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Role of apoptotic signaling pathway in metabolic disturbances occurring in liver tissue after cryopreservation: Study on rat precision-cut liver slices

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Abstract

Precision-cut liver slices in culture (PCLS) appears as a useful and widely used model for metabolic studies; the interest to develop an adequate cryopreservation procedure, which would allow maintaining cell integrity upon incubation, is needed to extend its use for human tissues. We have previously shown that cryopreservation of rat PCLS leads to caspase-3 activation and early alterations of their K^+ content upon incubation. In this study, we tested the hypothesis that counteracting intracellular K^+ loss and/or interfering with cell death signaling pathways could improve the viability of cryopreserved PCLS. PCLS were prepared from male Wistar rat liver and cryopreserved by rapid freezing before incubation. The addition of a caspase inhibitor–Z-DEVD-FMK (2.5 μM)–in the culture medium did not improve viability of cryopreserved PCLS. Incubation of cryopreserved PCLS in a K^+ rich medium (135 mM) increased K^+ content and avoided caspase-3 activation, but did not improve cell viability. Caspase-3 inhibition, a decrease in cell lysis, and improvement of glycogen content were observed in cryopreserved PCLS after addition of LiCl (100 mM) in the incubation medium. These results indicate that, even if caspase-3 activation is linked to the K^+ loss in cryopreserved PCLS, its inhibition does not allow restoring the metabolic capacities. LiCl, acting on a target upstream of caspase-3 inhibition, improves cell viability and allows glycogen accumulation when added in culture medium of cryopreserved PCLS; and could thus be considered as an interesting adjuvant in the culture of cryopreserved PCLS.

Keywords: Potassium; Lithium; Caspases; Cytochrome *c*; Precision-cut liver slices

Introduction

Precision-cut liver slices (PCLS) are widely used as in vitro tool to study toxicity and metabolism of xenobiotics (Bach et al., 1996; Lerche-Langrand and Toutain, 2000; Ekins et al., 2000; Martin et al., 2003). PCLS offer various advantages over isolated hepatocytes such as the preservation of tissue architecture, the maintenance of cell polarity and heterogeneity and the lack of use of hydrolytic enzymes. Cryopreservation of PCLS, that would allow storing precious tissues such as human samples i.e., is associated with a loss of cell viability upon thawing and further incubation. Despite the maintenance of drug metabolism capacity, several viability and functionality parameters are considerably altered in cryopreserved PCLS within 24 h of incubation (Martignoni et al., 2004; Vanhulle et al., 2001, 2003; Glöckner et al., 2001; Maas et al., 2000; De Graaf et al., 2000; Lupp et al., 2002; Sohlenius-Sternbeck et al., 2000; Martin et al., 2000). Mechanisms of cell death occurring in cryopreserved PCLS are poorly understood. Even if necrotic cells are often present in cryopreserved tissues, recent papers describe that cryopreservation may also be associated with apoptosis (Martin et al., 2000; Yagi et al., 2001; Fu et al., 2001; Baust et al., 2000). We have previously shown that ionic disturbances and an increase in caspase-3 activity occur after a few minutes of incubation of cryopreserved rat PCLS. Those phenomena are associated with mitochondrial alterations (decrease in PCLS O_2 consumption, a loss of coupling of O_2 consumption to ATP synthesis and mitochondrial calcium transport) and alterations in ATP-

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dependent anabolic pathways (Vanhulle et al., 2003). Caspase inhibition improves the viability of cryopreserved isolated hepatic and renal cells (Yagi et al., 2001; Baust et al., 2000). Furthermore, recent reports have implicated intracellular K^+ as an important regulator of apoptosis or necrosis in several cell types (Warny and Kelly, 1999; Thompson et al., 2001; Hughes et al., 1997; Bortner and Cidlowski, 1999; Maeno et al., 2000). An efflux of cellular K^+ – which can be prevented by incubating cells into a K^+ rich medium – is associated with caspase activation (Hughes et al., 1997) and mitochondrial alterations (Thompson et al., 2001; Bortner and Cidlowski, 1999). Moreover, in isolated or cultured cells, an apoptotic stimulus can be modulated by lithium, an inhibitor of the glycogen synthase kinase-3 (GSK-3) (Song et al., 2004; Phiel and Klein, 2001; Grimes and Jope, 2001).

Is it possible to counteract cell alterations occurring in cryopreserved PCLS by modulating those targets? In the present study, we have assessed the putative influence of boosting potassium level, or of modulation of caspase activity (Lithium chloride, addition of a broad-spectrum caspase inhibitor) on rat cryopreserved PCLS metabolic activities and cellular integrity.

Materials and methods

Chemicals

Williams' medium E and fetal calf serum (FCS) were purchased from Gibco BRL (Invitrogen™, Belgium). Dimethyl sulfoxide (DMSO) and enzymes were obtained from Sigma Chemicals (Belgium). L-Glutamine was purchased from ICN Biomedicals Inc. (Belgium) and radiolabeled chemicals from Amersham (Belgium). Z-DEVD-FMK was purchased from Bio-Rad (UK). Insulin (Actrapid HM®, Novo Nordisk, Denmark), dexamethasone phosphate (Decadron®, MSD, New Jersey, USA) and all other chemicals used were of the purest grade commercially available.

PCLS preparation and incubation

The liver of adult male Wistar rats (Harlan, Netherlands) weighing 280 to 320 g was perfused in situ with ice-cold Krebs–Ringer solution prior to removal. PCLS (about 250 μm thickness and 10 mm diameter) were prepared according to a procedure previously described (Vanhulle et al., 2001). Fresh slices were stored at least 30 min in ice-cold Williams' medium E supplemented with L-glutamine (2 mM), insulin (100 nM), dexamethasone (10 nM) and 10% FCS (v/v) under an atmosphere of carbogen (95% O_2/5% CO_2) until incubation at 37 °C. PCLS were further cultured into vials containing 2 ml Williams' medium E supplemented with L-glutamine (2 mM), insulin (100 nM), dexamethasone (10 nM) and glucose (final concentration of 25 mM) per slice, saturated with carbogen and placed in a shaking water bath at 37 °C. After 1 h of incubation at 37 °C, the incubation medium was changed. To assess the effects of K^+, Williams' medium E ($[K^+]=5$ mM and $[Na^+]=143$ mM) was replaced by a modified Krebs–Henseleit–Hepes buffer containing a high K^+ concentration (Hepes 20 mM, pH 7.4 (KOH), KCl 108.8 mM, KH_2PO_4 1.2 mM, $KHCO_3$ 25 mM, $MgSO_4$ 1.2 mM, $CaCl_2$ 1.3 mM and NaCl 5 mM).

PCLS cryopreservation and thawing

PCLS cryopreservation and thawing were performed as previously described (Vanhulle et al., 2001). Prior to freezing, PCLS were stored in ice-cold oxygenated Williams' Medium E supplemented with L-glutamine (2 mM), insulin (100 nM), dexamethasone (10 nM) and 10% DMSO (v/v) for 30 min. The PCLS were then put in cryogenic vials without any medium, followed by ultrarapid freezing immersion in liquid nitrogen. They were kept in liquid nitrogen until use. Two milliliters of Williams' Medium E supplemented with L-glutamine (2 mM), insulin (100 nM), dexamethasone (10 nM) and 10% FCS were added on frozen PCLS and the vials were placed into a water bath at 39 °C. After thawing, PCLS were washed in the same medium at 4 °C for 5 min and then incubated at 37 °C as the fresh ones.

Determination of PCLS viability and metabolic function

ATP content

PCLS were taken and sonicated in ice-cold 2% perchloric acid. Intracellular ATP content was measured on neutralized perchloric acid extracts using the ATP Bioluminescence Assay Kit CLS II from Boehringer (Germany).

Glycogen content

Glycogen content of PCLS was quantified by an enzymatic procedure as previously described (Vanhulle et al., 2001).

Protein synthesis

Protein synthesis by PCLS was assayed by measuring the incorporation of L-[U-^{14}C]leucine (175 μCi/mmol) into acid-precipitable proteins during the hour before sampling in the presence of the labeled precursor (Evdokimova et al., 2001).

K^+ content

PCLS were rinsed three times in ice-cold water and then sonicated in 3.5% perchloric acid. After centrifugation, the supernatant fraction was assayed for K^+ by flame photometry.

Lactate dehydrogenase (LDH) activity

The loss of membrane integrity was estimated by measuring LDH activity in the culture medium, according to Wroblesky and Ladue (1955).

Protein content

Protein content of PCLS was assessed by the method of Lowry et al. (1951).

Western immunoblotting

Western immunoblotting technique was performed in order to determinate the quantity of cytochrome *c* released from

mitochondria into the cytosol. Cytosolic extracts were obtained by ultra-centrifugation of PCLS homogenates (Bossy-Wetzel and Green, 1999). 8 μg cytosolic protein extracts were subjected to (SDS)-polyacrylamide gel electrophoresis (15% separating gel) followed by electroblotting towards nitrocellulose membrane. Non-specific binding was blocked by incubation in TBS buffer (pH 7.4) containing 5% (w/v) powdered milk protein for 1 h at 25 °C. The membrane was incubated for 2 h at 25 °C in TBS buffer containing powdered milk protein (1% w/v) and a mouse monoclonal anti-cytochrome *c* antibody (Santa Cruz Biotechnology, SanverTECH nv, Belgium) at 1:500 dilution. The secondary antibody, a horseradish peroxidase-coupled rabbit anti-mouse antibody (DAKO, Denmark), was incubated at 1:1250 dilution for 1 h at 25 °C. Immunodetection was performed using the ECL™ detection kit (Amersham, Belgium). Optical density of corresponding band was quantified using the program Image Master (Pharmacia Biotech Benelux, Roosendaal, Netherlands).

Determination of caspase-3 activity

Caspase-3 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.

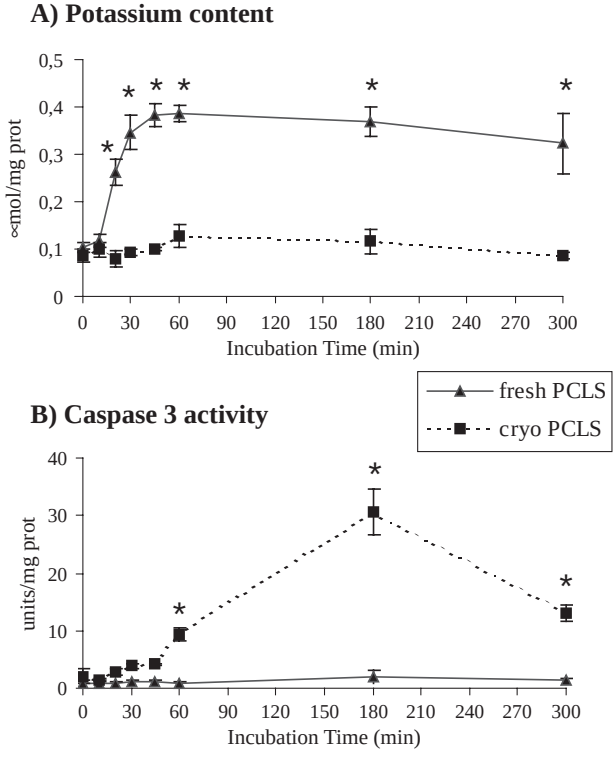

Fig. 1. Time-dependent evolution of K^+ content (A) and caspase-3 activity (B) in fresh and cryopreserved (cryo) PCLS incubated in Williams' medium E. Data are expressed as the mean ± S.E.M. ($n = 3$ or 4). The effect of cryopreservation treatment, the time effect and the interaction between cryopreservation treatment and time were significant for both K^+ content and caspase-3 activity ($p < 0.05$ two-way ANOVA). *Significantly different from fresh PCLS at one particular time point ($p < 0.05$ Bonferroni analysis).

Statistical analysis

All parameters were measured at least in duplicate on two slices obtained from the same rat. A mean value has been calculated for each experiment. The values presented in tables and figures correspond to the mean ± S.E.M. of at least 3 experiments (performed on 3 different rats). Statistical analysis of Fig. 1A and B was assessed by applying the two-factor analysis of variance (ANOVA) test and Bonferroni correction of *p* values was used for statistical comparison between results at a given time point. Other statistical analyses were performed by a one-way ANOVA followed by Tukey post hoc test, by using SPSS™ 9.0 statistical software. For all tests, $p < 0.05$ was considered statistically significant.

Results

Evolution of K^+ content, caspase-3 activity and cytochrome c cytosolic content in PCLS

Intracellular K^+ content was initially low in both fresh and cryopreserved PCLS incubated for 5 h in Williams' medium E (Fig. 1A). After 10 min, the K^+ content was progressively restored in fresh PCLS to reach normal values after 30 min of incubation, whereas it remained low in cryopreserved PCLS during the whole incubation period. On the contrary, caspase-3 activity remained low in fresh PCLS (±1 unit/mg proteins), whereas, in cryopreserved PCLS, it gradually increased to reach values 15 times higher than control values after 3 h of incubation (Fig. 1B). A further decrease in caspase-3 activity was observed in cryopreserved PCLS (4.41 ± 0.71 units/mg proteins after 7 h of incubation). Cytosolic cytochrome *c* content progressively increased with incubation time and was relatively similar in both fresh and cryopreserved PCLS during the first hour of incubation (Fig. 2). Despite an apparent higher cytosolic cytochrome *c* content in cryopreserved PCLS than in fresh PCLS after 3 and 5 h of incubation, this effect was not significant (Fig. 2B).

Influence of caspase inhibition on cellular metabolism and PCLS integrity

Fresh and cryopreserved PCLS were incubated in Williams' medium E in the absence or presence of Z-DEVD-FMK, a broad-spectrum and irreversible caspase inhibitor. Z-DEVD-FMK (2.5 μM) was added only during the preincubation period of 30 min at 4 °C and the first hour of incubation at 37 °C before medium change. This protocol allowed a continuous caspase-3 inhibition upon further incubation but avoided any adverse effects attributable to Z-DEVD-FMK during the incubation period: LDH leakage was similar in both control and Z-DEVD-FMK conditions after the hour of incubation (data not shown). Medium samples and PCLS analysis were performed after 3 h of incubation at 37 °C. Results are presented in Fig. 3. Caspase-3 activity in cryopreserved PCLS corresponded to 20.47 ± 5.34 and 1.54 ± 0.82 units/mg protein in the absence or in the presence of Z-DEVD-FMK,

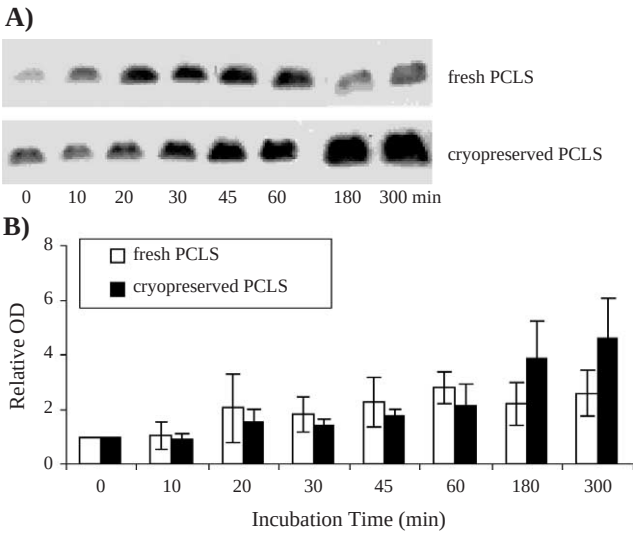

Fig. 2. Time-dependent evolution of cytochrome *c* cytosolic content in fresh and cryopreserved PCLS incubated in Williams' medium E, expressed as optical density (OD) of bands corresponding to cytochrome *c* appearing after western immunoblotting; (B) represents the mean of OD ratios measured at one time point versus time 0 for fresh and cryopreserved PCLS. Data are expressed as the mean ± S.E.M. ($n=3$). $p>0.05$ (one-way ANOVA).

respectively ($n=3$, Student's *t*-test: $p<0.05$). No significant modifications in LDH released in the medium, ATP content and protein synthesis could be attributed to the addition of Z-DEVD-FMK in the incubation medium of fresh PCLS. A significant increase in LDH released in the medium and a decrease in ATP content and protein synthesis were observed in cryopreserved PCLS as compared to fresh PCLS independently of the presence of Z-DEVD-FMK. The cytosolic content of cytochrome *c* was similar in cryopreserved PCLS incubated 3 h in the presence or absence of Z-DEVD-FMK (relative percentage of 114.7 ± .5, $n=3$).

Effect of a K^+ rich medium on caspase-3 activity and cell metabolism

The incubation in a K^+ rich medium led to a significant increase in the intracellular K^+ content in both fresh and cryopreserved PCLS (Table 1). Cellular lysis (LDH leakage) and caspase-3 activity were similar in both mediums in fresh PCLS (±1 unit/mg proteins). However, the incubation of fresh PCLS in a K^+ rich medium, when compared to the Williams' medium E, decreased their ATP content and protein synthesis and increased their glycogen content. Caspase-3 activation observed in cryopreserved PCLS incubated in Williams' medium E was prevented in the K^+ rich medium (Table 1). Cryopreservation of PCLS lowered ATP content, glycogen content and protein synthesis and increased cell lysis in both mediums (Fig. 4).

LiCl addition in the incubation medium

In preliminary experiments, we performed a dose–response analysis of LiCl (5 to 100 mM); 100 mM was chosen since it allowed a significant improvement of glycogen content and a decrease in caspase-3 activity. One set of experiments (Table 2) showed that 100 mM LiCl in the medium of cryopreserved PCLS during the whole incubation at 37 °C led to an increase in glycogen content and a decrease in LDH released in the medium and caspase-3 activity, without any effects on ATP content and protein synthesis after 3 h of incubation. However, when fresh PCLS were incubated in the same conditions, LiCl led to an activation of caspase-3, to a strong decrease in glycogen and ATP contents, and protein synthesis (Table 2). Therefore, a second set of experiments was performed, in which the addition of 100 mM LiCl was restricted to the first hour of incubation at 37 °C. The preincubation period of PCLS

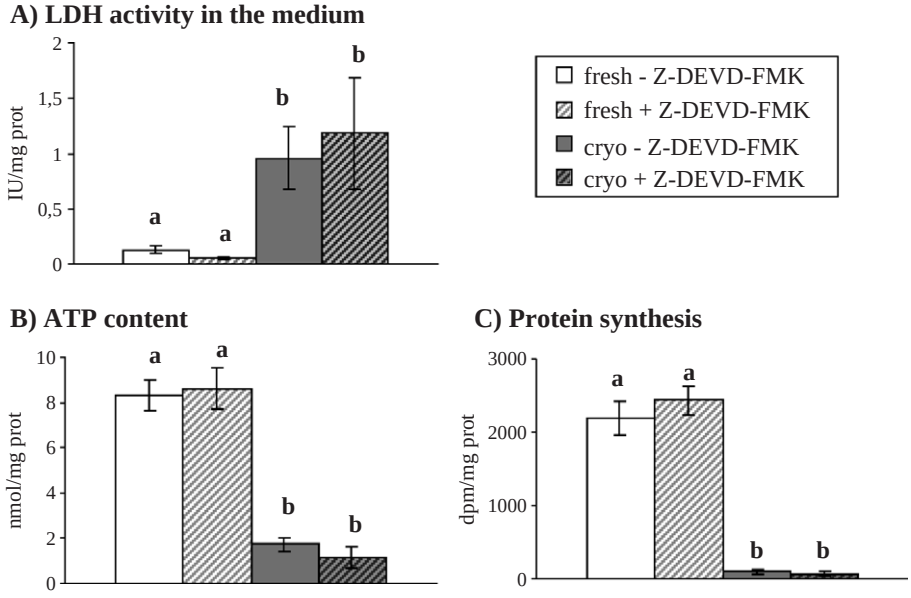

Fig. 3. LDH leakage into the medium (A), ATP content (B) and protein synthesis (C) in fresh and cryopreserved (cryo) PCLS incubated 3 h in Williams' medium E in the absence (−) or in the presence (+) of Z-DEVD-FMK. Data are expressed as the mean ± S.E.M. ($n=3$). Conditions not sharing the same letter are significantly different ($p<0.05$, one-way ANOVA).

Table 1
Effect of potassium rich medium on potassium content and caspase-3 activity in fresh and cryopreserved precision-cut liver slices

	Williams' medium E		K^+ rich medium	
	Fresh	Cryopreserved	Fresh	Cryopreserved
K^+ content (μmol/mg protein)	0.65 ± 0.10^a	0.13 ± 0.02^a	1.60 ± 0.21^b	2.23 ± 0.32^b
Caspase-3 activity (units/mg protein)	1.79 ± 0.14^a	15.74 ± 2.50^b	1.95 ± 0.16^a	0.91 ± 0.42^a

Fresh or cryopreserved PCLS were incubated in Williams' medium E or in a 5 mM K^+ or in a K^+ rich medium (135 mM K^+) during 3 h. Data are expressed as the mean ± S.E.M. ($n = 3$ or 4). ^{a,b}Conditions not sharing the same letter are significantly different ($p < 0.05$, one-way ANOVA).

30 min at 4 °C was performed in Williams' medium E without addition of LiCl. Functionality of fresh and cryopreserved PCLS was assessed after 3 and 7 h of incubation (Fig. 5). LiCl had no effect on LDH released in the medium, on glycogen content and on caspase-3 activity in fresh PCLS; only a slight decrease in ATP content and protein synthesis were present after 3 h, and not after 7 h of incubation. Interestingly, the incubation of cryopreserved PCLS with LiCl for 1 h significantly inhibited caspase-3 activation and decreased LDH release, without modifying glycogen and ATP content, and protein synthesis.

Discussion

Since PCLS appear as a useful and widely used model for metabolic studies (Martin et al., 2003; Lerche-Langrand and Toutain, 2000; Ekins et al., 2000; Olinga et al., 1997), the interest to develop an adequate cryopreservation procedure, which would allow maintaining cell integrity upon incubation, is needed. The freezing–thawing conditions used in the present study allowed maintaining key metabolic functions such as cytochrome P450-dependent oxidative metabolism in PCLS upon incubation after thawing (Vanhulle et al., 2001). However "energy" consuming and dependent processes such as drug conjugation, or lipid and protein synthesis, are considerably altered in cryopreserved PCLS within 24 h of incubation (Vanhulle et al., 2001, 2003; Glöckner et al., 2001; Maas et al., 2000; De Graaf et al., 2000; Lupp et al., 2002; Sohlenius-Sternbeck et al., 2000; Martin et al., 2000). Those disturbances can be initiated through freezing–thawing process but may be manifested during the post-thawing incubation conditions.

Mitochondrial alterations characterize both apoptosis and necrosis (Kroemer et al., 1998). Mitochondrial release of cytochrome *c* into cytoplasm is a mechanism proposed to be involved in caspase-3 activation. Once released, cytochrome *c* acts as a co-factor in the presence of dATP or ATP to stimulate the aggregation of Apaf-1, which can recruit and activate caspase-9 and subsequently caspase-3 via proteolytic processing (Blatt and Glick, 2001). In the present study, we have observed that cytosolic cytochrome *c* is relatively similar in both fresh and cryopreserved PCLS during the first hour of incubation. After 3 and 5 h of incubation, an apparent higher cytochrome *c* release is observed in cryopreserved PCLS as compared to fresh PCLS. Caspase-3 activation occurs only in cryopreserved PCLS within the first hour of incubation. We postulate that cytochrome *c* release is responsible for caspase-3

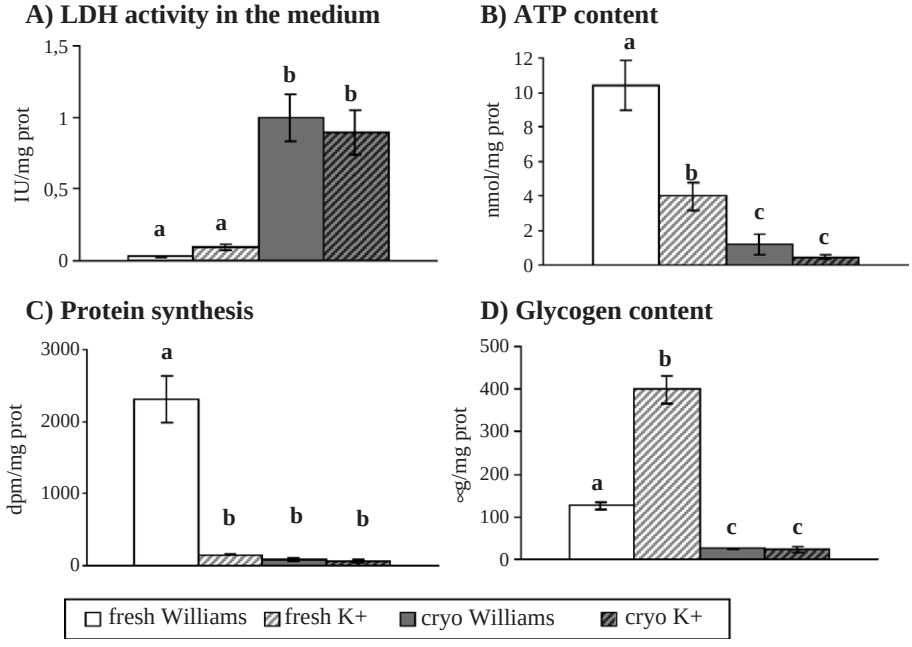

Fig. 4. LDH leakage into the medium (A), ATP content (B), protein synthesis (C) and glycogen content in fresh and cryopreserved (cryo) PCLS incubated 3 h in Williams' medium E or in a K^+ rich medium. Data are expressed as the mean ± S.E.M. ($n = 3$). Conditions not sharing the same letter are significantly different ($p < 0.05$, one-way ANOVA).

Table 2
Effect of lithium on functionality of fresh and cryopreserved precision-cut liver slices

	Williams' medium E without Li^+		Williams' medium E with Li^+	
	Fresh	Cryopreserved	Fresh	Cryopreserved
Caspase-3 activity (units/mg protein)	3.04±0.91[a]	14.09±2.48[b]	12.05±2.06[b]	2.21±.95[a]
LDH in medium (IU/mg protein)	0.072±0.028[a]	1.261±0.203[b]	0.066±0.018[a]	0.669±0.227[c]
ATP content (nmol/mg protein)	8.88±.27[a]	0.73±0.22[b]	2.29±0.18[c]	0.36±0.16[b]
Protein synthesis (dpm/mg protein)	2022.3±134.2[a]	68.0±13.3[b]	26.0±.6[b]	44.0±5.2[b]
Glycogen content (μg/mg protein)	207.5±50.6[a]	12.2±7.2[b]	93.5±32.1[c]	60.7±17.5[c]

Fresh or cryopreserved PCLS were incubated in Williams' medium E in the presence or in the absence of LiCl (100 mM) during 3 h. Data are expressed as the mean±S.E.M. ($n=3$ or 4). [a,b,c]Conditions not sharing the same letter are significantly different ($p<0.05$, one-way ANOVA).

activation in cryopreserved PCLS. Some studies describe a feedback amplification of cytochrome *c* release promoted by caspase activation (Chen et al., 2000; Xia et al., 2002). In our model, the inhibition of caspase activity (see below) had no effect on cytosolic cytochrome *c*.

Caspase inhibition has been shown to improve the viability of cryopreserved isolated hepatic and renal cells (Yagi et al., 2001; Baust et al., 2000). In the present study, caspase-3 inhibition was accomplished by addition of Z-DEVD-FMK into the incubation medium. No cytotoxicity could be attributed to Z-DEVD-FMK: it has no effects on LDH release, glycogen and ATP contents measured on fresh PCLS. We have shown that inhibition of caspase-3 did not ameliorate functionality of cryopreserved PCLS. Those results deny a key role of caspase-3 activation in the metabolic alterations occurring after cryopreservation.

If caspases are not an "essential" target per se, could ionic perturbations represent an important event triggering further cellular alterations? Some studies have suggested, in several cell types, that the loss of intracellular K^+ concentration enhanced cell death and that maintaining physiological K^+ level inhibits the activity of apoptotic nucleases and effector caspases (Warny and Kelly, 1999; Thompson et al., 2001; Hughes et al., 1997; Bortner and Cidlowski, 1999; Maeno et

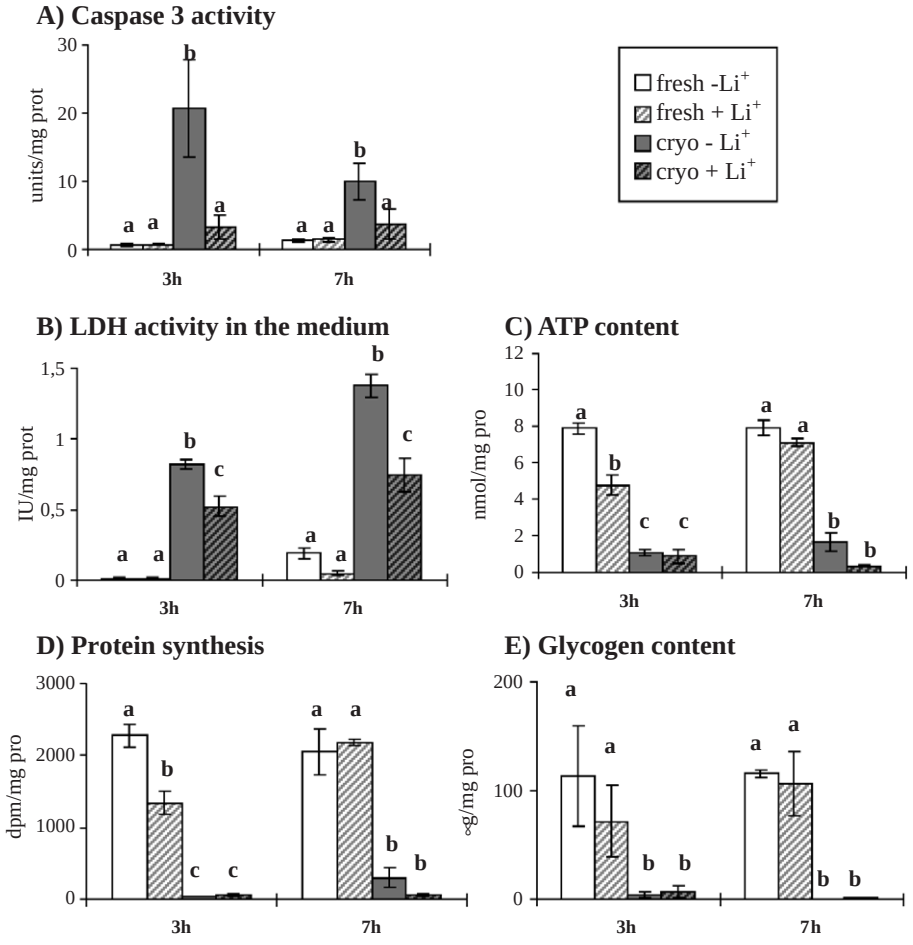

Fig. 5. Caspase-3 activity (A), LDH leakage into the medium (B), ATP content (C), protein synthesis (D) and glycogen content in fresh and cryopreserved (cryo) PCLS incubated 3 and 7 h in Williams' medium E in the absence (−) or presence (+) of 100 mM LiCl in the first hour. Data are expressed as the mean±S.E.M. ($n=3$). Conditions not sharing the same letter are significantly different ($p<0.05$, one-way ANOVA performed at each time).

al., 2000). We have previously described low intracellular K^+ level in cryopreserved PCLS as a consequence of low ATP level that always remains below the critical ATP level for Na^+/K^+-ATPase functionality (Vanhulle et al., 2003). In order to evaluate the relationship between cellular K^+ level and global PCLS functionality, PCLS were incubated in a K^+ rich medium. Incubation of fresh and cryopreserved PCLS in a K^+ rich medium significantly maintained the intracellular K^+ level and no caspase-3 activation was observed in cryopreserved PCLS, suggesting a link between low cellular K^+ content and caspase-3 activation as previously described in other cell types (Thompson et al., 2001; Hughes et al., 1997; Bortner and Cidlowski, 1999). Maintenance of physiological K^+ level in fresh PCLS incubated in the Williams' medium E could be implicated in the prevention of caspase-3 activation despite cytochrome *c* release; a lack of K^+ in cryopreserved PCLS could thus explain their difference in caspase activity. No improvement of cell lysis, ATP and glycogen contents and protein synthesis were observed in cryopreserved PCLS incubated in the K^+ rich medium after 3 h of incubation. It is important to note that no cytolytic effect was observed in fresh PCLS incubated in the K^+ rich medium but a significant increase of the glycogen was measured. This effect can be explained by the stimulatory effect of a K^+ rich medium on the uptake and phosphorylation of glucose and on the glycogen content, via the activation of the glycogen synthase as previously observed in isolated rat hepatocytes (Hue et al., 1975; Bontemps et al., 1978; Van Schaftingen and Vandercammen, 1992). The increase in glycogen synthesis may not be attributed to a condition promoting ATP-dependent anabolic processes, since ATP level–and protein synthesis–are both decreased in PCLS incubated in the presence of K^+. The stimulatory effect of K^+ on glycogen content does not occur in cryopreserved PCLS.

Lithium, a selective uncompetitive inhibitor of the GSK-3, is able to modulate cellular response to an apoptotic stimulus. GSK-3 phosphorylates and thereby regulates the function of many proteins such as glycogen synthase activity. Its activation facilitates a variety of apoptotic mechanisms in several cell types (Song et al., 2004; Phiel and Klein, 2001; Grimes and Jope, 2001). Lithium treatment during all the incubation has dramatic effects on fresh PCLS by increasing caspase-3 activity and lowering ATP and glycogen contents and protein synthesis. Therefore, we performed PCLS lithium treatment only during the first hour of incubation. Under those incubation conditions–avoiding alterations of functionality in fresh PCLS–lithium treatment led to caspase-3 inhibition and decreased cell lysis in cryopreserved PCLS. Lithium is not a direct inhibitor of caspase-3 activity (Mora et al., 2001), but could act by inhibition of cytochrome *c* translocation, by pro-apoptotic Bax down-regulation and anti-apoptotic Bcl-2 up-regulation by acting on GSK-3 (Ghribi et al., 2002; Chen and Chuang, 1999). The stimulatory effect of lithium on glycogen content, observed in cryopreserved PCLS, was observed only if lithium treatment was performed during the whole incubation time. This stimulatory effect combined to the absence of effect of the K^+ rich medium on glycogen content in cryopreserved PCLS suggest that GSK-3 could be activated after freezing–thawing conditions.

Conclusion

Counteracting caspase-3 activation and increasing K^+ content of cryopreserved PCLS do not lead to any improvement of cell viability. However, interesting results were obtained by adding lithium chloride in the medium for 1 h just after thawing: it decreased LDH release–a marker of cell membrane integrity–and lowered caspase-3 activity. We may propose that the addition of lithium chloride in the medium for a limited period of time after thawing can constitute a promising and easy way to improve cell viability inside the liver tissue.

Knowing that significant progresses have been made to improve PCLS viability by varying the amount of DMSO upon freezing (De Graaf et al., 2000), we would propose to combine an increase of DMSO in the freezing medium up to 18%, and the addition of lithium chloride in post-thawing incubation medium to get significant improvement of cell viability in cryopreserved PCLS.

Acknowledgments

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