such as drug conjugation, or lipid and protein synthesis – as confirmed in the present study - are rapidly lost upon incubation [3, 8-10].

We have shown, in this study, that ATP content of rat PCLS cryopreserved in 10% DMSO remains lower than in fresh PCLS all along the incubation period, a characteristic which persists after long term incubation (24 hours) [3]. The drop in ATP content in cryopreserved PCLS has also been described by [8] and Martin *et al* [24] but the mechanisms that could explain this observation had not been studied yet. ATP synthesis in
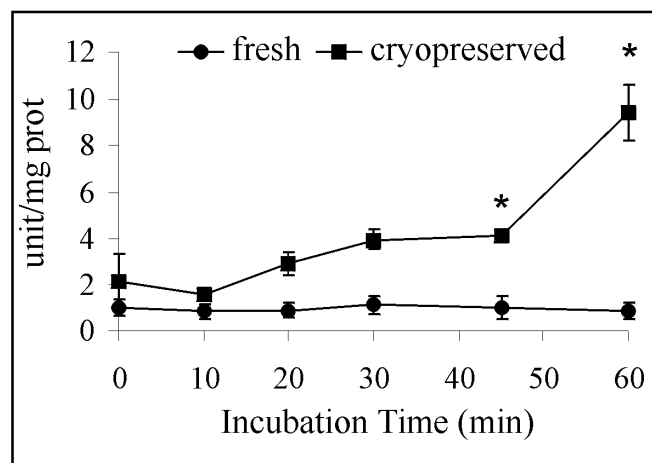
Fig. 7. Caspase-3-like activity in fresh PCLS and PCLS cryopreserved in 10% DMSO at various incubation times. Data are expressed as the mean ± SEM (n=4). Statistical analysis: ANOVA; treatment interaction: P<0.05; time-treatment interaction: P<0.05; Bonferroni analysis: * P<0.05 compared with fresh PCLS after the same incubation period.

the intact liver is supported by glycolysis and the mitochondrial oxidative phosphorylation [25]. We have shown that cryopreserved PCLS are able to take up extracellular glucose and to perform glycolysis to the same extent as the fresh ones. The decrease in glycogen content observed in cryopreserved PCLS suggests that freezing-thawing conditions favour metabolic pathways providing substrate, namely glucose-6-phosphate [26] for glycolysis. Despite this fact, we only observe a slight increase in ATP content in cryopreserved PCLS (+ 0.83 nmol/mg protein) from 0 to 30 min of incubation, that decreases later on in cryopreserved PCLS. Moreover, the addition of 25 mM glucose or 2 mM acetate as substrates in the medium did not allow any increase in ATP content in PCLS (data not shown). Total adenine nucleotide content gradually decreases in cryopreseved PCLS, thus suggesting the occurrence of nucleotide catabolism.

The difference between both fresh and cryopreserved PCLS in terms of alterations in ATP level might be explained by several mitochondrial alterations. F0F1-ATPase activity was not altered and even higher in cryopreserved than in control PCLS. The uncoupling and/or the alteration of the proton electrochemical gradient across the mitochondrial inner membrane could be implicated in the low ATP level. O_2 consumption by entire PCLS, which is representative of respiratory chain functionality is very low in both fresh and cryopreserved PCLS at the beginning of the incubation, reflecting the break of metabolic activity at 4°C [2]. After 30 min of incubation, both fresh and cryopreserved PCLS reach a maximal and similar level of oxygen consumption. Isolation of mitochondria from PCLS was performed in order to test the responsiveness of respiratory chain to substrates and inhibitors acting on the different complexes. Interestingly, during the period of linear increase in O_2 consumption (after 15 min of incubation), mitochondrial respiratory chain is functional as shown by the evolution of O_2 consumption through addition of substrates or "respiratory chain drivers" (succinate, ADP, $CaCl_2$), or by the inhibition of O_2 consumption by amytal or oligomycin in both fresh and cryopreserved PCLS. No studies were published before this one about mitochondrial performance after tissue cryopreservation. Only some studies report that rat liver mitochondria cryopreserved after isolation are able to respond in a qualitatively similar fashion in terms of oxygen consumption after addition of substrates (succinate) and phosphate acceptor (ADP) when compared to the response of fresh, control organelles [27]. After 60 min

of incubation, O_2 consumption by cryopreserved PCLS mitochondria did not increase after ADP, $CaCl_2$ and CCCP addition. This suggests a loss of mitochondrial membrane potential, which is involved in both H^+ and Ca^{++} transport across the mitochondrial membrane [28].

Mitochondrial injury has already been shown in cryopreserved porcine hepatocytes and this phenomenon seems to be linked to the activation of caspases leading to apoptosis, measured following 2 hours of incubation after thawing [29]. In our experiments, we show that caspase 3 activation is already present in cryopreserved PCLS after a few minutes of incubation.

The efflux of ions, primarily potassium, has been shown to play a necessary and pivotal role in cell shrinkage and apoptosis [30, 31]. We have observed that PCLS K^+ level at time 0 is low under both conditions, a phenomenon probably due to the passive movement of potassium out of the cell during PCLS preparation at 4°C. Only fresh PCLS were able after 10 min of incubation to restore their intracellular potassium content to reach their maximum after 30 min of incubation, whereas the intracellular potassium content of the cryopreserved PCLS remained low all along the incubation. The early ionic disturbances observed in cryopreserved PCLS are not due to alterations of Na^+/K^+-ATPase intrinsic activity, but rather to the fact that ATP level in cryopreserved PCLS always remains below the critical ATP level for Na^+/K^+-ATPase functionality [32, 33].

Whatever the mechanism, the lower K^+ level might in turn lead to mitochondrial alterations, namely by favouring cytochrome C release [34], or caspases activation [35]. If this hypothesis is confirmed in cryopreserved PCLS, our work will open a new field of investigation devoted to improve cell viability and functionality after PCLS freezing by modifying the slicing or incubation medium, i.e. by adding caspases inhibitors, a procedure which has been recently shown to improve cryopreserved pig hepatocytes survival [29] or through enrichment of K^+ content in slicing and/or incubation medium, which was also proven to depress chemical stress induced apoptosis in Jurkat T cells [34]. Finally, we propose that mitochondrial alterations could explain the low ATP level upon incubation. A specific study devoted to mitochondria functionality would be necessary to clarify this point.

De Graaf *et al* [9] have shown that cryopreservation in 18% DMSO, as compared to 12% DMSO concentration, allowed to increase potassium content and

the number of intact cells in PCLS, 4 hours after incubation. Our results are in accordance with the improvement of cell viability with 18% DMSO. After 1 hour of incubation, we observed a significant increase of potassium and ATP content; moreover, we have also shown a lower cell lysis and a decrease in caspase 3 activity, as compared to 10% DMSO condition. Further studies would also take into account the fact that 18% DMSO in the cryopreservation medium improves cell viability upon thawing, by a mechanism which would be studied further on. The combination of this condition (18% DMSO), together with scientifically-based improvement of incubation medium (enrichment of K^+ content? caspases inhibitors addition?) used after thawing, would help to strongly ameliorate cell viability in cryopreserved PCLS.

Abbreviations

CCCP (carbonylcyanide-*m*-chlorophenylhydrazone), CYP (cytochrome P450), FCS (fetal calf serum), LDH (lactate dehydrogenase), PCLS (precision-cut liver slices).

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