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Cryopreservation of Human Hepatocytes Alters the Mitochondrial Respiratory Chain Complex 1

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Transplantation of human hepatocytes has recently been demonstrated as a safe alternative to partially correct liver inborn errors of metabolism. Cryopreservation remains the most appropriate way of cell banking. However, mitochondrial-mediated apoptosis has been reported after cryopreservation and little is known on the involved molecular mechanisms. The aim of this study was to investigate mitochondrial functions of freshly isolated and cryopreserved/thawed hepatocytes from mice and humans. We report here that cryopreservation induced a dramatic drop of ATP levels in hepatocytes. The oxygen consumption rate of cryopreserved/thawed hepatocytes was significantly lower compared to fresh cells. In addition, the uncoupling effect of 2,4-dinitrophenol was lost, in parallel with a reduction of mitochondrial membrane potential. Furthermore, a decrease in mitochondrial respiratory rate was evidenced on permeabilized hepatocytes in the presence of substrate for the respiratory chain complex 1. Interestingly, this effect was less marked with a substrate for complex 2. Electron microscopy examination indicated that mitochondria were swollen and devoid of cristae after cryopreservation. These changes could explain the cytosolic release of the proapoptotic protein cytochrome c in cryopreserved cells. Nevertheless, no caspase 9-3 activation and only few apoptotic and necrotic cells were found, indicating that the subsequent cell death program was not yet evidenced. Our results demonstrate that cryopreservation of hepatocytes induced alteration of the mitochondrial machinery. They also suggest that, in addition to technical progress in the cryopreservation procedure, protection of the respiratory chain complex 1 should be considered to improve the quality of cryopreserved hepatocytes.

Key words: Hepatocyte; Cryopreservation; Liver cell transplantation; Mitochondria; Complex

INTRODUCTION

Partial correction of inborn errors of metabolism can be obtained after liver cell transplantation (LCT), leading to propose LCT as an alternative, or at least as a bridge, to orthotopic liver transplantation (e.g., to avoid irreversible brain damage in patients suffering from severe urea cycle disorders) (5,6,35,37,38). However, the use of freshly isolated cells is restricted by organ shortage and the limited quantity of cells that can be infused in one single session. At present, cryopreservation remains the only practical method for long-term storage and permits the development of a readily available cell bank, even in emergency cases (37). If a limited percentage of engrafted cells is sufficient to restore partially the deficient function, it is clear that many infused cells do not engraft into the recipient liver, lose viability, and are cleared by the reticulo-endothelial system. Initial high quality of cells is therefore crucial to enhance hepatocyte engraftment and subsequent repopulation of the recipient liver. Beyond their useful purpose in LCT, cryopreserved hepatocytes may also constitute an interesting tool in other domains, like toxicological studies and bioartificial liver, where high functional quality of cells is also required (1,36).

While induction of cell death was described following cryopreservation and thawing (C/T), after at least 12 h of culture, the mechanism by which cryostorage triggers cellular damage in isolated hepatocytes is still unclear (8). The mitochondrion is a key player in the initiation of cellular death (15), and recent studies highlighted its role in C/T-induced cellular damage (24,45). Indeed, disruption of mitochondrial membrane potential ($\Delta\Psi$) was reported following C/T (24). This mitochondrial

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damage is followed, within hours after thawing, by caspases' activation leading to DNA fragmentation and ultimately apoptosis (24). Because mitochondria are the major source of reactive oxygen species (ROS), induction of apoptosis by oxidative stress was also proposed to be involved in the impairment of hepatocytes after C/T (9).

The aim of this study was to investigate the effects of C/T on mitochondrial functions in hepatocytes isolated from mice and humans. Therefore, oxygen consumption rate (JO_2) was measured on intact and permeabilized hepatocytes before and after C/T. This approach was completed with morphological analysis of mitochondria by electron microscopy and determination of various parameters involved in the mitochondria-mediated cellular death. Based on our findings, we proposed that protection of the mitochondrial respiratory chain complex 1 could constitute an original way to prevent hepatocyte damages after C/T, and improve their functional capacity in LCT.

MATERIALS AND METHODS

Ethical Considerations

All experiments performed in animals and all procedures on human tissue have received approval from the university and hospital ethical review boards.

Animals

Male C57BL/6 mice were used at 8–12 weeks of age. All animals were housed in controlled light and temperature environment with ad libitum access to chow and water.

Cell Isolation and Cryopreservation/Thawing of Mouse and Human Hepatocytes

For mice, hepatocytes were isolated by collagenase A (Roche) (3,12). For humans, the hepatocytes isolation procedure was done on whole livers or liver segments not used for transplantation, as previously reported (35). Briefly, hepatocytes were isolated in the cell bank isolation facilities using the classical two-step perfusion method (collagenase P, Roche). Immediately after isolation, hepatocytes were resuspended either in serum-containing Williams' E medium (Invitrogen) or gently mixed at 5×10^6 cells/ml in a 3.6-ml freezing tube containing cryopreserving solution (University of Wisconsin solution, 25% fetal calf serum or human albumin, 10% DMSO, 11 mM glucose, 0.15 U insulin, 20 mg/L dexamethasone), as previously reported (1). The freezing protocol of murine hepatocytes consists of 20 min at −20°C followed by 2 h at −80°C before storing in liquid nitrogen as reported (21,22). In parallel, human hepatocytes were cryopreserved according to a computer-controlled cooling process previously described (37) by using a cryozon (Cryo 10 series, Planer Biomed).

After cryopreservation and storage for 24 h in liquid nitrogen, hepatocytes from humans or mice were thawed in a water bath at 37°C and cell suspension was supplemented with 11 mM glucose, 1 mM HCO_3^-, and a solution of human plasma proteins, as previously described (8). The cells were then low speed centrifuged twice to remove cryoprotectants and resuspended in serum-containing Williams' E medium.

Determination of Cell Viability

Cell viability was evaluated by the trypan blue dye exclusion test (0.4%, v/v), the lactate dehydrogenase (LDH) leakage enzymatic assay (Roche), and the 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2H tetrazolium bromide (MTT) assay (Sigma), as previously described (10,23). Intracellular ATP concentrations were measured using bioluminescence assay (Perkin Elmer).

Determination of Mitochondrial Oxygen Consumption Rate in Intact and Permeabilized Hepatocytes

Hepatocytes (4.5×10^6/ml) were incubated in a shaking water bath at 37°C in closed vials containing Williams' E medium saturated with a mixture of O_2/CO_2 (19:1). After 10 min, the cell suspension was transferred in a stirred oxygraph vessel equipped with a Clark oxygen electrode. The oxygen consumption rate (JO_2) was measured at 37°C before and after successive addition of 0.5 μM oligomycin (inhibitor of the F0 subunit of the ATP synthase), 150 μM 2,4-dinitrophenol (DNP), 0.15 μg/ml antimycin (inhibitor of mitochondrial respiratory chain complex 3), and 1 mM *N,N,N′,N′*-tetramethyl-1,4-phenylenediamine (TMPD) plus 5 mM ascorbate (substrate of the mitochondrial respiratory chain complex 4). For permeabilized hepatocytes, intact cells (10^6/ml) were incubated for 10 min as described above, then pelleted by centrifugation and carefully resuspended in medium (125 mM KCl, 20 mM Tris/HCl, 1 mM EGTA, and 5 mM Pi/Tris, pH 7.2) containing 200 μg/ml digitonin. After 3 min at 37°C, the permeabilized hepatocytes were transferred in the oxygraph vessel. As indicated, 5 mM glutamate/Tris plus 2.5 mM malate/Tris (substrate of the mitochondrial respiratory chain complex 1), or 5 mM succinate/Tris plus 0.5 mM malate/Tris (substrate of the mitochondrial respiratory chain complex 2), plus 1.25 μM rotenone (inhibitor of mitochondrial respiratory chain complex 1) were added. JO_2 was measured before and after the successive additions of 1 mM ADP/Tris, 0.5 μg/ml oligomycin, 50 μM DNP, 0.15 μg/ml antimycin, and 1 mM TMPD plus 5 mM ascorbate, permitting to determine the phosphorylating (state 3), the non-phosphorylating (state 4) and the uncoupled mitochondrial respiratory rate, and the maximal activity of cytochrome

oxidase, respectively. In all experiments, the antimycin-sensitive JO_2, which corresponds to mitochondrial respiration, was calculated by subtracting the antimycin-insensitive JO_2 from the total JO_2.

Determination of Mitochondrial Membrane Potential ($\Delta\Psi$)

Evaluation of $\Delta\Psi$ on intact cells was based on the uptake of the cationic fluorescence dye Rhodamine 123 (Rh 123), which accumulates into energized mitochondria proportionally to their negative inside membrane potential (28). Hepatocytes (2×10^6/ml) were incubated at 37°C in Williams' E medium supplemented with 1 μM Rh 123 and with or without 300 μM DNP. After 10 min of incubation, the cells were washed three times and the fluorescence was monitored (excitation and emission wavelengths at 498 and 524 nm, respectively).

Determination of Permeability Transition in Permeabilized Cells

Viable hepatocytes (2.5×10^6/ml) were incubated for 5 min in Williams' E as described above. The cells were then centrifuged and resuspended in buffer containing 250 mM sucrose, 10 mM MOPS, 1 mM Pi/Tris, and 50 μg/ml digitonin (pH 7.35). Measurements of free calcium (Ca^{2+}) were monitored spectrophotofluorimetrically in the presence of 0.25 μM Calcium Green-5N (excitation and emission wavelengths at 506 and 532 nm, respectively) (13). Cyclosporin A (CsA, 1 μM), the reference inhibitor of permeability transition pore (PTP) (28), or the vehicle (DMSO) were added to permeabilized hepatocytes and, after signal stabilization, pulses of 5 μl of 1 mM Ca^{2+} were successively added at 2-min intervals until the opening of the PTP, as indicated by the release of Ca^{2+} in the medium.

Determination of Cell Death

Western Blot Analysis. After cell fragmentation using the digitonin method (13), cytosolic and mitochondrial proteins (50 μg) were separated by SDS-PAGE and transferred to nitrocellulose membranes. Mitochondrial and cytosolic proteins were assayed by the Lowry method. Cytochrome c content was assessed in both compartments by immunoblot using specific primary antibody (Becton Dickinson) and secondary goat anti-mouse coupled to horseradish peroxidase. Quantification was performed by densitometry using Image J 1.32 (NIH).

Caspase Activity. Caspase 3 and caspase 9 activities were measured on total protein extract (50 μg) with specific substrate (Gentaur) using a spectrophotofluorometer (excitation and emission wavelengths at 360 and 480 nm, respectively).

DNA Fragmentation. DNA fragmentation was assessed by DNA agarose 1% gel electrophoresis after DNA purification using apoptotic DNA ladder Kit (Roche).

Flow Cytometry. Early apoptotic cell death was determined by flow cytometry using a double staining with fluorescein isothiocyanate (FITC)-stained annexin V and PE-APC-stained propidium iodide (Roche). Data acquisition (~10,000 cells) was carried out in a FACScalibur flow cytometer using CellQuest software (Becton Dickinson Biosciences).

Transmission Electron Microscopy

Hepatocytes were fixed in 0.1 M sodium cacodylate medium (pH 7.4) containing 2.5% glutaraldehyde for 48 h and then 4 h in 1% osmium tetroxyde before embedding in Epoxy Embedding Medium (Fluka). Ultrathin sections were stained with toluidine blue and uranyl acetate/lead citrate. Images were obtained with a Zeiss 109 transmission electron microscope (Carl Zeiss Inc.) at a magnification of 4140×.

Statistics

Results are expressed as mean ± SEM and significant differences were assessed by paired or unpaired Student's *t*-tests.

RESULTS

Evaluation of Cellular Viability Before and After Cryopreservation in Mouse and Human Hepatocytes

We first tested the effect of 24-h cryopreservation by a two-step process (–20°C/–80°C) on cellular viability evaluated by trypan blue exclusion test, lactate dehydrogenase (LDH) leakage, MTT reduction, and intracellular ATP concentration in mouse hepatocytes. The viability estimated by the classical trypan blue assay was significantly reduced after C/T, while no apparent modifications were observed on the release of LDH and on MTT reduction (Table 1). Moreover, a dramatic decrease in intracellular ATP concentration was evidenced follow-

Table 1. Evaluation of Cellular Viability of Freshly Isolated and Cryopreserved/Thawed Mouse Hepatocytes

	Fresh	Cryopreserved/ Thawed
Trypan blue (% viable cells)	92.6 ± 1.0	52.6 ± 4.4*
LDH extra/LDH intra	0.27 ± 0.02	0.28 ± 0.07
MTT test (a.u.)	0.6 ± 0.0	0.5 ± 0.1
ATP (nmol 10^6 cells^{-1})	3.6 ± 1.6	0.1 ± 0.0*

The extracellular and intracellular LDH contents were comparable between freshly isolated and cryopreserved/thawed hepatocytes. The results are expressed as mean ± SEM ($n = 5$).
*$p < 0.05$ compared with fresh cells.

ing C/T. A similar fall in ATP was also observed using computer-controlled cooling process on both mouse (data not shown) and human hepatocytes (2.8 ± 0.1 vs. 0.4 ± 0.0 nmol 10^6 cells^{-1}, $p < 0.05$, $n = 5$).

Cryopreservation Decreases Cellular Respiration in Mouse and Human Hepatocytes

In order to investigate if modifications of mitochondrial oxidative phosphorylation (OXPHOS) could explain the intracellular ATP drop following C/T, we measured the cellular JO_2 of fresh and cryopreserved/thawed hepatocytes from mice and humans (Table 2). Under basal conditions (i.e., in the absence of any drug addition), JO_2 of cryopreserved/thawed hepatocytes was significantly reduced compared with fresh cells in both mice and humans (Table 2). This difference was not present after addition of oligomycin. Indeed, the calculated oligomycin-sensitive JO_2 was reduced following C/T in both species (7.4 ± 2.3 vs. 17.5 ± 2.9 nmol O_2 min^{-1} 10^6 cells^{-1} in mice and 3.3 ± 0.5 vs. 7.8 ± 1.2 nmol O_2 min^{-1} 10^6 cells^{-1} in human hepatocytes, $p < 0.05$), suggesting that the effect of C/T was likely due to the alteration of a mitochondrial process linked to ATP synthesis.

Interestingly, while DNP addition induced stimulation of JO_2 in freshly isolated cells, this uncoupling effect was abolished following C/T in murine and human (Table 2) hepatocytes. Furthermore, the mitochondrial $\Delta\Psi$ evaluated by the uptake of Rh 123 was severely reduced following C/T in basal conditions (Fig. 1). The addition of DNP to the cells, which was able to significantly decrease $\Delta\Psi$ in fresh mice hepatocytes, had no effect in cryopreserved/thawed hepatocytes, confirming an almost total $\Delta\Psi$ collapse in these cells.

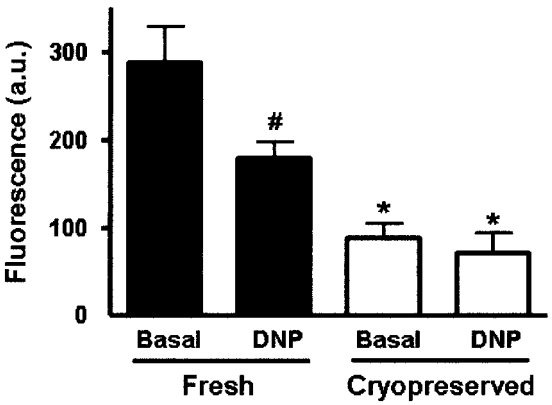

Figure 1. Effect of cryopreservation/thawing on mitochondrial membrane potential in isolated mouse hepatocytes. The mitochondrial membrane potential ($\Delta\Psi$) was evaluated on freshly isolated (filled bars) and cryopreserved/thawed (open bars) hepatocytes by the uptake of the cationic probe Rhodamine 123 (Rh 123). Cells were incubated at 37°C for 10 min in Williams' E medium containing Rh 123 and in the presence or not of DNP. After 10 min of incubation, the cells were then carefully washed and the fluorescence corresponding to the $\Delta\Psi$-driven mitochondrial accumulation of the probe was monitored using spectrophotofluorimeter. Results were expressed as mean ± SEM ($n = 3$). *$p < 0.05$ compared with basal, #$p < 0.05$ compared with fresh cells.

The maximal activity of cytochrome c oxidase was measured by recording JO_2 in the presence of saturating concentrations of TMPD/ascorbate and after addition of antimycin. No decrease in the mitochondrial respiratory chain complex 4 activity was observed following C/T (Table 2). Comparable results on cellular respiration after C/T of mouse hepatocytes were also obtained using a computer-controlled cooling process (data not shown).

Table 2. Oxygen Consumption Rate of Freshly Isolated and Cryopreserved/Thawed Hepatocytes

	JO_2 (nmol O_2 min^{-1} 10^6 cells^{-1})			
	Basal	Oligomycin	DNP	TMPD/Asc
Human				
Fresh	10.6 ± 1.1	1.9 ± 0.7	14.0 ± 1.4	49.1 ± 3.3
Cryopreserved/thawed	5.3 ± 1.1*	2.0 ± 0.8	4.2 ± 1.1*	40.2 ± 4.7
Mouse				
Fresh	25.0 ± 4.9	7.3 ± 0.4	40.1 ± 6.3	215.7 ± 23.6
Cryopreserved/thawed	12.3 ± 3.5*	6.5 ± 0.3	7.5 ± 1.4*	190.3 ± 18.7

Freshly isolated and cryopreserved/thawed hepatocytes (4.5×10^6 cells/ml) from human or mouse were incubated in closed vials at 37°C in Williams' E medium saturated with a mixture of O_2/CO_2 (19:1). After 10 min, oxygen consumption rate (JO_2) was measured in an oxygraph vessel coupled with Clark electrode before and after the successive additions of oligomycin, DNP, antimycin, and TMPD + ascorbate. The antimycin-sensitive JO_2 was calculated and the results are expressed as mean ± SEM ($n = 5$).
*$p < 0.05$ compared with fresh cells.

Cryopreservation Decreases Mitochondrial Oxygen Consumption Rate in the Presence of Respiratory Chain Complex 1 Substrate

We further investigated mitochondrial respiratory rate after digitonin permeabilization of hepatocytes, allowing mitochondrial OXPHOS to be investigated in situ. Antimycin- and oligomycin-sensitive mitochondrial respiratory rate measured in fresh and cryopreserved/thawed hepatocytes from both mice and humans are presented in Table 3. In the presence of glutamate/malate, a significant decrease in mitochondrial state 4 JO_2 (i.e., in nonphosphorylating condition) was evidenced following C/T in both murine and human cells (Table 3). This lower mitochondrial JO_2 persisted in the presence of ADP (state 3) or in an uncoupled state (DNP addition). However, as already found in intact cells, C/T did not affect the respiratory rate in the presence of TMPD-ascorbate.

In contrast, when mitochondria were energized by succinate/malate, no difference was observed in state 4 respiratory rate (Table 3). However, state 3 and the uncoupled state remained affected in murine and human cryopreserved/thawed hepatocytes. These results on in situ mitochondria confirmed those obtained in intact cells, showing that C/T induced mitochondrial alteration and $\Delta\Psi$ collapse, but also demonstrated that the mitochondrial respiratory chain complex 1 was more affected than complex 2. This latter finding was evidenced by an increase in complex 2-to-complex 1 respiratory rate ratio (Table 3).

Cryopreservation Alters Mitochondrial Ultrastructures

In order to investigate if the alteration of mitochondrial machinery was related to mechanical disruption of mitochondrial ultrastructures following C/T, we performed electron microscopy. Mitochondria were severely damaged following C/T in mouse (Fig. 2A, B) and human (Fig. 2E, F) hepatocytes. Indeed, while mitochondrial content was not affected (Fig. 2C, G), those organelles exhibited a partial loss of cristae and appeared swollen (Fig. 2D, H) compared to fresh hepatocytes in both mice and humans.

Table 3. Oxygen Consumption Rate in Permeabilized Hepatocytes From Freshly Isolated and Cryopreserved/Thawed Hepatocytes

	JO_2 (nmol atoms O_2 min^{-1} 10^6 $cells^{-1}$)			
	State 4	State 3	DNP	TMPD/Asc
Human				
Glutamate/malate				
Fresh	3.0 ± 0.6	26.6 ± 9.7	32.5 ± 16.2	134.9 ± 43.8
Cryopreserved	1.2 ± 0.6*	3.3 ± 1.3*	1.1 ± 0.6*	93.7 ± 29.3
Succinate/malate				
Fresh	13.8 ± 2.5	61.1 ± 14.5	70.4 ± 18.1	143.1 ± 31.3
Cryopreserved	15.0 ± 3.2	25.3 ± 6.6*	5.0 ± 2.6*	100.0 ± 20.4
Complex 2/complex 1				
Fresh	5.9 ± 1.8	3.3 ± 0.8	2.6 ± 1.0	1.1 ± 0.3
Cryopreserved	52.3 ± 28.8*	14.4 ± 5.9*	27.3 ± 16.1*	1.2 ± 0.4
Mouse				
Glutamate/malate				
Fresh	9.1 ± 1.2	51.3 ± 4.2	40.5 ± 2.3	331.7 ± 20.4
Cryopreserved	3.5 ± 0.4*	13.4 ± 6.3*	11.4 ± 3.7*	295.4 ± 25.6
Succinate/malate				
Fresh	49.4 ± 6.5	90.2 ± 12.3	102.9 ± 21.3	275.4 ± 12.4
Cryopreserved	43.2 ± 7.8	71.3 ± 24.4	58.4 ± 23.7*	236.7 ± 14.7
Complex 2/complex 1				
Fresh	5.4 ± 1.3	1.7 ± 1.0	2.5 ± 0.8	0.8 ± 0.2
Cryopreserved	12.3 ± 3.6*	5.3 ± 1.2*	5.1 ± 1.9*	0.8 ± 0.1

Permeabilization of freshly isolated or cryopreserved hepatocytes (10^6 cells/ml) from human or mice was achieved by digitonin after a 10-min preincubation in Williams' E medium as described in the legend of Figure 3. Permeabilized cells were then incubated in the presence of either glutamate + malate or succinate + malate + rotenone. The mitochondrial respiratory rate was measured before and after the successive additions of ADP/Tris, oligomycin, DNP, antimycin, and TMPD + ascorbate. The antimycin- and oligomycin-sensitive JO_2 was calculated and the results are expressed in nmol O_2 min^{-1} 10^6 $hepatocytes^{-1}$ as means ± SEM ($n = 5$).
*$p < 0.05$ compared to fresh cells.

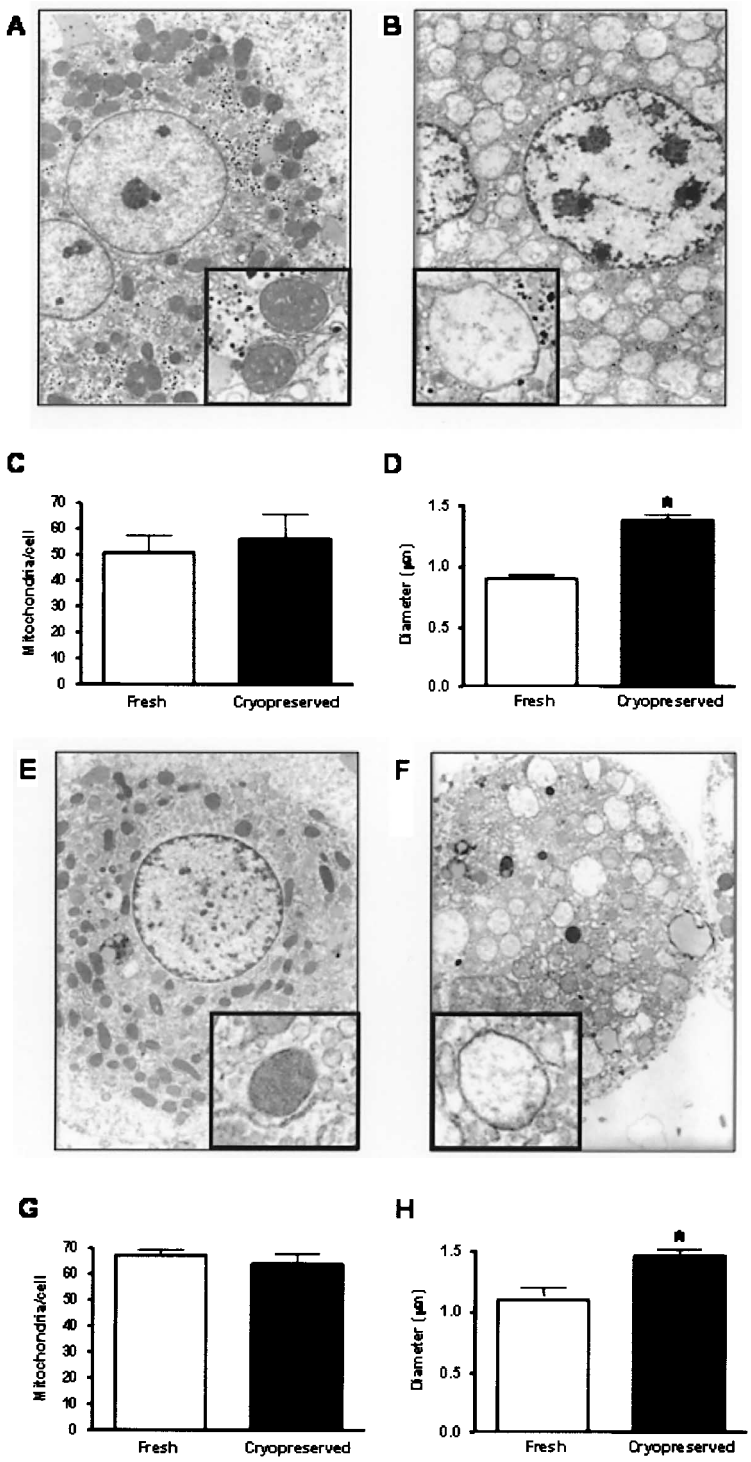

Figure 2. Morphological alterations induced by cryopreservation/thawing in mice and human hepatocytes. The morphology of mouse (A–D) or human (E–H) hepatocytes was evaluated before and after cryopreservation/thawing by electron microscopy as described in Materials and Methods. Typical images of freshly isolated (A, E) and cryopreserved/thawed (B, F) hepatocytes are shown at a magnification of 4140× and are representative of three experiments. The number (C, G) and diameter (D, H) of mitochondria were assessed in both fresh and cryopreserved hepatocytes. More than 500 mitochondria were counted and analyzed on random fields in each condition. $*p < 0.05$ compared with fresh cells.

Lack of Mitochondrial Calcium Uptake After Cryopreservation

The regulation of the mitochondrial PTP, which is involved in the mitochondrial-mediated apoptosis, was assessed in digitonin-permeabilized hepatocytes (Fig. 3). PTP opening was induced by repetitive addition of Ca^{2+} pulses until induction of permeability transition, as assessed by fast release of Ca^{2+}. In fresh murine and human cells, in situ mitochondria took up and retained Ca^{2+} (Fig. 3A, C, trace a). In the presence of CsA, the Ca^{2+} requirement for achieving the permeability transition was significantly increased in both mouse (+31%, $p < 0.01$) (Fig. 3A trace b, B) and human fresh hepatocytes (+86%, $p < 0.01$) (Fig. 3C trace b, D). However, no calcium retention was evidenced in cryopreserved/thawed cells (Fig. 3A, C, lower panel) and CsA had no effect.

The Cytosolic Release of Cytochrome c Following Cryopreservation Was Not Associated With Caspases Activation, DNA Fragmentation, and Apoptosis

Because the PTP, together with other factors, is involved in the release of cytochrome c from mitochondria to the cytoplasm, the content of this proapoptotic protein involved in the commitment to cell death was assessed in both compartments by Western blotting. While cytochrome c was hardly detectable in the cytoplasm of fresh murine hepatocytes, C/T significantly increased its cytosolic level (Fig. 4A). However, the mitochondrial content of cytochrome c was not significantly affected (data not shown), indicating that only a small fraction was released into the cytoplasm following cryopreservation/thawing. Despite cytochrome c release, caspase 3 and 9 activities (Fig. 4B) and genomic DNA fragmentation (Fig. 4C) were not modified after C/T.

Finally, the effect of C/T on the number of necrotic and apoptotic cells was evaluated by annexin V/propidium iodide staining. As shown in Figure 4D, C/T had no apparent effect. Indeed, only few apoptotic cells were detected and necrosis was rather low, estimated at 4.3 ± 0.1% in both fresh and cryopreserved/thawed hepatocytes.

DISCUSSION

The scarcity of liver donors is a major obstacle to the general application of LCT. However, this limitation could be overcome by the use of cryopreserved hepato-

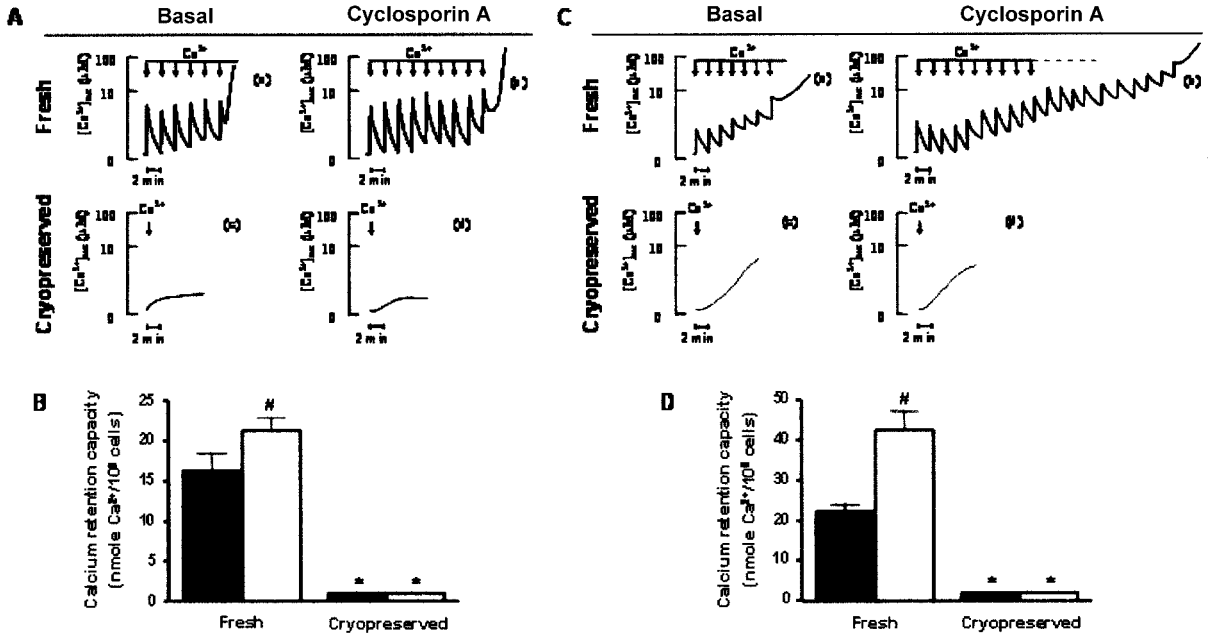

Figure 3. Effect of cryopreservation/thawing on mitochondrial permeability transition in permeabilized mouse or human hepatocytes. After 10 min of preincubation in Williams' E medium at 37°C, mouse (A, B) or human (C, D) hepatocytes (5×10^6 cells) were added in a medium containing 250 mM sucrose, 10 mM MOPS, 1 mM Pi-Tris, 25°C (pH 7.35). The medium was supplemented with succinate, Calcium Green-5N followed by the addition of vehicle (A and C, traces a and c) or CsA (A and C, traces b and d). Experiments were started 3 min after permeabilization with digitonin. Where indicated, 5 µl of 1 mM Ca^{2+} pulses were added every 2 min (arrows) until opening of PTP, as observed by the release of Ca^{2+} into the medium. Typical experiments are shown in (A) and (C) and comparison of the effect of cryopreservation/thawing on the Ca^{2+} retention capacity of permeabilized mouse hepatocytes is presented in (B) and (D) (filled bars, basal; open bars, cyclosporin A) as mean ± SEM ($n = 3$). *$p < 0.05$ compared with fresh cells, #$p < 0.05$ compared with basal.

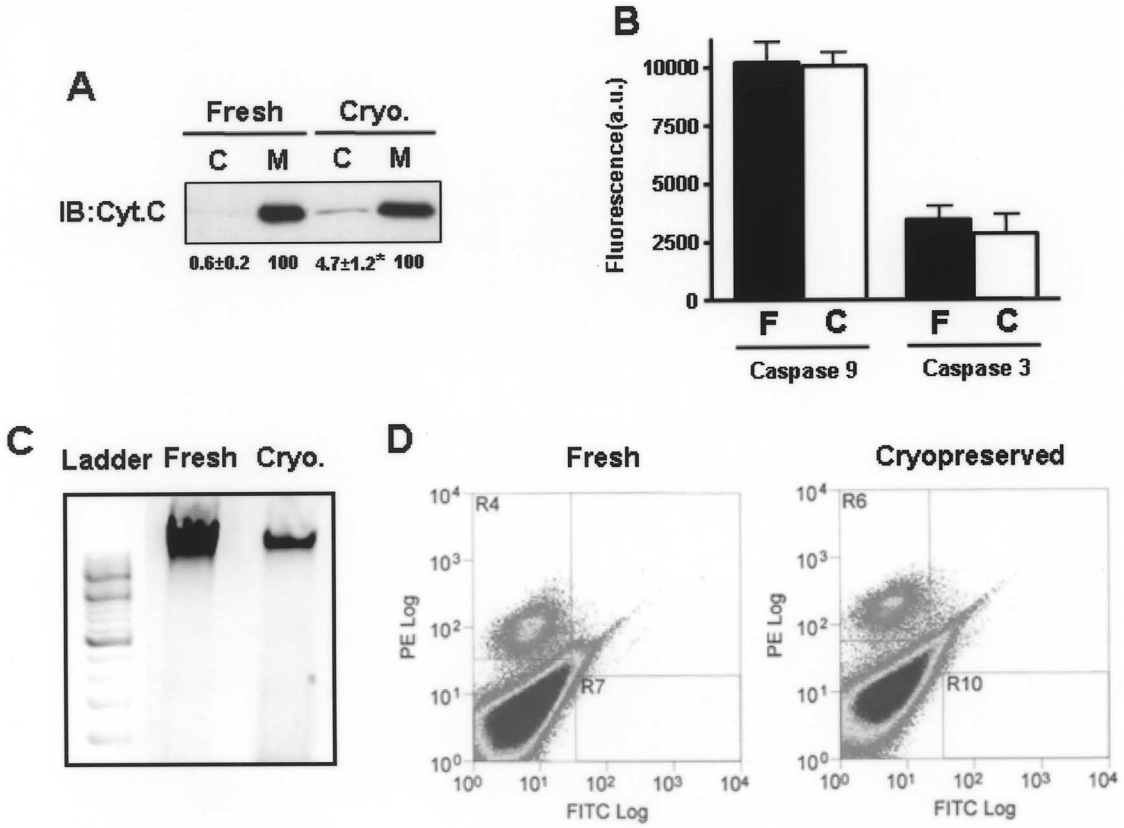

Figure 4. Effect of cryopreservation/thawing on cellular death-related parameters in isolated mice hepatocytes. (A) Cytochrome c was detected by Western blot on both cytosolic (C) and mitochondrial (M) compartment of fresh and cryopreserved/thawed hepatocytes after digitonin fractionation. Typical experiments are shown and the relative distribution of cytosolic cytochrome c compared to mitochondria is indicated below and is the mean ± SEM ($n = 3$). *$p < 0.05$ compared with fresh cells. (B) Caspase 9 and 3 activities were measured on freshly isolated (F) and cryopreserved/thawed (C) hepatocytes. The results are presented as means ± SEM ($n = 3$). #$p < 0.05$ compared with nontreated cells. (C) DNA fragmentation was assessed following DNA purification on both freshly isolated (F) and cryopreserved/thawed (C) hepatocytes. Typical results are shown (negative image) and are representative of three experiments. (D) Early apoptotic cell death was determined on fresh and cryopreserved/thawed hepatocytes by flow cytometry using a double staining with fluorescein isothiocyanate (FITC)-stained annexin V and PE-APC-stained propidium iodide (Roche). Annexin V-stained cells that were propidium iodide negative were considered as apoptotic (R7 and R10 boxes) and propidium iodide-stained cells that were Annexin V negative were considered as necrotic (R4 and R6 boxes). Typical results are shown and are representative of three experiments.

cytes (16), which have been reported to be able to repopulate part of the recipient liver of mice with genetically induced hepatic disease (17) and of children with inborn errors of metabolism (37). However, the quality of thawed hepatocytes leaves much to be desired, even though the conditions of the most efficient cryopreservation have been extensively studied (21,22). For instance, several studies have shown an increased rate of mitochondria-related cell death after cryopreservation of liver cells (8,24,45), in line with the widely described difficulty to freeze differentiated cells.

The aim of this study was to investigate the effect of C/T on hepatocytes from both mice and humans, principally on mitochondrial functions, to further provide new trails to improve viability and functionality following cryostorage. The important finding of our work was that C/T altered the mitochondrial machinery, notably at the level of the respiratory chain complex 1. This was observed on both mouse and human hepatocytes and whatever the cryopreservation procedure used.

Our study of cell viability after C/T provided apparent conflicting results (Table 1). The extracellular-to-intracellular LDH ratio was not modified by C/T, suggesting that the plasma membrane integrity was not sufficiently altered and is probably a late event following more subtle cellular damages. Moreover, no modification of MTT reduction was evidenced following C/T. The MTT test measures several mitochondrial dehydrogenases activities (25) and is an indirect marker of mitochondrial content rather than a viability test itself. In-

deed, these results are redundant with those obtained by oxygraphy on the maximal activity of mitochondrial cytochrome c oxidase (complex 4), which is also generally used to assess the mitochondrial content in intact and permeabilized cells (27,30). By contrast, intracellular ATP concentration was dramatically decreased after C/T, in relation with a reduced viability estimated by the trypan blue exclusion test. Taken together, our results indicate that LDH and MTT tests do not adequately assess viability and that intracellular ATP concentration is probably the most sensitive parameter to detect early cellular damages related to cryostorage (44).

We found that C/T decreased cellular respiration. This effect was evidenced on oligomycin-sensitive respiration, suggesting that it could result from alteration of a mitochondrial process linked to ATP synthesis rather than an intrinsic modification of mitochondrial membrane proton permeability (leak). Moreover, $\Delta\Psi$ was reduced after C/T, as previously reported (24,45), and the uncoupling effect of DNP was abolished. The cationic agent DNP is a protonophore, which increases the proton permeability of the mitochondrial membrane and thus decreases $\Delta\Psi$. Uncoupling stimulates mitochondrial respiration and abolishes ATP synthesis (14, 39). Because functional proton pumps (complexes 1, 3, and 4) and mitochondrial substrate subservience are required for mitochondrial OXPHOS, alteration of one or more of these complexes and/or a decrease in mitochondrial substrate supply linked to the $\Delta\Psi$ drop could explain the absence of uncoupling effect of DNP.

In permeabilized hepatocytes (in situ mitochondria), the activity of the mitochondrial complexes 1 and 2 can be tested in the presence of appropriate substrates. The basal mitochondrial respiratory rate (state 4, which is due to passive reentry of protons through the mitochondrial inner membrane) increases upon addition of ADP (state 3, respiration coupled with ATP synthesis) and is further enhanced by the uncoupler DNP in freshly isolated cells. With glutamate/malate as substrate for complex 1, a marked impairment of mitochondrial OXPHOS following C/T was observed under state 4, state 3, and uncoupled conditions. Interestingly, the inhibition of state 4 respiration was not present with complex 2 substrate, whereas both reduction of state 3 and abolition of the uncoupling effect of DNP were still present. The difference in inhibition of state 4 respiration depending on the substrates indicates that C/T altered complex 1, but not complex 2. Furthermore, the inhibition of state 3 respiratory rate and the lack of uncoupling effect of DNP, whatever the substrates used to energize the mitochondria, suggest that other modifications of the respiratory chain did occur. The fact that state 4 in presence of succinate was not affected after C/T indicated that complexes 2, 3, and 4 were unaffected. By contrast, complex 5 (ATP synthase) was also probably altered because state 3 respiration was decreased whatever the substrate used. Similar results were recently reported on mitochondria isolated from cryopreserved/thawed precision-cut rat liver slices (42) and on permeabilized myocardial fibers from rat heart transplant after cold ischemia-reperfusion (19). Complex 1 and 5 have been identified as the two more fragile mitochondrial respiratory chain complexes (11,19,31,32,43). Indeed, the activity of complex 1 is rapidly altered following cold ischemia of whole organs (19,31), by a still unknown mechanism. The respiratory chain complex 1 is one of the largest known membrane proteins complexes (4) and also the major source of mitochondrial ROS (20). Thus, specific alterations by C/T of complex 1 subunit(s), which consist of the hydrophilic domain containing the redox centers of the enzyme (33), and/or deregulation of ROS production leading to oxidative stress could constitute one of the starting points of the C/T-induced decrease in JO_2 and $\Delta\Psi$, leading to ATP depletion.

Using mitochondrial calcium challenge, which is known to induce the opening of the PTP, we demonstrated that mitochondrial calcium uptake and retention were abolished after C/T. This result might be related to the decrease in $\Delta\Psi$, because the calcium uptake by the mitochondria is electrogenic (2,29), and/or to an increased mitochondrial permeability, due to opening of the PTP. In agreement with the latter hypothesis and previous observations (24,45), cytosolic cytochrome c increased in cryopreserved/thawed hepatocytes, suggesting that C/T induced mitochondrial permeability transition, which is known to be involved, at least in part, in the mitochondrial release of this proapoptotic protein. Classically, the formation of apoptosome by the ATP-dependent oligomerization of cytochrome c with Apaf-1 allows the recruitment of caspases 9 and the subsequent activation of caspase 3, leading to the commitment of the apoptotic cascade (15). However, in our conditions, the mitochondrial release of cytochrome c was neither associated with activation of caspases 9 and 3, nor with DNA fragmentation and apoptosis. To explain this unexpected result, we could suggest that the massive drop of ATP induced by C/T could have prevented caspases activation by an impairment of the ATP-dependent formation of the apoptosome (34). In addition, these apoptosis-related parameters were measured in isolated hepatocytes immediately after thawing. Thus, in these conditions, the early effect of C/T ($\Delta\Psi$ drop, PTP opening, and cytochrome c release) could be evidenced but experimental evidence for apoptosis probably required longer incubation period. Indeed, caspase 3 activation and apoptotic cells were reported in porcine cryopreserved hepatocytes but only several hours after post-thawing on primary culture (24,45).

Improvement of viability and attachment efficiency were recently reported for rat hepatocytes' preincubated with cytoprotectants prior to cryopreservation (40,41). However, in our conditions, we failed to demonstrate any improvement of the various mitochondrial parameters affected by cryostorage in the presence of PTP inhibitor (CsA), antioxidant molecules (*N*-acetyl-L-cysteine, ascorbic acid, or ascorbic 2-glucoside acid) or by changing the concentration of DMSO, albumin, glycerol, and/or glucose in the cryopreservation medium (data not shown). However, protection of the ischemia-induced alteration of the mitochondrial respiratory chain complex 1 has been demonstrated in liver following preincubation with bilobalide (18) or melatonin (7). Thus, the use of those compounds in the hepatocyte cryopreservation medium might be considered as potential interesting cytoprotectants for future investigation.

Finally, cellular impairments could be explained by alteration of mitochondria induced by water crystallization during cryopreservation. Indeed, while matrix swelling could be the consequence of the opening of the PTP leading to osmotic damages, the massive alteration of mitochondrial ultrastructures evidenced by electron microscopy in cryopreserved/thawed hepatocytes (Fig. 4B, F) could also suggest mechanical disruption related to ice crystal formation. Thus, it appears that technical improvement of cryopreservation remains one of the cornerstones of LCT in the future, permitting to limit or prevent irreversible damages due to ice crystallization.

In summary, we report here that C/T induces loss of mitochondrial function and severe ATP depletion in hepatocytes. While ATP synthase damages are probably present, this cryopreservation-induced deleterious effect is related to a predominant alteration of the mitochondrial respiratory chain complex 1, leading to significant decrease in cellular JO_2 and mitochondrial $\Delta\Psi$. These results suggest that, in addition to technical progress in the cryopreservation process, physical and/or chemical protection of the mitochondrial respiratory chain complex 1 may be considered as a new approach to improve the quality of hepatocytes following cryopreservation.

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