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Hepatocyte Suspension for Liver Cell Transplantation:

Consequences of Cryopreservation/Thawing

and

Evaluation of the Infusion Related Pro-coagulant Activity

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A Magali,

A maman,

A papa

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Ce que l'on conçoit bien s'énonce clairement, Et les mots pour le dire arrivent aisément (Nicolas Boileau, 1636-1711)

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Summary

Liver cell transplantation provides clinical benefit to patients with congenital metabolic abnormalities and currently represents an alternative to orthotopic liver transplantation or at least an interim measure for unstable patients awaiting transplantation. Our team and others have already demonstrated that transplanted hepatocytes can achieve metabolic control in the short or medium term. The quality of transplanted cells remains the first limiting factor for the success of liver cell transplantation. Because the use of freshly isolated cells is restricted by contemporary organ donation, cryopreservation remains necessary for long-term storage and permanent availability of the cells.

In this thesis, we have first reviewed and discussed established hepatocyte cryopreservation protocols, especially the cooling procedure, and have focussed on the *in vitro* and *in vivo* assays used for the evaluation of post-thawing hepatocyte quality.

Amongst 9 cell transplanted patients in our center, several received exclusively or predominantly cryopreserved/thawed hepatocytes. We demonstrated post-transplantation benefits of using these cells in control patients with congentital abnormalities in the urea cycle, particularly with respect to clear evidence of cell engraftment and *de novo* appearance of enzyme activity. However, despite these clinical benefits, we found an *in vitro* relationship between the low post-thawing quality of cryopreserved /thawed hepatocytes and an alteration in their mitochondrial function. This post-thawing mitochondrial damage was already evident after the first –20°C cryopreservation step of our protocol, suggesting it occurs early in the process, around the nucleation point, by intracellular ice formation. Cellular impairment could therefore be possibly explained by mechanical alteration of mitochondria due to water crystallisation during the cryopreservation process or thawing procedure. We also observed a poor efficacy of cryopreserved/thawed hepatocytes (as compared to freshly isolated cells) when used liver engraftment in two mice transplantation models. The marked reductions in intracellular ATP concentrations and the decreases in oxygen consumption by hepatocytes were therefore used as markers for the evaluation of the effects of several compounds

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such as bilobalide, hyperosmotic or anti-oxidant molecules, pore transition permeability inhibitors, and for the evaluation of the resistance of selected hepatocyte subtypes to cryopreservation protocols.

We also demonstrated that isolated hepatocytes exert tissue factor-dependent pro-coagulant activity, which may contribute to the early loss of infused cells. We observed that the addition of N-acetyl-L-cysteine to hepatocyte suspensions inhibits coagulation activation.

In conclusion, this work has identified several ways to improve the clinical benefit of liver cell transplantation, including new cryopreservation strategies, such as vitrification. In addition, modulation of the pro-coagulant activity induced by cell infusion with N-acetyl-L-cysteine might beneficially enhance cell engraftment.

Résumé

La transplantation d'hépatocytes est une nouvelle approche thérapeutique pour le traitement des maladies métaboliques. Elle peut être proposée en alternative à la transplantation de foie entier ou, à tout le moins, en attente de celle-ci chez les patients instables, à risque de décompensation métabolique. Les essais cliniques effectués chez 9 patients aux cliniques St Luc ainsi que ceux publiés dans la littérature démontrent l'intérêt de la transplantation de cellules hépatiques à court et moyen terme.

La qualité de la suspension cellulaire transplantée reste le premier facteur limitant pour le développement clinique de la technique. La cryopréservation reste le moyen le plus approprié pour la conservation à long terme des cellules. Elle permet de constituer une banque de cellules pouvant être utilisées à tout moment.

Nous avons d'abord analysé les protocoles de cryopréservation décrits dans la litérature, ainsi que leurs limites tant au niveau de la préservation de la qualité cellulaire après décongélation *in vitro* qu'après transplantation *in vivo*.

Dans ce travail, nous avons démontré l'intérêt d'utiliser des cellules cryopréservées/décongelées, afin de stabiliser des patients atteints de maladies du cycle de l'urée, avant la greffe de foie entier. Les tests de contrôle de qualité effectués sur ces cellules ont cependant montré une altération aux niveaux biochimique et cellulaire, après décongélation. Nous avons ainsi démontré une chute des concentrations intracellulaires d'ATP, signe d'une atteinte mitochondriale. Nos travaux ont également permis de mettre en évidence une diminution de la consommation d'oxygène des hépatocytes en suspension, due plus particulièrement à une atteinte du complexe 1 de la chaîne respiratoire. Cette atteinte mitochondriale peut déjà être observée après l'incubation de la suspension cellulaire à -20°C. Aux alentours de cette température critique se fait le passage de l'état aqueux à l'état cristallin suggérant que les dégâts mitochondriaux observés sont dès lors vraisembablement dus à la formation de glace intracellulaire durant le processus de cryopréservation ou de décongélation. Diverses tentatives visant à améliorer les paramètres

mitochondriaux affectés par le processus de congélation/décongélation par l'addition d'agents protecteurs du complexe 1 (Bilobalide), d' inhibiteurs du pore de transition de perméabilité (Ciclosporine A), d' anti-oxydants ou encore de solutions hyperosmotiques à la solution de cryopréservation, n'ont pas permis d'améliorer la qualité cellulaire. Le tri de sous-types de populations hépatocytaires ou l'isolement de foies hépatectomisés n'ont pas permis de révéler de différences de capacité de résistance à la cryopréservation.

Toujours dans le but d'améliorer le rendement de la transplantation d'hépatocytes et d'augmenter l'efficacité d'implantation dans le parenchyme receveur, nous avons démontré dans la deuxième thèse capacité hépatocytes partie de la la des isolés (fraîchement isolés ou cryopréservés/décongelés) à induire un phénomène de coagulation dépendant du facteur tissulaire. Cette activité pro-coagulante, inhibée in vitro par lea N-acetyl-L-cystéine, pourrait être le point de départ d'une réaction inflammatoire aspécifique influençant ainsi la réussite de la transplantation cellulaire.

En conclusion, nous proposons dans ce travail différentes stratégies en vue de l'amélioration du rendement de la thérapie cellulaire. La vitrification, autre technique de cryopréservation, permettrait d'éviter la formation d'eau intracellulaire. Enfin la modulation de l'activité pro-coagulante par la N-acetyl-L-cystéine, due à la transplantation cellulaire, constitue une piste intéressante pour essayer d'améliorer l'implantation des cellules transplantées et ainsi le rendement de la greffe.

Abbreviations

acute liver failure (ALF), cryopreservation/thawing (C/T), cytochrome P450 (CYP), deoxyribonucleic acid (DNA), differential scanning calorimetry (DSC), dimethylsulfoxide (DMSO), fetal calf serum (FCS), hypothermosol (HTS), intracellular ice formation (IIF), lactate dehydrogenase (LDH), liver cell transplantation (LCT), mitochondrial membrane potential ($\Delta\Psi$), oxygen consumption rate (JO_2), phosphate-buffered saline (PBS), reactive oxygen species (ROS), ribonucleic acid (RNA), university of Wisconsin (UW), 2,4-dinitrophenol (DNP), N, N, N', N'tetramethyl-1,4-phenylenediamine (TMPD), 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2H tetrazolium bromide (MTT), 3-0-methyl glucose (3OMG), 50% of hepatocytes which showed IIF (50TIIF).



1. Foreword



1. Foreword

The liver performs many functions that are essential for life, the most crucial being its role in body metabolism. Congenital metabolic abnormalities occur in approximately 1 in every 900 live births. Most of these conditions are rare, but any physician will encounter patients affected by one or other of these diseases. Management of patients with metabolic diseases is often complex and includes the use of orphan medications, highly specific diets and special education. The quality of life of both the patients and their families is often poor, due to anorexia, naso-gastric feeding, poor variability of diet and severe diet restriction, social eviction, special education requirements and frequent hospitalizations. Liver transplantation for such patients has become a very successful procedure, with over 90% of children now achieving long term survival (110). However, transplantation for a non-life-threatening condition. In addition to short-term complications, chronic graft hepatitis, fibrosis and progressive cirrhosis may develop later in a significant proportion of transplanted children (41). Furthermore, shortage of donor organs for this procedure remains a major obstacle, with about 15% of patients in need of liver transplantation currently dying while waiting for a donor graft (117).

Liver cell transplantation (LCT) can be used to restore both congenital or acquired deficienies in liver function, and currently represents the most promising alternative (2,34,74,90,91). Unlike organ transplantation, this procedure is less radical, less invasive, potentially less expensive and fully reversible. Indeed, case reports have shown that the technique is feasible and well tolerated in humans (24,43,57).

The LCT procedure involves the transfer of an isolated hepatocyte suspension [freshly isolated or cryopreserved/thawed (C/T)] into the diseased liver via the portal vein system. The technique requires intact vascular access to the liver, in order to allow the infusion of hepatocytes into the recipient hepatic sinusoids for subsequent engraftment within the recipient liver parenchyma. Infused hepatocytes cause both portal hypertension and transient ischaemia-reperfusion injury. The

portal hypertension is usually resolved within 2–3 hours with no obvious long term detrimental effects; even microcirculatory abnormalities disappear within 12 hours (50). Initial microcirculatory disruption in the liver, followed by opening of additional vascular channels is thought to account for this transient response. However, in addition to these transient changes, the interference in blood flow due to the infusion is sufficient to cause local ischaemia reperfusion events. Ischaemia reperfusion is also associated with activation of Kupffer cells, and consequently release of cytokines. Activated Kupffer cells may play a positive role by increasing endothelial permeability and improving the access of transplanted cells to the liver plate. Cells become adherent to the endothelial cells separate to allow transplanted cells to pass into the liver plate. After a few hours, the process is terminated and the plasma membrane structures are reconstituted so that the liver plate is remodelled to accommodate the transplanted cells and polarity is restored (51). However, activated Kupffer cells may also act to increase clearance of 'foreign' cells (66).

The hepatocytes are obtained from cadaveric donor livers using a two-step collagenase perfusion technique, and can either be infused immediately after isolation, if kept in a cold solution of University of Wisconsin (104), or cryopreserved for further transplantation. Because LCT is still in its development phase, there is no access to the conventional pool of organ donors within the organ allocation systems and the availability of freshly isolated cells therefore does not match with simultaneous recipient presentation. Therefore, the major challenge is to maintain isolated hepatocytes viable and functional until a recipient candidate presents predominantly through cryopreservation and storage. Cryopreservation of hepatocytes therefore represents an essential step for the success of LCT.

This technique still faces many hurdles and efforts currently focus on improving the technique to enable validation of its clinical use. Research is underway to identify ways in which to improve the viability and quality of hepatocytes after cryopreservation. Having reviewed the state of the art with respect to "C/T and hepatocytes" (Section 2.1.1), this thesis will present the study of the

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consequences of C/T on hepatocyte suspension quality used for LCT as the main theme (Section 4.1). As preventing the rejection and/or loss of transplanted freshly isolated or C/T hepatocytes to increase durability of the results remains another challenge, we also investigated the pro-coagulant effects of the infused hepatocyte suspension, as the starting-point for an aspecific inflammatory reaction (Section 4.2).



2. Introduction



2. Introduction

2.1 The consequences of cryopreservation/thawing on hepatocytes used for Liver Cell Transplantation

The use of freshly isolated cells remains restricted by both organ shortage and the limited quantity of cells that can be infused in a single session. The success of LCT, as evaluated by engraftment yield and liver repopulation capabilities, also remains closely dependent on the quality of the transplanted hepatocytes. Cryopreservation remains the only practical method for hepatocyte long-term storage and many studies have been undertaken to identify an efficient protocol which will preserve the quality of the hepatocytes after thawing. Nevertheless, an international panel of experts recognised in 1999 that "research should continue to improve the cryopreservation procedures" (76).

The incorporation of C/T hepatocytes in both toxicological studies and the bioartificial liver has led to major advances being identified for the C/T process, namely in the maintainance of high metabolic quality of the cells, especially with respect to the important phase I and II metabolism enzyme pathways and response to inducers (78,116). Current C/T protocols and the subsequent analysis of the post-thawing quality were reviewed in both *in vivo* and *in vitro* models. However, although abundant, the provided data on isolated hepatocytes still remain conflicting.

2.1.1 State of the art

2.1.1.1 Classical factors for a successful hepatocyte cryopreservation protocol

In this chapter, we describe the steps, proposed in the literature, as required for an efficient cryopreservation protocol.

2.1.1.1.1 Isolation and pre-culture

An initial high cell quality is essential prior to cryopreservation. Key factors that compromise the quality of the hepatocytes, prior to C/T, include high fat content in the liver, prolonged storage after organ removal and prolonged warm ischemia (76).

Liver isolation is performed using two-step collagenase perfusion (taking around 10 minutes/step) at 37°C. The first step involves the use of a solution containing a calcium chelating agent, which removes calcium ions and disrupts the desmosomal structures. This is followed by a second solution which contains the digestive enzyme, collagenase, and calcium which is essential for its activity. After mechanical disaggregation, filtration and low speed centrifugation, the obtained isolated hepatocyte suspension is removed (86).

Detachment from the extracellular matrix, which is a principal occurrence during hepatocyte isolation, has been described as promoting apoptosis. This early cell death (known as anoikis) can not be totally rescued by the *in vitro* culture of hepatocytes under attached conditions and cells which undergo this process will die in the hours following the isolation procedure (108,138).

To promote recovery from this cell trauma and to improve their post-isolation quality, freshly porcine isolated hepatocytes were pre-incubated at 37°C for different periods of time before cryopreservation. These data demonstrated that 24 hours pre-culture in a spinner bioreactor led to higher albumin production after C/T as compared to both non pre-cultured and 48 hours pre-cultured porcine hepatocytes. Nevertheless, the albumin production level remained markedly lower following cryopreservation as compared to freshly isolated cells, even with 24 hours pre-incubation $(0.21 \pm 0.24 \text{ mg/ml/h vs} 0.88 \pm 0.62 \text{ mg/ml/h}) (23,58).$

The effect of pre-culture before cryopreservation was also investigated in rat, dog and human hepatocytes. Gomez-Lechon et al. obtained high quality post-thawing cells as evaluated by viability assays, adaptation of hepatocytes to culture, drug-metabolizing capability and cytochrome P450 (CYP) induction (47). However, these results were not confirmed by Lloyd et al. using pig hepatocytes cultured in a bioartificial liver (78).

2.1.1.1.2 Freezing solution

2.1.1.1.2.1 University of Wisconsin

University of Wisconsin (UW) solution is the gold standard cryopreservation medium for isolated hepatocytes. Originally developed as a cold storage solution for transplant organs, UW is

intracellular-like solution in composition and its important components have been widely investigated. Molecules such as Lactobionate (100 mM), a large molecular weight anion, which is impermeable to most membranes and capable of suppressing hypothermia-induced cell swelling, or Raffinose (30 mM), which provides additional osmotic support, have been proposed as the principal cryoprotectants of the UW solution. In addition, dexamethasone stabilises cell membranes (73,115). In an interesting study, four different media were compared for the cryopreservation of rat hepatocytes: Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum and 12% dimethylsulfoxide (DMSO); UW solution supplemented with 12% DMSO and 2 other commercial solutions (Cell Banker). Parameters including viability, plating efficiency, lactate dehydrogenase (LDH) release, ammonia removal test, and lentiviral gene transfer were highly maintained when hepatocytes were cryopreserved with UW solution (5). The effectiveness of UW solution as a cryopreservative agent, suggests that both metabolic and ultrastructural factors may be important in the effective cryopreservation of primary isolated hepatocytes (1).

2.1.1.1.2.2 HypoThermosol

HypoThermosol (HTS), a dextran-based intracellular-type solution, has also been used, as a carrier solution of the freezing medium. Freshly isolated rat hepatocytes cryopreserved in HTS supplemented with 10% DMSO showed high viability, long-term hepatospecific function, and a favourable response to cytokine challenge post-thawing as compared to supplemented culture media (114). If further studies confirm these results, then a decrease in the DMSO levels within the cryopreservation solution might be possible. There are currently no data comparing UW and HTS available in the literature (6,7).

2.1.1.1.2.3 Type of cryoprotectant

Two classes of cryoprotectants are described, permeating (DMSO, glycerol) or non permeating [polymers (Dextran), oligosaccharides (Trehalose) and sugars such as glucose, sucrose or fructose]. The non permeating cryoprotectants will be discussed further in Section 4.1.3.2.

DMSO is an important polar permeating aprotic solvent which is less toxic than other members of this class. The use of DMSO in medicine dates from around 1963, when a team of workers at the University of Oregon Medical School discovered that it could penetrate the skin and other membranes without damaging them and could carry other compounds into a biological system. As a cryoprotectant, DMSO is able to enter cells and reduce injury by moderating the increase in solute concentration during the freezing process. In most studies, DMSO has been identified as being the ideal cryoprotectant, notably by resulting in the best plating efficiency (16,44,56,80,100,109,111,114,122). A final concentration of 10% DMSO is typically described and although there are some exceptions, a higher concentration has a potential toxicity due to high osmolarities (55). The rate of addition of the cryoprotectant also appears important for the outcome of cryopreservation; some authors have advocated adding the permeating cryoprotectants slowly to cell suspensions in order to avoid damage related to osmotic shock and to a lesser extent cellular dehydratation (55,80,130). Freezing should also be started as soon as possible after the addition of the cryoprotectant to reduce the possibility of toxicity; it has been recommended that DMSO should be added at 4°C, as a DMSO-associated toxicity was demonstrated at 25°C and 37°C. Some authors proposed to add permeating cryoprotectants slowly to the cell suspension in order to avoid damages related to osmotic shock and cellular dehydratation (80,130) which seems to be a minor point (55). The cryoprotective effects of glycerol and glycine betaine are based on a mechanism similar to the well documented stabilization of complex enzymes against dissociation into subunits at high salt concentrations by preventing dissociation of the peripheral cold labile proteins. The binding of glycine betaine and glycerol molecules is limited to the water lipid interface of thylakoid membranes (96).

2.1.1.1.2.4 Serum

Human application does not tolerate the use of animal origin products because of possible zoonosis contamination or an immune response to animal proteins (103), but fetal calf serum (FCS) or human albumin are classical ingredients of experimental cryopreservation solutions. A wide range of

concentrations (10 to 90%) have been used and in most published studies, no significant differences were noted while varying the percentage of serum (1,16,37,42,80,111,118).

Nevertheless, some authors have successfully cryopreserved porcine hepatocytes without serum and noted that after thawing, the addition of conditioned medium derived from hepatic non-parenchymal cells improved attachment and function of hepatocytes [urea production, CYP activity (89)].

2.1.1.1.3 Concentration of hepatocytes and vial type

In most published studies, hepatocyte concentrations varied from 10^6 to 10^7 cells/ml of cryopreservation medium (124). Lloyd et al. did not find a significant superiority of any concentration whilst evaluating pig cell return post-cryopreservation, hepatocyte attachment, LDH leakage, bilirubin conjugation or CYP 3A4 activity (78). However De Loecker et al. reported that cell viability increased as cell density decreased (estimated by the viability trypan blue test) (29,30). High cell density may increase membrane-membrane contacts and cell damage. Therefore, unless high cell density will save space and is of interest for cell banking, cryopreservation at a low density (less than 10^7 cell/ml) is recommended. No new data have been published recently as hepatocyte concentration is considered a minor factor in achieving the best post-thawing quality.

Vial type may also potentially influence post-thawing cell quality. Although few data are available in the literature, 50ml bags appear to give better post-thawing hepatocyte quality than 100 ml bags (134).

2.1.1.1.4 Cooling process

Slow freezing protocols are considered to be the best strategies for cryopreservation of mature hepatocytes. Early methods included the use of an isopropanolol cooler device, placed in a -80° C freezer, giving a constant temperature decrease of -1° C/min down to -70 or -80° C (20). Other slow freezing protocols have been described in the literature, varying temperatures from -1 to -5° C/min up to -40° or -80° C, before storage at -196° C (79). A decrease in temperature at -1.9 C/min from 4 to -30° C and then -30 C/min from -30 to -150° C has been adopted by many

authors (31,37,107). More specific techniques have been described by Diener et al. and Hengstler et al. (35,54,130). In their studies, freshly isolated rat hepatocytes were submitted to several protocols, where the temperatures of the vial and of the cryopreserving solution were controlled. Initially, shock freezing in liquid nitrogen dramatically decreased cell viability, despite the presence of 10% DMSO. A slow freezing protocol of -2° C/ min led to much better viability than a cooling rate of -38°C/min. While using this protocol, they determined that at approximately -20° C, the cell suspension became supercooled. Indeed, when crystallisation commenced, the latent heat of fusion was released and the cell sample warmed. This release of heat was considered potentially deleterious; therefore they developed a freezing program with shock cooling. Their protocol achieved the best post-thaw hepatocyte quality (86% viability vs 79% achieved by the slow linear protocol). It has been reported that the same cooling shock can be obtained by clamping the vials, with forceps cooled in liquid nitrogen (28). However, Lloyd et al. (LDH release, cell return, attachment, and biochemical assays) and our team (personal contribution) have failed to show any difference between computer-controlled freezing rate, the Nalgen propan-2-ol device or simply using -20° C and -80° C freezers (78).

Storage of hepatocytes at -20° C or -80° C remains deleterious for cell function as several proteases might remain active at these temperatures. However, at -130° C, no chemical reaction may occur as there is no more thermal energy and furthermore, as water exists in the vitreous or crystalline states at this temperature, no liquid water is present. Therefore, $-140/-150^{\circ}$ C° is the minimum acceptable temperature for long-term storage of cryopreserved hepatocytes (31,92,100). At -140° C (vapour phase of liquid nitrogen) or -196° C (liquid phase of liquid nitrogen), cells can be stored for long term periods (31,37,76). However, it is the passage of water from one state to another that is the critical point for cell quality rather than the duration of the storage.

A summary of several cooling process described in the literature is presented in Table 1.

Species	Cryoprotectant	Freeze rate	Storage	References
			Temperature	
Human	DMSO	-1°C/min	-80°C	(20)
Rat	DMSO	-1° C to -38° C (with	Liquid nitrogen	(28)
		cooling shock) then		
		liquid nitrogen		
Dog, monkey,	DMSO	-1.9°C/min from 4	Liquid nitrogen	(31)
human		to -30° C, then $-$		
		30° C from -30° C to		
		-150°C		
Rat	DMSO	138°C/min	Liquid nitrogen	(35)
		2. −2°C/min		
		3. Slow variable		
		4.Optimized variable		
		rate		
Human	DMSO	-1.9°C/min from 4	Liquid nitrogen	(37)
		to -30° C, then		
		-30°C from -30°C		
		to -150°C		
Rat	DMSO	Cooling in 10 min	Liquid nitrogen	(54)
		down to 0°C, 8 min		
		at 0°C, in 4 min		
		down to -8° C, in 0.1		
		min down to -28° C,		
		in 2 min down to		
		–33°C, in 2 min up		
		to –28°C, in 16 min		
		down to -60° C, in 4		
		min down to -100°C		
		(variable rate)		
Human	DMSO	Variable rate	Liquid nitrogen	(76)
Dog, monkey,	DMSO	-1.9°C/min from 4	Liquid nitrogen	(107)
human		to -30° C, then		
		-30°C from -30°C		
		to -150°C		
Rat	DMSO	Variable rate	Liquid nitrogen	(130)
Pig	DMSO	1. (35) optimised	Liquid nitrogen	(134)
		2. Modified variable		

Table 1: Summary of freeze rate comparison studies

2.1.1.1.5 Thawing procedure

Rapid thawing at 37°C to minimize cellular damage due to reformation of intracellular ice has been found to enhance viability. The critical point of this procedure is again to avoid the deleterious phenomenon of intracellular ice formation (IIF). As for cooling, a slow dilution of the cryoprotectant at 4°C is recommended, to avoid osmotic shock damage and toxicity due to the cryoprotectant (55).


2.1.1.3 In vitro evaluation of hepatocyte quality after cryopreservation/thawing

2.1.1.3.1 Plating

The *in vitro* attachment of isolated hepatocytes to plastic dishes is widely used for the evaluation of their quality. Low plating efficiency which is often documented in cryopreserved cells, remains a major problem because engraftment of the transplanted hepatocytes in the recipient liver parenchyma is also dependent on these proteins interacting with the extracellular matrix (5,73,92,106,121). Structural membrane damage observed after cryopreservation may contribute to such alteration. Dhawan et al. recently demonstrated that the process of cryopreservation leads to downregulation of cell adhesion molecules at the gene and cellular level (127). However, preculture of hepatocytes or the addition of trehalose/oliosaccharides to the freezing medium seemed to improve the attachment of cells to substrates such as collagen (69,87,120).

2.1.1.3.2 Hepato-specific functions

Conjugation and secretion of biliary acids is maintained following C/T of human hepatocytes. The uptake activity of taurocholate in C/T hepatocytes was found to range from 10 to 200% of that observed in freshly isolated cells immediately after thawing (105).

The characterization of freshly isolated and C/T monkey hepatocytes demonstrated that various hepato-specific functions were maintained but at a lower level of activity. Indeed, in this study, the ability for synthesis, (proteins, glucose, glucose-6 phosphatase activity) was decreased after deep freeze storage (16). Concerning protein synthesis, data from the literature show that this important hepatic function is often impaired in hepatocytes after C/T. For example, albumin production in cryopreserved human hepatocytes was shown to be about half that of freshly isolated hepatocytes (28).

Glycogen synthesis in cryopreserved porcine hepatocytes was found to be reduced to about 30% after 24 hours and to 47% after 48 hours of culture, as compared to freshly isolated hepatocytes. Reduced basal levels of glycogen and of glycogen synthesis could be explained by an increased energy demand in cryopreserved hepatocytes required for the repair damage caused by

cryopreservation. Glycogenolysis was reduced to about 50% in cryopreserved hepatocytes and gluconeogenesis to about 40% of glucose production in freshly isolated hepatocytes at day 1 and 2 post-thawing. Incubation with glucagon (90 min) increased the glucose production from glycogenolysis and gluconeogenesis in both freshly isolated and cryopreserved hepatocytes (81). According to the literature, urea production also seems to be reduced following C/T (16).

Species	Hepato-specific functions	Relative assays	Impairment following C/T	Reference
Human	Taurocholate and estradiol uptake	Radioactivity counted using a liquid scintillation counter	No	(105)
Pig	 Protein synthesis Urea synthesis Glucose synthesis Glucose-6 phosphatase activity 	 Leucine incorporation into proteins, NH4Cl incubation and measurement of urea concentration in supernatant 2) Incubation with fructose and measurement of glucose concentration in supernatant 3) Quantitation of phosphoric acid formed after addition of glucose-6- phosphate 	Yes	(16)
Human	Protein synthesis	Incorporation of radiolabeled isoleucine into proteins	Yes	(28)
Pig	 1) Glycogen synthesis 2) Glycogenolysis 3) Gluconeogenesis 	 Incubation with insulin and glucose and glycogen formed evaluated after digestion by aminoglucosidase and measurement of glucose Incubation with glucagon, evaluated by glucose release. Incubation with glycerol (glucose precursor) and glucagon, evaluated by glucose release. 	Yes	(81)

Table 2: Hepato-specific function and their relative assays

2.1.1.3.3 Drug-metabolizing enzyme activities

There are no apparent significant changes in drug metabolizing enzyme activities between freshly isolated and cryopreserved hepatocytes for the major drug-metabolizing pathways. The cryopreservation of human hepatocytes isolated from 17 donors did not alter their capability to metabolize substrates for the major CYP isoforms (CYP1A2, CYP2A6, CYP2C9, CYP2C19, CYP2D6, and CYP3A4) as well as the phase II enzymes UDP glucuronyltransferase, and 7-HC sulfation for sulfotransferase, as compared to freshly isolated hepatocytes (77). Consensus regarding the phase II enzymes remains unclear. Indeed, phase I drug-metabolizing enzyme activities analyzed in cryopreserved human, rat, and mouse hepatocytes are very similar to those of freshly isolated hepatocytes, while phase II enzyme activities are affected by cryopreservation (116). Other studies show better stability of drug metabolizing activities in monkey than in rodent hepatocytes. After thawing, Phase I and Phase II activities (CYP, ethoxycoumarin-O-deethylase, aldrin epoxidase, epoxide hydrolase, glutathione transferase, glutathione reductase and glutathione peroxidase) were well preserved (32). The documented decrease in the activity of phase II enzymes may be related to the loss of the corresponding cofactors although a hypothesis of physical cell alteration is not excluded (116). As the microsomal membrane may offer some protection (20), the cytosolic enzymes are probably more sensitive to freeze-associated damage (notably glutathione Stransferase), because of greater exposure to ice crystal damage,.

2.1.1.3.4 CYP induction

If C/T hepatocytes cultures are sensitive to CYP inducers (rifampicin, rifabutin, phenobarbital, omeprazole, beta-naphthoflavone) the induced activity remains lower as compared to freshly isolated cells with an increased delay induction time (54,67,76,82,98,101,106).

2.1.1.3.5 Hormone responsiveness

Few data are available regarding hormone responsiveness of C/T hepatocytes. In a dog model, freshly isolated hepatocytes showed significant responses to both α_1 - and β_2 -adrenergic agonists with little indication of dedifferentiation in these cultures, while cryopreserved cells showed no

response to either agonist (107). This indicates alteration in cell membrane to receptor expression and/or their intracellular transduction systems.

2.1.1.3.6 Gene expression

Cryopreservation could also potentially affect the differentiation state of isolated hepatocytes. Recently published results showed that the gene expression pattern in freshly isolated human hepatocytes was similar to that of the intact liver. Furthermore, gene expression in isolated human hepatocytes was not affected by cold storage and cryopreservation. However, the gene expression was profoundly affected in monolayer cultures after plating. Specifically, the gene expression changes, observed in cultured suspensions of human hepatocytes, are involved in cellular processes such as phase I/II metabolism, cell membrane polarity, fatty acid and lipid metabolism, apoptosis, and proteasomal protein recycling. An oxidative stress response has been suggested as being partially involved in these changes (99).

A summary of *in vitro* evaluation of post-thawing hepatocyte quality is presented in Table 3.

Species	Cryopreservation protocol	Impairment following C/T	No impairment following C/T	References	
Rat and human	Pre-incubation 20°c, –70°c, liquid nitrogen	Plating	CYP induction	(106)	
Porcine	Slow freezing protocol up to -80°c	Trypan blue exclusion test Plating Ammonia clearance		(73)	
Rat	Slow freezing protocol up to -80°c	Trypan blue exclusion test Plating Ammonia clearance		(5)	
Human	20% DMSO, 40% FCS Slow freezing protocol	Trypan blue exclusion test Plating LDH release MTT	ATP Urea synthesis	(92)	
Porcine	Freezing boxes or slow freezing protocol	CYP Glycogen synthesis Glycogenolysis Gluconeogenesis	Plating	(81)	
Rat and mouse	Slow freezing protocol	Plating Uptake of neutral red Protein synthesis		(121)	
Porcine	Immediate cryopreservation Serum free	Protein synthesis Gluconeogenesis CYP activity Urea synthesis	Trypan blue exclusion test	(16)	
Monolayer culture post-thawing	Not available	Protein synthesis		(28)	
Human	Storage in liquid nitrogen		Conjugation and secretion of biliary acids	(105)	
Human	Slow freezing protocol		CYP activity Phase II enzymes	(77)	
Human, rat and mouse	Slow freezing protocol	Phase II enzymes	Phase I enzymes	(116)	
Monkey	Slow freezing protocol	LDH release Plating	Phase I enzymes Phase II enzymes	(32)	
Human	Storing at -80°c	Cytosolic enzymes	CYP activity	(20)	
Rat	Slow freezing protocol	CYP induction		(54)	
Human	Slow freezing protocol	CYP induction		(67)	
Rat	Slow freezing protocol		CYP induction	(82)	
Human	Not available	CYP induction		(98)	
Human	Not available	CYP induction		(101)	
Human, dog and monkey	Slow freezing protocol	Hormone responsiveness		(107)	
Human	Pre-incubation Freezing boxes or slow freezing protocol		Gene expression	(99)	

Table 3: In vitro evaluation of post-thawing quality of hepatocytes

2.1.1.4 In vivo models

Data published in the literature differ about the efficiency of C/T hepatocytes to engraft and repopulate the recipient liver. In the eighties, Fuller et al. described a rat model in which fewer cryopreserved hepatocytes cells were detected 1 month post-transplantation in the recipient liver after intrasplenic transplantation, as compared to freshly isolated cells (46). This was confirmed in Nagase analbuminemic rats after the evaluation of engraftment and albumin production in C/T hepatocytes transplanted rats. If clusters of C/T cells were detected in the recipient liver, their size and their number were significantly lower as compared to freshly isolated hepatocytes. In addition, no significant production of albumin was detected in the C/T transplanted rats. In rats treated with D-galactosamine, the survival of rats transplanted with C/T hepatocytes following intrasplenic transplantation improved to 60% after 7 days (as compared to 100% with freshly isolated hepatocytes) (25,26).

C/T hepatocytes have been shown to possess identical clonal replicative potential to that of freshly isolated cells indicating that they could provide an interesting cell source for LCT. Indeed, C/T hepatocytes constituting 0.1% of total adult hepatocyte number recipient ones could repopulate a mean of 32% of recipient liver parenchyma (61). Furthermore, transplantation of woodchuck hepatocytes into the liver of urokinase-type plasminogen activator/recombination activation gene-2 mice, a model of liver regeneration, demonstrated that cryopreserved cells retained the ability to divide and to extensively repopulate a xenogenic liver. Notably, *in vivo* susceptibility to infection with woodchuck hepatitis B virus and proliferative capacity of C/T woodchuck hepatocytes in recipient mice were identical to those observed with freshly isolated hepatocytes (21).

Dunn et al. showed in the Dalmatian dog model that sequential intrasplenic LCT provided a significant but transient (22 days) correction of urinary uric acid excretion that was similar to either freshly isolated or C/T hepatocytes (38). Papalois et al. demonstrated that simple cryopreserved pig hepatocytes, at -20°C, have sufficient viability after 1 month of storage to support the hepatic function of animals with severe acute liver failure (93).

The efficiency of C/T human hepatocytes has also been evaluated in NOD/SCID mice model. In this study, Cho et al. demonstrated that following transplantation (engraftment in the peritoneal cavity and the liver), cryopreserved human liver cells retained hepatic function (glycogen storage, glucose-6 phosphatase activity) and proliferated in response to liver injury by carbon tetrachloride (17). The above informations are summarized in Table 4.

LCT model	LCT protocol	Effect post-LCT with C/T hepatocytes	References
Rats	Intrasplenic autologous	-Low detection level	(46)
Analbuminenic rats	Intrasplenic rat hepatocytes transplantation	-Low engraftment efficacy -No albumin production	(25,26)
Mice with transgene induced liver	Intrahepatic mice hepatocytes transplantation	-Identical clonal replicative potential	(61)
Urokinase-type plasminogen activator transgenic mice	Intrasplenic woodchuck hepatocytes transplantation	-Identical proliferative capacity	(21)
Dalmatian dog	Multiple intrasplenic healthy mongrels hepatocytes transplantation	-Identical transient correction in urinary uric acid excretion	(38)
Toxic acute liver failure (rats)	Intrasplenic porcine xenotransplantation	-Identical hepatic function support	(93)
Carbone tetrachloride treated NOD/SCID mice	Intrasplenic or intraperitoneally human hepatocytes transplantation	-Retain hepatic function -Proliferate	(17)

Table 4: In vivo evaluation of the quality of post-thawed hepatocytes

2.2 Study of the pro-coagulant activity of the cell preparation

Haemostasis is maintained by the activation of the coagulation cascade upon vessel injury. The coagulation cascade is triggered by two distinct pathways: either tissue factor-dependent (the extrinsic pathway) or tissue factor-independent (the intrinsic pathway, contact activation), where the tissue factor is a 47-kDa transmembrane glycoprotein. Tissue factor is constitutively expressed by cells in the adventitia of blood vessels. It is found in richly vascularised tissues such as the cerebral cortex, renal glomeruli, and lungs. Normally, cells exposed to blood (such as endothelial cells and monocytes) do not express tissue factor, but certain inflammatory stimuli including lipopolysaccharide, immune complexes and cytokines can induce tissue factor expression in these cells. Tissue factor is strictly regulated by a tissue factor pathway inhibitor in the blood. Both pathways converge at the activation of the coagulation factor X to factor Xa in the generation of thrombin. Thrombin cleaves fibringen to fibrin which induces the formation with platelets of an insoluble thrombus in order to stop the bleeding (18). Additionally, tissue factor appears to promote inflammation with thrombin and is known to activate protease-activated receptors (19). This activation of protease-activated receptors elicits the production of proinflammatory cytokines and promotes leukocyte rolling in venules. Bennet et al. have described a thrombotic reaction following islet cell transplantation, which is elicited when islets come into direct contact with ABOcompatible blood (8). This phenomenon has been called instant blood-mediated inflammatory response and is characterized by the activation of both the coagulation and the complement systems and by a rapid binding of activated platelets to the islet surface. More recent studies performed in Sweden associate instant blood-mediated inflammatory response with early beta cell activity (88). To our knowledge, pro-coagulant activity related to tissue factor has not yet been evaluated in other

cell transplantation models and more specifically for the liver.

3. Aims of the study

3. Aims of the study

We evaluated the use of C/T hepatocytes in LCT to stabilize patients with urea cycle disorders who were awaiting orthotopic liver transplantation. The ability of C/T hepatocytes to engraft the recipient liver and to improve the impaired metabolic parameters was investigated. For a variety of metabolic disorders, a small percentage (<10%) of enzyme activity allows the modification of the phenotype from a severe to a moderate or even mild form of the disease. However, a higher proportion of liver cell engraftment is sought.

We assumed that successful engraftment was closely related to the liver cell quality and remaining C/T hepatocytes, and for disponibility reasons, the appropriate cell source. Based on our preliminary viability results obtained with C/T hepatocyte suspension used in two urea cycle disorders patients, which showed marked reductions in ATP concentrations, we studied in detail the mitochondrial biochemical functions of C/T hepatocytes as compared to freshly isolated ones. Thereafter, we determined the timing of the mitochondrial damage and investigated strategies to improve these parameters mainly by changes in the cryopreservation medium composition or selection of hepatocyte sub-populations that may be more resistant to the freezing related damage.

Another approach to improve cell engraftment involved studying early mechanisms of cell rejection and cell death occuring in LCT. It was demonstrated that pancreatic islet infusions were responsible for a tissue factor pro-coagulant activity, which can be the starting-point of an unspecific inflammatory reaction which can impair the long-term success of transplantation. We therefore evaluated tissue factor dependent pro-coagulant activity in both freshly isolated and C/T hepatocyte suspensions and their modulation by pharmacological agents. This approach will help us to understand the early mechanisms of cell rejection and cell death that may occur in LCT and hence may lead to design strategies for the improvement of cell engraftment.

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4. Results and discussion



4. **Results and discussion**

4.1 Consequences of cryopreservation/thawing on hepatocytes used for liver cell transplantation

4.1.1 Results

4.1.1.1 Cryopreserved/thawed cells used to bridge unstable urea cycle disorder patients to orthotopic liver transplantation

Limited information is available about the potential use of C/T hepatocytes in humans for metabolic disorders. All published case reports, to our knowledge, have infused at least some proportion of freshly isolated hepatocytes, preventing any conclusion regarding the respective efficiency of C/T versus freshly isolated cells. The effect of LCT in acute liver failure (ALF) was evaluated by Bilir et al. in five patients with severe ALF who underwent intrasplenic and/or intrahepatic LCT with C/T hepatocytes. Three of the 5 patients who survived 48 hours after LCT had substantial improvement in encephalopathy scores, arterial ammonia levels, and pro-thrombin times. Clinical improvement was paralleled by an increase in aminopyrine and caffeine clearance. All 3 patients lived substantially longer than expected based on clinical experience after LCT but eventually died. Postmortem examination showed the presence of transplanted hepatocytes in the liver and spleen by light microscopy and FISH analyses (119). However, in ALF, the demonstration of the LCT efficacy remains difficult. Studies into inherited metabolic diseases have also used freshly isolated hepatocytes.

In Sections 4.1.1.1.1 and 4.1.1.1.2 (articles 1 and 2) we demonstrate the potential of C/T hepatocytes to stabilize unstable, metabolic decompensation in urea cycle disorder patients while waiting for a liver graft.

4.1.1.1.1 Cryopreserved liver cell transplantation controls ornithine transcarbamylasedeficient patient whilst awaiting liver transplantation

In this Section (Article 1), we demonstrate that cryopreserved cells allowed metabolic control and urea synthesis in a male ornithine transcarbamylase-deficient patient who was waiting for orthotopic liver transplantation. This patient was stabilized during the entire waiting time leading up to the transplantation. In addition, a patient from King's College Hospital (UK), with an inherited factor VII deficiency, was exclusively transplanted with C/T hepatocytes and experienced a transient 20% reduction in factor VII therapy requirement (34).

Article 1: Cryopreserved Liver Cell Transplantation controls Ornithine Transcarbamylase deficient patient while waiting Liver Transplantation. American Journal of Transplantation, 2005 Aug; 5(8):2058-61.

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Case Report

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Cryopreserved Liver Cell Transplantation Controls Ornithine Transcarbamylase Deficient Patient While Awaiting Liver Transplantation

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Liver cell transplantation was performed in a child with urea cycle disorder poorly equilibrated by conventional therapy as a bridge to transplantation. A 14-month-old boy with ornithine transcarbamylase (OTC) deficiency received 0.24 billion viable cryopreserved cells/kg over 16 weeks. Tacrolimus and steroids were given as immunosuppressive treatment while the patient was kept on the pre-cell transplant therapy. Mean blood ammonia level decreased significantly following the seven first infusions, while urea levels started to increase from undetectable values. After those seven infusions, an ammonium peak up to 263 µg/dL, clinically well tolerated, was observed. Interestingly, blood urea levels increased continuously to reach 25 mg/dL, after the last three infusions. Eventually, he benefited from elective orthotopic liver transplantation (OLT) and the post-surgical course was uneventful. We conclude that use of cryopreserved cells allowed short- to medium-term metabolic control and urea synthesis in this male OTC-deficient patient while waiting for OLT.

Key words: Children, liver cell transplantation, metabolic disease, pediatric medicine, urea cycle

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Introduction

The urea cycle is the final and crucial pathway for the metabolism of waste nitrogen in humans. Hence, an alteration of this pathway causes an accumulation of ammonia, which has been described to cause several deleterious effects of the brain, for instance neurological damage and cognitive deficits. Urea-genesis is liver-based and hepatocyte is the single cell able to express the ornithine transcarbamylase (OTC) gene, and by this way fully elimi-

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nate ammonia. Male patients with OTC deficiency, which is a severe form of urea cycle disorder presenting from birth, are at high risk of severe brain damage due to hyperammonemia. Management by diet protein restriction and use of ammonium scavengers are not always sufficient to control the disease and stabilize the patient (1), and orthotopic liver transplantation (OLT) becomes then the ultimate treatment to avoid repeated encephalopathic episodes and irreversible brain damage (2).

Rapid transplantation is limited by the organ shortage and long waiting time, during which irreversible brain damage may occur and jeopardize the long-term psychomotor development.

Liver cell transplantation (LCT) has been shown to restore partial metabolic activity in various animal models of inborn errors of metabolism (3,4). Because human trials have already reported short or medium metabolic effects in different inborn metabolic diseases, such as Crigler–Najjar disease (5), Glycogen storage type 1a disease (6) and Refsum disease (7), this alternative therapy has been proposed as a bridge during his waiting time for OLT. Moreover, in LCT, cells can be made available without competing with whole organs, by using resected segments of reduced livers or even segment four obtained from split livers (8). One additional advantage of LCT is the possibility to cryopreserve cells that are available for infusions when required.

In a previously reported OTC patient, Horslen et al. have recently demonstrated that using fresh and cryopreserved cells, isolated from 10 different livers, only a short-term (11 days) metabolic stabilization has been observed with a normal protein intake during this short period. The child was subsequently transplanted after this LCT trial (9).

In the current study, we show that LCT with cryopreserved cells can be successful to stabilize an OTC-deficient patient for as long as 6 months, providing metabolic control and urea synthesis while waiting for OLT.

Patient and Methods

A 14-month-old boy (10 kg) with a urea cycle disorder was referred to our unit for liver transplantation. He had experienced numerous attacks of hyperammonemia since birth, when he presented with acute encephalopathy and ammonium level of 548 µg/dL. He had positive orotic aciduria and a liver biopsy confirmed OTC deficiency (enzymatic activity assay). The child had impaired psychomotor development and was maintained on a restricted protein intake (1 g/kg/day), sodium benzoate therapy (250 mg/kg/day) and arginine/citrulline supplementation. He was hospital bound most of the time because of recurrent episodes of encephalopathy and hyperammonemia.

His parents were not considered as living donor candidates, and the boy was registered in the Eurotransplant waiting list. In view of his unstable condition, and following parental information and institution review board approval, a program of LCT was proposed in order to stabilize the child while waiting, as a bridge to transplantation. No contra-indication to LCT was found, and left to right cardiac shunt was amongst others excluded.

A port-a-cath device was inserted surgically in the portal system according to the technique described elsewhere (10) and allowing repetitive infusions. The last injection was made after direct portal puncture during surgery to remove the port-a-cath, which was no more in the portal system. During the ninth cell injections with port-a-cath, an ultrasound was performed before and after each infusion to control the catheter position. Moreover, contrast Doppler ultrasound was applied in order to follow flow direction before infusions, while the portal pressure was monitored before and after infusions. Central venous pressure, blood pressure, pulse rate, temperature and oxygen saturation were also monitored during infusions.

Cell isolation

Cell isolation procedure has been reported earlier (7). Briefly, hepatocytes were isolated at the liver cell transplantation center at the St. Luc Hospital, Université Catholique de Louvain using the classical two-step perfusion method under good manufactory practice guidelines. In this case, 5.4 billion hepatocytes with a viability of 89% estimated by the trypan blue exclusion (TBE) method (plating: 42%) were isolated from one ABO compatible 19-year-old boy reduced-liver (no fatty infiltration) recovered postmotem. Serologic tests for hepatitis B, hepatitis C, HIV and CMV were negative.

Cryopreservation and thawing

Isolated cells were frozen in an University of Wisconsin solution with 5 mM Hepes, 20 mg/L dexamethasone, 40 ||IU/L insulin, 1 M glucose, 3.75% human albumin and 10% DMSO by controlled rate freezing as demonstrated by a consensus on liver cells cryopreservation (11). After cryopreservation in liquid nitrogen, cells were rapidly thawed in a 37°C water bath and washed twice in a stable solution of plasmatic proteins (85% of albumin) containing bicarbonate (0.84 mg/L), glucose (2.5 g/L) and 10 IU/L heparin. After two centrifugations at 1200 rpm and 4°C, cells were resuspended in the same solution at the desired concentration for injection. Viability of the thawed cells was estimated by TBE method and additional functional tests including intracellular adenosine triphosphate assay and tetrazolium salts reduction test (succinate deshydrogenase activity) were also performed. The infused cell suspensions were constituted by ~95% of albumin positive cells (with hepatocyte morphology) as demonstrated by immunocytochemical studies. In primary cultures, these cells were also able to produce albumin and to synthesize urea as demonstrated by biochemical assays (data not shown).

Cell infusion

The child received 3.5 billion cryopreserved cells (2.4 billion viable cryopreserved cells). The median viability as determined by TBE method was 70% (range: 50–84%). The cells were given in 10 successive infusions over 16 weeks. Each infused cell suspension contained 30 to a maximum of 100 \times 10⁶ cells/kg body weight, to reach 5–10% of the total liver cells number (5,6,12). A first series of seven infusions (71% of total infused cells) was performed during 1 month when he arrived in our unit. Three last infusions

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were performed in 15 days while he came back to hospital for follow-up 2.5 months later.

He was maintained on the same dietary and medical management as before. Nevertheless, the controlled diet was probably not followed during the period between the two steps of infusions. Immune suppression included steroids given at a dose of 1 mg/kg progressively tapered to reach 0.25 mg/kg at 1 month and tacrolimus (Prograft, Fujisawa, Berlin, Germany) to reach a through level of 6–8 ng/mL. Before each cell infusion, intravenous hydrocortisone (10 mg/kg) was administered. The patient was maintained under the same immunosuppressive and metabolic treatment until a post-mortem graft became available 6 months after the first infusion and 2 months after the last one. The post-OLT immunosuppressive treatment included tacrolimus monotherapy according to our immunosuppressive OLT protocol. To evaluate immunization against infused cells, donor-HLA-antibodies were measured 2 months after the end of the infusions just before OLT. The patient had orthotopic transplant 6 months after the first infusion.

Biochemical monitoring

Due to the deleterious effect of hyperammonemia episodes, plasma ammonia was followed on a regular basis before and after infusions. Blood urea nitrogen was monitored as a final product of the urea cycle activity. Additional ammonia measures were done whenever the patient was unwell.

The engraftment of the transplanted cells was not followed on liver biopsies to avoid unnecessary invasive exams, as this therapy was proposed as a bridge to liver transplantation.

The explanted liver was not analyzed for cell engraftment due to lack of informed consent.

Psychomotor development

The patient was evaluated using the early cognitive development rating scales (EEDCP, 2000).

Statistical analysis

The data were analyzed using the Student's t- test (paired).

Results

The infusions were perfectly tolerated without any acute adverse events. Clinical parameters remained within normal range and no important changes in liver function tests were observed (Table 1). Pre- and post-cell-transplant Doppler ultrasound of the portal system and hepatic artery neither show any change of portal pressure nor blood flow.

Before LCT, several episodes of hyperammonemia occurred reaching 548 μ g/dL at birth (7 days of live) and 422 μ g/dL 5 months later. The patient had recurrent episodes of severe psychomotor decompensation, while under diet and treatment. During first month, the patient received a first series of seven infusions with a total of 1.7 billion viable cells. During this period, analysis of blood ammonia (μ g/dL) showed a significant decrease in its mean level 71.4 \pm 7.7 (median: 73; range: 38–131) (n = 32) as compared to the

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Table 1: Ammonia, AST (normal AST laboratory values: 14–63 IU/L) and ALT levels (normal ALT laboratory values: 6–33 lu/L) were followed during the pre-transplant period, first series of infusions, post first series period, second series of infusions and the last period, free of infusions, while waiting OLT. Data are presented as mean ± SD. Slight increase in AST and ALT levels was recorded during the two infusions periods with a maximum of 151 and 130 IU/L, respectively. These values returned to control levels 2 days after the last infusion of each series

	Pre-transplant	1st series	Post 1st series	2nd series	Post 2nd series
Ammonia levels (µg/dL)	118.0 ± 11.7	71.4 ± 7.7	110.9 ± 39.3	82.7 ± 27.5	138.6 ± 4.9
AST (IU/L)	23.5 ± 2.6 (n = 11)	76.0 ± 13.8 (n = 24)	54.6 ± 34.4 (n = 11)	82.8 ± 32.6 (n = 7)	38.5 ± 20.3 (n = 12)
ALT (IU/L)	$36.8 \pm 2.7 \ (n = 11)$	57.7 ± 11.8 (n = 24)	$40.5 \pm 11.9 \ (n = 11)$	59.7 ± 31.6 (n = 7)	$39.3 \pm 7.9 (n = 12)$

mean value before LCT 118.0 \pm 11.7 (p = 0.0048) (median: 96; range: 25–548) (n = 58).

After the first series of infusions, when he left hospital, transient hyperammonemia episodes, clinically well tolerated, up to 263 µg/dL (2 months after the seventh infusion) were detected biochemically, related to decreased compliance to treatment and diet or to possible rejection of the cells (mean ammonia for this period: 110.9 ± 39.3) (median: 101.5; range: 43-263) (n = 13). A second series of three infusions was then made in 15 days. Mean ammonia levels during this period decreased to 82.7 \pm 27.5 with no statistical significance (p = 0.514) (median: 80; range: 41-132) (n = 6). Later, respectively, 5 days and 2 months after the last infusion, two hyperammonemia episodes occurred up to 338μ g/dL during intercurrent infection, being well tolerated clinically (mean ammonia levels of this period: 138.6 \pm 4.9) (median: 110; range: 46–338) (n = 24). During these two episodes, the diet was reduced to 0.5 g/kg until ammonia levels returned again to normal values. The rest of the treatment was not modified.

In parallel, we evaluate the levels of urea as a probable indicator of metabolically active engrafted cells. Urea starts to become detectable 1 month after the first infusion, at the end of the first infusion series (two peaks up to 13 mg/dL). These significant urea levels were maintained up to 2 months before decreasing to reach undetectable values. Importantly, urea levels increased again after the last infusions and reached the highest value of 25 mg/dL. The post-LCT average was 12.3 ± 0.6 (n = 40) as compared to undetectable pre-LCT values (n = 12) (p = 0.0008) (Figure 1).

From the end of the first infusion series, the child psychomotor development markedly improved, according to EEDCP scales, with acquisition of sitting position and speech, which had disappeared earlier. No psychomotor decompensation occurred during post-LCT hyperammonemia episodes unlike pre-LCT episodes.

Following LCT and before the planned OLT, there was no evidence of sensitization to donor HLA's as shown by negative panel reactive antibodies. Six months after first infusion, when a post-mortem graft was available, he was liver transplanted. Following OLT, ammonia and urea levels re-

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Figure 1: Follow-up of urea levels in an OTC deficient patient. In the pre-transplant period, the urea levels were undetectable. The first series of infusions (seven infusions in 1 month) was followed by three additional infusions performed in 15 days (second series of infusions). Infusion of cryopreserved cells is symbolized by a black arrow.

turned within the normal range, and the post-transplant course was uneventful.

Discussion

While waiting for OLT, this patient received safely 0.24 billion viable cryopreserved cells/kg. Cells were infused over a period of 16 weeks in two different series. Single donor origin aimed to avoid immune sensitization in view of the planned OLT.

We demonstrate that cryopreserved cells can be used for elective LCT, and exert metabolic control. Indeed, the first series of infusion (1 month—71% of all infused cells) led to the control of ammonia levels, which remained within normal ranges. This immediate and transient result confirms the data obtained by Horslen et al. (9) using a larger number of cells from different donors as well as prior observations from our center showing that metabolic control can be obtained immediately following infusion of a large cell mass (7).

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Our patient required, however, a second series of infusion when ammonia levels increased again. This secondary loss of effect was probably due to non-permanent engraftment or rejection of a part of the infused cells. Indeed, animal studies have shown immunogenicity of infused murine hepatocytes (13). The increased ammonia levels may also be explained by a decreased compliance to diet following the first infusions, as the patient was not hospital bound in this period. Nevertheless, the port-a-cath access allowed us to plan additional infusions, leading again to normalization of ammonia levels during the infusion period.

Whereas the ammonia levels can be influenced by several parameters, urea synthesis is a more significant indicator of metabolically active engrafted cells. Urea levels increased progressively in blood to reach the highest detected value 5 months after the first infusion. This result indicates that donor liver cells were engrafted and had become metabolically active. This allowed to stabilize the patient further and to protect him against encephalopathic episodes while waiting for OLT.

Previous animal studies have demonstrated that the efficiency of cryopreserved cells can be comparable to that of freshly isolated cells. Fresh or cryopreserved cells resulted in similar decrease in urinary uric acid excretion in dalmatian dogs (14). Liver repopulation with cryopreserved/thawed hepatocytes was demonstrated in mice model (15,16). The cryopreserved cells have also the ability to retain hepatic function and to respond to liver injury as it was demonstrated in immunodeficient mice (17). In humans, Baccarani et al. described use of cryopreserved cells in a bioartificial liver as a source of human liver cells to bridge a patient, affected by fulminant hepatic failure, to emergency liver transplantation (18). Nevertheless, exclusive use of cryopreserved cells was to our knowledge not yet reported in LCT.

We conclude that cryopreserved/thawed hepatocytes can be safely infused to achieve metabolic control. We showed immediate efficacy in ammonia levels and medium-term efficacy in urea synthesis as well as improved psychomotor development. LCT should be considered as a bridge to transplantation for OTC deficiency patients to avoid neurological decompensation.

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4.1.1.1.2 Sustained engraftment and tissue enzyme activity after liver cell transplantation for argininosuccinate lyase deficiency

In this Section (Article 2), we focused on the engraftment capacity of C/T hepatocytes (64% of total infused cells), in parallel to metabolic improvement and psychomotor development. We were able to demonstrate sustained engraftment of donor liver cells in repeated biopsies, correlated with recovery of argininosuccinate lyase activity, originally absent, measurable in different biopsy samples. In parallel, LCT improved the clinical and biochemical conditions of the patient, transforming a severe neonatal to the equivalent of moderate late-onset disease as observed in patients with residual, around 1%, enzyme activity.



Article 2: Sustained engraftment and tissue enzyme activity after liver cell transplantation for argininosuccinate lyase deficiency. Gastroenterology, 2006 Apr; 130(4):1317-23.

CASE REPORT

Sustained Engraftment and Tissue Enzyme Activity After Liver Cell Transplantation for Argininosuccinate Lyase Deficiency

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Background & Aims: Donor cell engraftment with expression of enzyme activity is the goal of liver cell transplantation for inborn errors of liver metabolism with a view to achieving sustained metabolic control. Methods: Sequential hepatic cell transplantations using male and female cells were performed in a 3.5-year-old girl with argininosuccinate lyase deficiency over a period of 5 months. Beside clinical, psychomotor, and metabolic follow-up, engraftment was analyzed in repeated liver biopsies (2.5, 5, 8, and 12 months after first infusion) by fluorescence in situ hybridization for the Y-chromosome and by measurement of tissue enzyme activity. Results: Metabolic control was achieved together with psychomotor catch-up, changing the clinical phenotype from a severe neonatal one to a moderate late-onset type. The child was no longer hospitalized and was able to attend normal school. Sustained engraftment of male donor liver cells was shown in repeated biopsies, reaching 19% at 8 months and 12.5% at the 12-month followup. XXYY tetraploid donor cells were mainly detected during the infusion period (2.5- and 5-month biopsies), whereas in the follow-up 8-month and 1-year biopsies, diploid donor cell subpopulations had become dominant. Moreover, argininosuccinate lyase activity, originally absent, became measurable in 2 different biopsy samples at 8 months, reaching 3% of control activity, indicating in situ metabolic effect and supporting the clinical evolution to a moderate form of the disease. Conclusions: Liver cell transplantation can achieve donor cell engraftment in humans in a significant proportion, leading to sustained metabolic and clinical control with psychomotor catch-up.

L iver cell transplantation (LCT) can provide partial metabolic control in various human inborn metabolic diseases.¹⁻⁴ Beside the demonstration of a metabolic effect and its durability, the challenge of LCT in man is the demonstration of long-term engraftment and repopulation of the recipient's liver by donor hepatocytes expressing the deficient enzyme in parallel to metabolic improvement.

In urea-cycle disorders, the neurologic outcome after orthotopic liver transplantation (OLT) is closely related to sequelae of hyperammonemic episodes occurring before transplantation.^{5,6} Waiting time on the pretransplant list currently exceeds 1 year in the Eurotransplant network, and LCT may avoid further brain damage caused by recurrent hyperammonemia episodes.⁷ Such metabolic control can be achieved with cryopreserved cells, making scheduled sequential infusions possible.⁸ In neonatal onset argininosuccinate lyase (ASL) deficiency, patients have an absence of enzyme activity, being at particular risk of hyperammonemic brain damage, whereas patients with late-onset ASL deficiency express residual activity and are at lower risk.

Hereafter, we report long-term chimerism of donor and recipient hepatocytes after LCT in a neonatal form of ASL deficiency, with parallel phenotypic changes in metabolic control, clinical control, and psychomotor catchup.

Patient and Methods

Case

A 42-month-old girl weighing 13.5 kg had ASL deficiency with secondary psychomotor retardation because of recurrent episodes of hyperammonemia. The diagnosis of ureacycle disorder was established shortly after birth after hyperammonemic coma (NH₄⁺: 700 μ g/dL, normal levels <125 μ g/dL) associated with significant argininosuccinic acid (ASA) levels in serum and urine. Complete absence of ASL activity

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Abbreviations used in this paper: ASA, argininosuccinic acid; ASL, argininosuccinate lyase; CK-7, cytokeratin-7; FISH, fluorescence in situ hybridization; LCT, liver cell transplantation; OLT, orthotopic liver transplantation.

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was confirmed on a liver biopsy (Laboratoire de Biochimie Médicale, Hopital Necker, Paris, France). There was no histological fibrosis.

The child was treated by appropriate dietary protein restriction (1 g/kg/d), phenylbutyrate (300 mg/kg/d) (Ammonaps; Orphan Europe, Paris, France), and arginine supplementation (3×500 mg/d), but she still experienced episodes of hyperammonemia with peaks >400 µg/dL during her 3 first years of life. The child was registered on the Eurotransplant liver transplant waiting list, and LCT was considered with the aim to improve metabolic control and avoid further brain damage while waiting for a graft.

The hospital ethical review board approved the project. No contraindication to LCT was found.

Cell Handling

Liver cells were isolated from 3 different cadaveric donor livers as previously described.^{3–8} Viability estimated by the trypan blue exclusion technique reached 81% to 90%. Other quality control tests of fresh and cryopreserved cells were performed as reported earlier.⁸

Cells were infused when either fresh or after cryopreservation and thawing.⁸ A Port-a-Cath system (Smiths Medical, St Paul, MN) was placed in the portal system for easy access and sequential infusions.⁹

First series of infusions. The patient received 1.7 billion cryopreserved/thawed cells from a 19-year-old male postmortem donor liver in 7 infusions over 1 month.

Second series of infusions. Two and a half months from the first infusion, the patient received the last cell infusion from the first liver (0.3 billion cells). Furthermore, the next day, she received 0.7 billion fresh female liver cells obtained from a 55-year-old postmortem donor.

Third series of infusions. Two months later, because the Port-a-Cath was no longer in the portal system, the patient received under direct portal percutaneous puncture 1 billion fresh cells from a male 8-year-old postmortem donor. To reach 9% of her total hepatic mass, during surgery performed to remove the Port-a-Cath, she received a last infusion of 1 billion cryopreserved cells obtained from the same donor (last infusion of the third series) 1 week later.

The total number of transplanted cells thus reached 0.35 billion/kg infused over \sim 5 months. All infusions were well tolerated. Doppler ultrasound of the portal system did not show any flow change.

Immune suppression treatment included tacrolimus (blood through level of 6 to 8 ng/mL) and prednisolone, progressively tapered to reach 0.25 mg/kg/d, according to the local OLT transplant protocol. Before each cell infusion, intravenous hydrocortisone (10 mg/kg) was administered. The pre-LCT metabolic treatment and diet were maintained during the whole posttransplant period.

Biochemical Monitoring

The ammonia level in plasma was measured by using an ammonia reagent by a timed endpoint method (Synchron LX System, Fullerton, CA) (nl $<125 \ \mu g/dL$). Blood and urine ASA were analyzed by ion (cation)-exchange resin chromatography. Liver function tests and alpha-fetoprotein levels were routinely followed up.

Liver Biopsies: Fluorescence In Situ Hybridization

Liver biopsies (n = 5) were performed 1, 2.5, 5, 8, and 12 months after the first infusion (3 and 7 months after last infusion for the 2 latter biopsies). The fluorescence in situ hybridization (FISH) analysis was performed on liver-biopsy fingerprints mounted on slides. Transplanted male cells were detected by using a probe specific to the SRY gene labelled with a SpectrumOrange (LSI SRY), with a CEP X SpectrumGreen controle probe, and centromeric probes specific to autosomes (CEP12, CEP16, and CEP18; Vysis Product, Downers Grove, IL). Hybridized slides were examined by using either a DMRB microscope (Leica) or an axioplan 2 microscope (Zeiss, Berkshire, UK) equipped with single-bypass filters for excitation of DAPI (Sigma, Bornem, Belgium), FITC, and rhodamine; double bypass filters for excitation of FITC/rhodamine; and triple bypass filters for excitation of DAPI/FITC/rhodamine. Images were captured by using a Photometrics (Downingtown, PA) camera and processed by Isis software (Metasystem, Germany). For each hybridization, 200 cells in interphase were analyzed. The cutoff level for each probe was determined by scoring 200 intact nuclei in biopsies obtained from 4 healthy donors plus 1 biopsy of the patient before LCT to set the baseline based on the mean plus 3 standard deviations. The thresholds of detection for the 1-, 2.5-, and 5-month biopsies (hybridization with probes for chromosomes X and Y) were 4.0% for XY cells, 6.9% for XXY cells, and 2.1% for XXYY cells, respectively.

To evaluate cell ploidy, the 8-month and 1-year biopsies were incubated with probes specific for the X, Y, and 18 chromosomes. The thresholds of detection were 4.2% for 18,18, XXY and 0% for 18, 18, XXYY subpopulations. According to previous protocols,¹⁰ albumin and cytokeratin-7 (CK-7) staining was performed on liver biopsy fingerprints to determine the hepatocyte lineage of the cells found on the fingerprints.

Tissue Enzyme Activity

The ASL activity was evaluated on hepatic biopsies by conversion of arginine (Sigma) and [¹⁴C]-fumarate (Sigma) in [¹⁴C]-ASA. [¹⁴C]-fumarate and [¹⁴C]-ASA were separated by exchange resin chromatography and the [¹⁴C]-ASA quantified. A normal ASL activity was measured as a positive control of the reaction, whereas argininosuccinate synthase activity was evaluated as a control of quality of the biopsy.

Psychomotor Development

The patient's psychomotor development was evaluated by the same psychologist before and after LCT in the pediatric neurology unit using the early cognitive development rating scales (2000).



Statistical Analysis

The data were analyzed using the Student t test (unpaired test for post-LCT "ammonia level population" compared with pre-LCT "ammonia level population," mean \pm standard deviation).

Results

Metabolic Follow-up

Ammonia levels. Three severe reported episodes of hyperammonemia (>400 μ g/dL) occurred before LCT, even under appropriate metabolic treatment (Figure 1). Mean ammonia levels obtained during the 3.5 years before infusions were 249.37 ± 173.09 μ g/dL (n = 16) and decreased after LCT to 118.27 ± 50.18 μ g/dL (n = 81) (P < .0001) (Figure 1).

During the infusion period, ammonia levels remained within the normal limits (average ammonia levels: 99.62 \pm 41.52 µg/dL, n = 48, P < .0001), except for 1 value up to 305 µg/dL clinically well tolerated during an infective episode. After the last infusions, ammonia levels fluctuated during 4 months (average ammonia levels: 165.25 \pm 51.51 µg/dL, n = 20, P = .0462). For the last 6-month follow-up period, ammonia levels eventually remained within the normal laboratory limits range even during a gastroenteritis episode (average ammonia levels; 114.85 \pm 51.56 µg/dL, n = 13, P = .0101).

Blood and urine argininosuccinic acid. There were no statistically significant differences in blood and urine mean ASA levels: serum levels changed from 389 \pm 138.9 µmol/L (n = 4) to 244.5 \pm 123.8 µmol/L (n = 17, **P** = .0626), whereas urine levels changed from 14,246.50 \pm 775.30 mmol/mol creatinine (n = 2) before infusions to 12,311.73 \pm 6347.79 mmol/mol creatinine (n = 11) after infusions.

Liver-function tests and alpha-fetoprotein. Liver tests, serum aspartate aminotransferase, and alanine aminotransferase were slightly increased before infusions $(1.5 \times \text{upper limit of normal})$. No significant alterations were noted in liver-function tests, and alpha-fetoprotein levels remained within normal values throughout the study period (data not shown).

FISH and Enzyme Activity

Infusion period. FISH analysis did not detect Ypositive cells on the 1-month biopsy. XX/XXYY chimerism appeared on the 2.5-month biopsy, with 4.7% Y-positive cells (blank 2.1%) (Figure 2A and B). Secondary hybridization with probes for autosomes 12 and 16 shows by position correlation the tetraploid (4N) status of those cells (Figure 2C). Five months after the first infusion, 4N XXYY cell population was 5.5% (blank 2%) and a marginal percentage of 2N XXY cells appeared. Y-chromosome real-time semiquantitative polymerase chain reaction confirmed presence of the Y chromosome (data not shown).

Follow-up (infusion free) period. Three months after the last infusion, 2N XXY cells reached 14% (vs blank: 4.2% with triple hybridization) and 2N XXYY cells 5% (vs blank: 0%) (Figure 3A and B). Those 2



Figure 2. (A and B) FISH Y chromosome on liver cells obtained by biopsy performed 2.5 months post-LCT in the ASL-deficient patient showing 4.7% of XXYY cells. Red: SRY (chromosome Y) and green: DXZ1 (chromosome X-CEP X). (C) Hybridization with centromeric probes specific of autosomes (CEP12, CEP16) of the 2.5-month biopsy fingerprints where XXYY cells were found in significant proportion, contiming the tetraploid status of XXYY cells. Red: CEP12 (chromosome 12) and green: CEP16 (chromosome 16).

subpopulations had 1 pair of chromosomes 18. Albumin and CK-7 staining on liver biopsies showed that 95% of the analyzed cells were albumin positive and CK-7 negative (Figure 4A). Five percent of the analyzed cells were albumin negative and CK-7 positive (Figure 4B). Further FISH analysis showed that by position correlation, the Y-positive cells were albumin positive and CK-7 negative, confirming their hepatocyte lineage. The last biopsy, taken 1 year after the first infusion (7 months free from infusion), showed durable presence of 11% 2N XXY cells (vs blank: 4.2%) and 1.5% 2N XXYY cells (vs blank: 0%).

FISH on donor cells. FISH for chromosomes X, Y, and 18 was also applied on the male-infused liver cells showing 8% tetraploidy from the first male donor. Tetraploid cells were absent from the second male donor. No Y-positive cells were found in the infused female liver cells.

Tissue enzyme activity. Liver-tissue ASL activity was measured on 2 samples of the 8-month biopsy whose fingerprints had shown the Y-positive FISH staining. ASL activity reached 0.15 and 0.34 nmol/ $L \cdot min^{-1} \cdot mg^{-1}$, respectively (vs 11.70 and 9.00 nmol/ $L \cdot min^{-1} \cdot mg^{-1}$ as ASL positive controls; ie, 1.3% and 3.8% of control activity). Argininosuccinate synthase activity was measured as quality control of the biopsy and reached normal values of 12.80 and 11.10 nmol/ $L \cdot min^{-1} \cdot mg^{-1}$, respectively, similar to controls. The ASL activity of the 1-year biopsy again reached 2.6% of control activity (0.78 vs 30.4 nmol/L \cdot min⁻¹ \cdot mg⁻¹ as ASL positive control). The pre-LCT ASL activity was undetectable, whereas argininosuccinate synthase activity was normal (6.90 nmol/L \cdot min⁻¹ \cdot mg⁻¹).

Clinical Evolution and Psychomotor Development

The child's psychomotor development was markedly impaired before LCT; upright position was acquired at the age of 20 months and she had no acquisition of speech. Her developmental age at 3.5 years was evaluated at 20 months by using the Psyche Educational Profile Revised (PEP-R) test for global motility, language, personal-social behavior, and adaptive behavior.

After LCT, psychomotor development improved remarkably according to both parents and the nursing team. Within the year after infusions, the child acquired fecal and urinary continence, was able to dress alone, was able to perform some difficult puzzle exercises, and entered elementary school. Psychomotor evaluation (PEP-R) performed by the same psychologist, 14 months after the first infusion, showed a 13-month catch-up in 8 months time for global evaluation and an 18-month catch-up in 8 months time for evaluation of items in development.

Discussion

The most important finding of this study is the demonstration of successfully engrafted donor hepatocytes in 4 successive biopsies taken up to 1 year after the first infusion and up to 7 months after the last infusion. The high percentage and durable presence of Y-positive cells are significant achievements in comparison with results obtained in successful animal studies without artificial regenerative stimulus.^{11,12} Moreover, the obtained percentage of engrafted donor cells measured does not account for the female donor cells infused, not detectable by the FISH technique used. This is the first



Figure 3. (A and B) FISH Y-18 chromosomes on liver cells obtained by biopsy performed 8 months post-LCT in the ASL-deficient patient indicating a significant percentage of 2N XXY, 2N XXYY. Red: SRY (chromosome Y), green: DXZ1 (chromosome X-CEP X), and blue: CEP 18 (chromosome 18).

Figure 4. (A) Albumin (in green) and CK-7 (in red) staining of cell fingerprints from Y-positive biopsies indicating a 95% panel of albumin-positive, CK-7 negative cells (4',6-diamidino-2-phenylindole nuclei staining in blue). (*B*) Five percent of cells being albumin negative and CK-7 positive on cell fingerprints from Y-positive biopsies (4',6-diamidino-2phenylindole nuclei staining in blue).



demonstration with a direct proof of long-term liver cell engraftment in humans. Human studies have reported indirect arguments of around 1% of engrafted donor cells, more precisely donor DNA, in early posttransplant biopsies by using donor Y-real-time polymerase chain reaction in a Refsum patient and short tandem repeats analysis in 2 adult patients with acute hepatic failure.^{3,13} These observations were made soon after the infusions and did not enable conclusions to be drawn concerning stable and long-term engraftment.

It was important to confirm in our patient that engrafted cells had a hepatocytic phenotype because infused human liver cells are a mixed suspension of different cell types.^{14–19} Coimmunostaining showed that cells found on several biopsies were predominantly albumin positive, CK-7 negative, and had a hepatic morphology.

Transplanted adult rat hepatocytes in mixed suspension can rapidly engraft, maintain normal function, and survive in the liver up to 1 year after LCT.²⁰ These cells maintain a growth potential and are able to replicate in the recipient's liver in an animal model.²¹ Mouse adult hepatocytes are capable of repopulation with up to 69 cell doublings without loss of function and without loss of differentiation or dysplasia.²²

We detected a significant percentage of tetraploid cell population in the successive biopsies taken 2.5 and 5 months after the first infusion. A second diploid subpopulation XXY appeared at month 5 and became more prominent in the last 2 biopsies, whereas the 4N cells disappeared. Diploid XXYY-accompanying cells emerged in the 8-month and 1-year biopsies. Epstein²³ showed that up to 80% of the nuclei of adult mammalian liver parenchymal cells are polyploid. Beginning in young adulthood, average hepatocyte DNA content increases because of polyploidization, whereas diploid hepatocytes become a minority. Polyploidization is a general strategy of cell growth that enables an increase in metabolic output, cell mass, and cell size.24,25 This may constitute an alternative to cell division. Weglarz et al26 showed also that mouse polyploid hepatocytes are potential progenitors for regenerating foci in damaged liver after LCT. Furthermore, in the hereditary mouse model of tyrosinemia, small hepatocytes separated by cell density have lower repopulation capacity compared with medium-sized and large cells, showing that regular, binucleated, and large-differentiated hepatocytes have the best repopulation potential.27 Tetraploid hepatocytes found in significant proportions in donor cells before and after LCT may suggest that these cells' suspension have an engraftment advantage. However, this hypothesis is not supported by other studies in animal models after regenerative stimuli.28,29 The 2N XXY and 2N XXYY cells found in our patient, after cell infusions from 2 male livers, could occur from division of the tetraploid cells obtained from the first male-infused cells. Another possibility is that we miss the tetraploid genotype of those 2 subpopulations because of technical limitations. We cannot exclude either that the 4N XXYY cells and the 2N XXY/2N XXYY cell mosaicism found later were not obtained by fusion between host and male donor cells. However, there is no indication from animal studies that mature hepatocytes fuse with native host cells.

In parallel, LCT significantly improved the child's clinical and biochemical conditions. Ammonia levels decreased significantly during the infusion period except during an intercurrent infective episode. This early effect was interpreted as being linked to the important infused cell mass, as already observed in our previous Refsum patient.³ Ammonia levels afterwards fluctuated during 4 months, possibly because of the decrease of immediate mass effect.^{3,8} Later on, 9–15 months after the first infusion, ammonia levels remained permanently below the toxicity threshold, even during an episode of gastro-

enteritis, suggesting efficient engraftment and repopulation. These results correlated with the detection of tissue enzyme activity, which was sufficient to maintain ammonium levels within the normal values, whereas ASA was still produced in significant amounts by a recipient's liver cells.

The patient's clinical condition was transformed from a severe neonatal form to the equivalent of moderate late-onset disease as observed in patients with residual enzyme activity, which could also be measured around 1%.^{30,31} These late-onset patients, with normal psychomotor development, also excrete high concentrations of ASA in urine, leading authors to conclude that this compound is not responsible for the neurotoxicity.^{30,31} Nevertheless, other reports state that despite ammonium metabolic control, the long-term neurologic outcome of ASL-deficient patients remains unsatisfactory,³² justifying the planned OLT.

Furthermore, liver histology of an ASL-deficient patient shows often diseased pale hepatocytes with abnormal glycogen deposition and progressive fibrosis.³⁵ The success of LCT in this particular case of ASL may possibly be explained by a proliferative advantage of the transplanted cells on the diseased host hepatocytes.

In conclusion, the FISH technique can be applied to show engraftment after LCT in cases of sex-mismatched LCT.^{34,35} LCT in humans can procure persisting engraftment of transplanted cells parallel to tissue enzyme activity, transforming a severe disease into a moderate form of this urea-cycle disorder. This led to significant psychomotor and clinical progress in our patient. Future developments should aim to increase engraftment by procuring selective advantage to the transplanted cells in human LCT.

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4.1.1.2 *In vitro* quality of cryopreserved/thawed hepatocytes

4.1.1.2.1 Cryopreservation of human hepatocytes alters the mitochondrial respiratory-chain complex 1

The dramatic reduction in ATP levels observed following C/T in human hepatocytes used for LCT recipient patients led us to study in detail mitochondrial functions, this key cellular organelle being the principal ATP producer under aerobic conditions. We demonstrated in this Section (Article 3) a functional and morphological impairment of mitochondria following C/T. These changes could explain the cytosolic release of the pro-apoptotic protein cytochrome c in cryopreserved cells, the probable starting-point of cellular death which occurs within hours following thawing.



Article 3: Cryopreservation of human hepatocytes alters the mitochondrial respiratory-chain complex 1. Cell Transplantation, 2007; 16(4):409-19.



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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 × 106 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 HCO₃⁻, 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 serumcontaining Williams' E medium.

Determination of Cell Viability

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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 (4.5×106/ml) were incubated in a shaking water bath at 37°C in closed vials containing Williams' E medium saturated with a mixture of O2/CO2 (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 (JO2) 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-1,4phenylenediamine (TMPD) plus 5 mM ascorbate (substrate of the mitochondrial respiratory chain complex 4). For permeabilized hepatocytes, intact cells (106/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. JO2 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 nonphosphorylating (state 4) and the uncoupled mitochondrial respiratory rate, and the maximal activity of cytochrome oxidase, respectively. In all experiments, the antimycinsensitive JO_2 , which corresponds to mitochondrial respiration, was calculated by subtracting the antimycininsensitive 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 μ M Rh 123 and with or without 300 μ M 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 $(2.5 \times 10^6/\text{ml})$ 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 (Ca²⁺) 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 Ca²⁺ were successively added at 2-min intervals until the opening of the PTP, as indicated by the release of Ca²⁺ in the medium.

Determination of Cell Death

Western Blot Analysis. After cell fragmentation using the digitonin method (13), cytosolic and mitochondrial proteins (50 μ g) 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 μ g) 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 \pm 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 \pm 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 JO2 of fresh and cryopreserved/thawed hepatocytes from mice and humans (Table 2). Under basal conditions (i.e., in the absence of any drug addition), JO2 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 JO2 was reduced following C/T in both species $(7.4 \pm 2.3 \text{ vs. } 17.5 \pm 2.9 \text{ nmol } O_2$ min-1 106 cells-1 in mice and 3.3 ± 0.5 vs. 7.8 ± 1.2 nmol $O_2 \text{ min}^{-1} 10^6 \text{ cells}^{-1}$ 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

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	$JO_2 \text{ (nmol } O_2 \text{ min}^{-1} 10^6 \text{ cells}^{-1}\text{)}$			
	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	$4.2 \pm 1.1^{*}$	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 $(4.5 \times 10^6 \text{ cells/ml})$ from human or mouse were incubated in closed vials at 37°C in Williams' E medium saturated with a mixture of O₂/CO₂ (19:1). After 10 min, oxygen consumption rate (*J*O₂) 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 *J*O₂ 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 TMPDascorbate.

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

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	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 \pm 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 Ca²⁺ pulses until induction of permeability transition, as assessed by fast release of Ca²⁺. In fresh murine and human cells, in situ mitochondria took up and retained Ca²⁺ (Fig. 3A, C, trace a). In the presence of CsA, the Ca²⁺ 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 $4.3 \pm$ 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 Ca²⁺ pulses were added every 2 min (arrows) until opening of PTP, as observed by the release of Ca²⁺ into the medium. Typical experiments are shown in (A) and (C) and comparison of the effect of cryopreservation/thawing on the Ca²⁺ retention capacity of permeabilized mouse hepatocytes is presented in (B) and (D) (filled bars, basal; open bars, cyclosporin A) as mean \pm SEM (n = 3). *p < 0.05 compared with fresh cells, #p < 0.05 compared with basal.



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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 \pm 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 \pm 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 \pm 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 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-tointracellular 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. Indeed, 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 ATPdependent 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 (ΔΨ 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 postthawing 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 ischemiainduced 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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4.1.1.2.2 Kinetics study of complex 1 impairment during the cryopreservation process

4.1.1.2.2.1 Introduction

Crystallisation starts around -20° C, which constitutes the nucleation point. Work from Diener et al. showed that around this temperature, the cell suspension becomes supercooled, the latent heat of fusion is released and the cell sample is warmed (35) which may be deleterious for the cell quality. We studied the kinetics of the mitochondrial damage described in Section 4.1.1.2.1 by evaluating intracellular ATP levels, trypan blue viability, and oxygen consumption of thawed mouse hepatocytes after the -20° C or -80° C step of the cryopreservation process. The results were compared to those obtained using freshly isolated cells and C/T hepatocytes data.

4.1.1.2.2.2 Results and discussion

Mouse hepatocytes were isolated and cryopreserved according to the protocols described in Section 4.1.1.2.1. The freezing-protocol of hepatocytes consisted of 20 min at -20° C followed by 2 h at -80°C before storage in liquid nitrogen. After the -20° C step, -80° C step and cryopreservation-storage for 24 hours in liquid nitrogen, the hepatocytes were thawed.

As shown in Tables 3-6, the mitochondrial damage following C/T, ATP depletion, oxygen consumption and complex 1 impairment, was already detectable after the -20° C step, and of course thawing (Tables 5-6-7-8). A dramatic decrease in intracellular ATP concentration was indeed observed following the -20° C and -80° C steps (Table 5). Under basal conditions (i.e., in the absence of any drug addition), oxygen consumption rate (*J*O2) of -20° C and -80° C C/T hepatocytes was significantly reduced compared with fresh cells (Table 6). This difference was not present after addition of oligomycin. The addition of 2, 4 dinitrophenol (DNP) induced stimulation of *J*O2 in freshly isolated cells, but this uncoupling effect was abolished following -20° C and -80° C C/T. The maximal activity of cytochrome c oxidase was measured by recording *J*O2 in the presence of saturating concentrations of N, N, N', N'-tetramethyl-1,4-phenylenediamine (TMPD)/ascorbate and after addition of antimycin. No decrease in the mitochondrial respiratory chain complex 4 activity was observed following -20° C and -80° C C/T.

In the presence of glutamate/malate, a significant decrease in mitochondrial state 4 JO2 (i.e., in non phosphorylating condition) was evidenced following -20° C and -80° C C/T (Table 7). This lower mitochondrial JO2 persisted in the presence of ADP (state 3) or in an uncoupled state (DNP addition). However, as already found in intact cells, -20° C and -80° C 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 8). However, state 3 and the uncoupled state remained affected in -20° C and -80° C C/T hepatocytes.

These results suggest that the cryopreservation damage occurred early in the process, around the nucleation point and IIF. IIF may also occur during the thawing process. Cellular impairments could therefore possibly be explained by mechanical alteration of mitochondria induced by water crystallisation during the cryopreservation process or thawing procedure.

	ATP (nmol.10 ⁶ cells ⁻¹)	Trypan Blue (% viability)	
Fresh	2.7 ± 0.8	85.4 ± 5.6	
Cryopreserved	0.1 ± 0.0 *	59.5 ± 3.3*	
-20°C	0.1 ± 0.0 *	69.4 ± 5.2*	
-80°C	0.1 ± 0.0 *	61.5 ± 4.3*	

Table 5: Evaluation of cellular viability of freshly isolated and cryopreserved/thawed ($-20^{\circ}C$, $-80^{\circ}C$, liquid nitrogen) mouse hepatocytes

Results are expressed as mean \pm S.E.M. (n=5). *p<0.05 compared with fresh cells.

	Basal	Oligomycin	DNP	TMPD/Asc
Fresh	18.3 ± 4.5	7.3 ± 1.2	30.8 ± 4.5	130.7 ± 15.9
Cryopreserved	8.1 ± 2.3*	4.9 ± 1 .7	7.1 ± 2.3*	125.6 ± 13.6
-20°C	10.3 ± 3.5*	5.3 ± 1.6	11.1 ± 3.5*	129.3 ± 12.6
-80°C	7.5 ± 1.7*	4.8 ± 0.9	7.3 ± 3.3*	120.7 ± 9.3

Table 6: Oxygen consumption rate of freshly isolated and cryopreserved/thawed (-20°C, -80°C, liquid nitrogen) mouse hepatocytes

Freshly isolated and cryopreserved/thawed human hepatocytes (4.5 10^6 cells/ml) were incubated in closed vials at 37 °C in Williams' E medium saturated with a mixture of O₂/CO₂ (19:1). After 10 min, oxygen consumption rate (JO_2 , nmol O₂.min⁻¹.10⁶ cells⁻¹) was measured in an oxygraph vessel coupled with Clark electrode before and after the successive additions of 0.5 µM oligomycin, 150 µM DNP, 0.15 µg/ml antimycin and 1 mM TMPD+5 mM ascorbate. The antimycin-sensitive JO_2 was calculated and the results expressed as mean ± S.E.M. (n=3). * p<0.05 compared with fresh cells.

	State 4	State 3	DNP	TMPD/Asc
Fresh	15.6 ± 3.7	40.9 ± 7 .4	38.8 ± 2.3	199.6 ± 17.8
Cryopreserved	6.5 ± 2.1*	13.9 ± 7.4*	12.6 ± 4.1*	183.2 ± 15.8
-20°C	8.3 ± 3.3*	18.9 ± 6.8 *	15.5 ± 3.2*	189.1 ± 13.6
-80°C	5.9 ± 2.0*	13.8 ± 2.3*	11.6 ± 3.9*	191.6 ± 10.8

Table 7: Oxygen consumption rate in permeabilized mouse hepatocytes (freshly isolated and cryopreserved/thawed (-20° C, -80° C, liquid nitrogen)) energised by glutamate/malate.

Permeabilization of freshly isolated or cryopreserved hepatocytes (10^6 cells/ml) was achieved by digitonin after a 10min pre-incubation in Williams' E medium as described in legend of Figure 3. Permeabilized cells were then incubated in the presence of 5 mM glutamate + 2.5 mM malate. The mitochondrial respiratory rate (JO_2 , nmol O_2 .min⁻¹. 10^6 cells⁻¹) was measured before and after the successive additions of 1 mM ADP/Tris, 0.5 μ M oligomycin, 50 μ M DNP, 0.15 μ g/ml antimycin and 1 mM TMPD+5 mM ascorbate. The antimycin- and oligomycin-sensitive JO_2 was calculated and the results expressed in nmol O_2 .min⁻¹. 10^6 hepatocytes⁻¹ as means \pm S.E.M. (n=3). * p<0.05 compared to fresh cells.

	State 4	State 3	DNP	TMPD/Asc
Fresh	35.1 ± 7.9	62.9 ± 9.6	69.3 ± 15.3	250.3 ± 6.6
Cryopreserved	31.5 ± 5.8	55.2 ± 6.3	30.4 ± 4.6*	252.8 ± 11.2
-20°C	32.3 ± 4.7	50.7 ± 4.9	29.6 ± 3.5*	210.8 ± 7.2
-80°C	34.8 ± 3.5	57.3 ± 5.9	28.3 ± 7.1*	215.1 ± 6.8

Table 8: Oxygen consumption rate in permeabilized mouse hepatocytes (freshly isolated and cryopreserved/thawed (-20° C, -80° C, liquid nitrogen)) energised by succinate/malate.

Permeabilization of freshly isolated or cryopreserved hepatocytes (10^6 cells/ml) was achieved by digitonin after a 10min pre-incubation in Williams' E medium as described in legend of Figure 3. Permeabilized cells were then incubated in the presence of 5 mM succinate + 0.5 mM malate + 1.25 μ M rotenone. The mitochondrial respiratory rate (JO_2 , nmol O_2 .min⁻¹.10⁶ cells⁻¹) was measured before and after the successive additions of 1 mM ADP/Tris, 0.5 μ M oligomycin, 50 μ M DNP, 0.15 μ g/ml antimycin and 1 mM TMPD+5 mM ascorbate. The antimycin- and oligomycin-sensitive JO_2 was calculated and the results expressed in nmol O_2 .min⁻¹.10⁶ hepatocytes⁻¹ as means \pm S.E.M. (n=3). * p<0.05 compared to fresh cells. 4.1.1.3 In vivo evaluation of cryopreserved/thawed hepatocytes

4.1.1.3.1 Introduction

Since data from the literature are conflicting about the post-thawing quality of C/T hepatocytes *in vivo*, we evaluated their potential to engraft in an animal model of LCT (metabolic disease) in comparison with freshly isolated hepatocytes. In a mdr2 -/- mice model, corresponding to the progressive familial intrahepatic cholestasis type 3 (MDR3 deficiency), we evaluated the potential of C/T hepatocytes to produce phospholipids in bile (33,60).

4.1.1.3.2 Material and Methods

4.1.1.3.2.1 Animals

See Material and Methods (Section 4.1.1.2.1).

Mice from the mdr2-/- strain (gift from Professor Elferink–Amsterdam Liver Center, Amsterdam, Netherlands) were used at 8-12 weeks of age in LCT recipients.

4.1.1.3.2.2 Isolation and cryopreservation/thawing of mouse hepatocytes

See Material and Methods (Section 4.1.1.2.1). C/T hepatocytes used for transplantation showed a marked decrease in ATP concentration as compared to freshly isolated cells.

4.1.1.3.2.3 Intrasplenic hepatocyte transplantation

Freshly isolated or C/T ($2*10^6/200 \ \mu l$ PBS) mouse hepatocytes, from wild-type mice were transplanted via intrasplenic injection on 8 to 12 week old female mdr2 -/- mice (n=3). Mice transplanted with physiological serum were used as negative controls.

4.1.1.3.2.4 Phospholipids dosage

We evaluated the bile phospholipids levels using a phospholipids B assay kit (Wako Chemicals, Neuss, Germany) at four weeks post-transplantation. Total cholesterol levels were measured by a routine technique using a Beckman automatic analyzer (Beckman, Namur, Belgium).

4.1.1.3.3 Results and discussion

As reported in Table 9, in the MDR3 equivalent mdr2 -/- mice model, we were able to detect phospholipids in the bile samples from the freshly isolated hepatocytes transplanted mice (14.3 +/-

	Phospholipids (nmol/min/100g)	Total cholesterol (nmol/min/100g)	
Wild type	42.1 ± 2.3	0.4 ± 0.1	
mdr2 -/- Tx Physio	$0.0 \pm 0.0*$	0.4 ± 0.2	
mdr2 -/- Tx fresh	14.3 ± 1.0*	0.3 ± 0.1	
mdr2 -/- Tx C/T	$0.0 \pm 0.0*$	0.4 ± 0.1	

1.0 vs 0 nmol/min 100g tissue in negative control (physiological serum transplanted mice), 34% of positive control (wild type mice)), but not with C/T hepatocytes transplanted mice.

Table 9: Biliary total cholesterol and phospholipids levels in freshly isolated or C/T hepatocytes transplanted mdr2 -/- mice

Our results confirmed the poor quality of the C/T hepatocytes *in vivo* when compared to freshly isolated cells. In the mdr2-/- mice model, C/T hepatocytes were unable to allow recovery of the deficient function, i.e. production of phospholipids in bile.

4.1.1.4 Improvement of mitochondrial parameters impaired after cryopreservation/thawing

4.1.1.4.1 Evaluation of cryopreservation medium supplements

4.1.1.4.1.1 Introduction

Based on the results presented in Section 4.1.1.2.1, we first evaluated if C/T induced mitochondria damage could be prevented by supplementation of the cryopreservation medium with pharmacological agents. We tested a complex 1 protector, anti-oxidant molecules, pore transition permeability inhibitors or hyperosmotic solutions. Ginkgo biloba extract demonstrates anti-ischemic properties attributable to the terpenoid fraction, mainly due to the presence of bilobalide. By protecting complex I and III activities, bilobalide allows mitochondria to maintain their respiratory activity under ischemic conditions as long as some oxygen is present, thus delaying the onset of ischemia-induced damage (12,62-64). We also evaluated the influence of potential cryoprotectants added to the cryopreservation medium: anti-oxidant molecules, as complex 1 is a major reactive oxygen species (ROS) producer, (ascorbic acid, N-acetyl-L-cysteine) (45); mitochondrial permeability transition pore inhibitors (Ciclosporin A) (95) or hyperosmotic sucrose (50, 100 and 200 mM) (125,126) to dehydrate cells before the critical –20°C temperature.

Hepatocytes were isolated and cryopreserved according to the protocols described in Section 4.1.1.2.1. Isolation and/or cryopreservation media were supplemented by bilobalide (1 μ g/ml). We also tested the effect of 1 hour bilobalide (1 μ g/ml) pre-incubation at 37°C following the isolation procedure on the C/T induced damage. Hyperosmotic sucrose (50, 100 and 200 mM), anti-oxidant molecules such as ascorbic acid (1mM) and N-acetyl-L-cysteine (5 mM) or permeability transition pore inhibitors (Ciclosporin A 0.1 μ M) were added to the cryopreservation medium. Freshly isolated and C/T hepatocytes with non supplemented media were used as controls. Intracellular ATP levels and *J*O2 in both intact and permeabilized hepatocytes were evaluated according to the protocols described in Section 4.1.1.2.1.

4.1.1.4.1.2 Results and discussion

We failed to see any improvement in mitochondrial parameters by the inclusion of bilobalide irrespective of the conditions used, drug in isolation, pre-culture and/or cryopreservation media (Tables 10-11-12-13).

In addition, no improvement in intracellular ATP was observed following the addition of other "cryoprotectants" to the cryopreservation medium (Table 10). *J*O2 of intact hepatocytes remained affected by C/T with all the "cryoprotectants" used (Table 11). State 4, state 3 and uncoupled state of permeabilized hepatocytes energized by glutamate/malate were also decreased after C/T in all the tested conditions (Table 12). When mitochondria were energized by succinate/malate, no difference was observed in state 4 respiratory rate (Table 13). However, state 3 and the uncoupled state were affected in supplemented cryopreservation medium C/T hepatocytes.

By using classical methods of C/T recommended in the literature, we failed to improve the mitochondrial parameters. In conclusion, based on our preliminary results, none of the proposed cryoprotectants, avoided the C/T related impairments of hepatocytes. A mechanical alteration due to IIF is probably responsible for the observed damages.

	ATP (nmol.10 ⁶ cells ⁻¹)	Trypan blue (% viability)
Fresh	3.2 ± 0.9	89.5 ± 2.4
Cryopreserved	0.1 ± 0.0 *	65.8 ± 3.9*
Bilobalide	0.1 ± 0.0 *	61.5 ± 4.6*
Bilobalide (+ isolation)	0.1 ± 0.0 *	67.8 ± 2.1 *
Bilobalide (+isolation, +pre-	0.1 ± 0.0 *	58.4 ± 2.6 *
culture)		
Ascorbic acid 1 mM	0.1 ± 0.0 *	62.7 ± 5.2*
N-acetyl-L-cysteine 5mM	0.1 ± 0.0 *	68.7 ± 7.3*
Ciclosporin A 0.1µM	0.1 ± 0.0 *	60.3 ± 4.5*
Sucrose 50 mM	0.1 ± 0.0 *	30.9 ± 4.8*
Sucrose 100 mM	0.1 ± 0.0 *	25.8 ± 1.2*
Sucrose 200 mM	0.1 ± 0.0 *	20.4 ± 1.6*

Table 10: Evaluation of cellular viability of freshly isolated and cryopreserved/thawed mouse hepatocytes with or without "cryoprotectants"

Results are expressed as mean \pm S.E.M. (n=30 for fresh and cryopreserved samples, n=3 for cryoprotectants samples). *p<0.05 compared with fresh cells.

	Basal	Oligomycin	DNP	TMPD/Asc
Fresh	20.3 ± 3.3	5.2 ± 1.0	41.6 ± 6.7	176.4 ± 15.9
Cryopreserved	10.7 ± 3.5*	4.8 ± 0.9	8.2 ± 1.8*	165.2 ± 17.3
Bilobalide	9.6 ± 32.3*	4.4 ± 1.4	7.8 ± 1.3 *	169.3 ± 14.2
Bilobalide	8.3 ± 4.1*	3.1 ± 0.8	6.9 ± 1.1*	158.1 ± 9.1
(+ isolation)				
Bilobalide	11.5 ± 2.1*	4.9 ± 0.8	8.9 ± 0.8*	176.4 ± 10.3
(+isolation, + pre-culture)				
Ascorbic acid 1 mM	11.6 ± 3.4*	5.2 ± 1.4	7.8 ± 1.3*	171.0 ± 18.6
N-acetyl-L-cysteine 5mM	11.0 ± 2.8*	4.9 ± 1.6	9.0 ± 1.5*	166.1 ± 13.8
Ciclosporin A 0.1 µM	9.8 ± 2.6*	4.1 ± 0.6	7.3 ± 1.8 *	175.2 ± 12.6
Sucrose 50 mM	6.7 ± 1.2*	5.1 ± 1 .0	5.3 ± 0.9*	149.2 ± 9.1
Sucrose 100 mM	5.6 ± 2.8*	4.5 ± 1.1	5.1 ± 0.9*	165.2 ± 14.3
Sucrose 200 mM	4.5 ± 1.0*	3.5 ± 0.7	4.4 ± 0.6*	157.1 ± 18.4

Table 11: Oxygen consumption rate of freshly isolated and cryopreserved/thawed mouse hepatocytes with or without "cryoprotectants"

Freshly isolated and cryopreserved/thawed human hepatocytes (4.5 10^6 cells/ml) were incubated in closed vials at 37 °C in Williams' E medium saturated with a mixture of O₂/CO₂ (19:1). After 10 min, oxygen consumption rate (*J*O₂, nmol O₂.min⁻¹.10⁶ cells⁻¹) was measured in an oxygraph vessel coupled with Clark electrode before and after the successive additions of 0.5 µM oligomycin, 150 µM DNP, 0.15 µg/ml antimycin and 1 mM TMPD+5 mM ascorbate. The antimycin-sensitive *J*O₂ was calculated and the results expressed as mean ± S.E.M. (n=30 for fresh and cryopreserved samples, n=3 for cryoprotectants samples). * p<0.05 compared with fresh cells.

	State 4	State 3	DNP	TMPD/Asc
Fresh	13.1 ± 1.8	51.7 ± 2.0	42.1 ± 3.5	321.9 ± 24.3
Cryopreserved	3.8 ± 0.5*	20.7 ± 5.3*	10.9 ± 3.1 *	298.2 ± 20.0
Bilobalide	3.9 ± 1.4*	21.6 ± 4.9*	12.1 ± 4.5*	310.5 ± 16.3
Bilobalide	4.1 ± 0.6*	17.6 ± 4.3*	13.1 ± 4.7 *	308.7 ± 19.0
(+ isolation)				
Bilobalide	4.0 ± 0.9*	23.0 ± 6.8*	12.6 ± 2.5 *	311.9 ± 10.8
(+isolation, + pre-culture)				
Ascorbic acid 1 mM	4.2 ± 0.6*	22.1 ± 3.7*	13.9 ± 3.8 *	301.6 ± 16.9
N-acetyl-L-cysteine 5mM	4.1 ± 0.5*	19.7 ± 5.0*	11.6 ± 2.8 *	264.9 ± 12.5
Ciclosporin A 0.1 µM	4.3 ± 0.9*	24.7 ± 2.3 *	12.3 ± 3.5 *	301.3 ± 21.5
Sucrose 50 mM	3.2 ± 0.4*	19.2 ± 4.9*	8.2 ± 2.5*	274.2 ± 13.2
Sucrose 100 mM	2.9 ± 0.9*	17.7 ± 3.9*	7.8 ± 2.9 *	312.6 ± 21.6
Sucrose 200 mM	2.8 ± 1.0*	16.5 ± 3.3*	7.5 ± 2.4*	327.4 ± 16.4

Table 12: Oxygen consumption rate in permeabilized hepatocytes energised by glutamate/malate from freshly isolated and cryopreserved/thawed mouse hepatocytes with or without "cryoprotectants"

Permeabilization of freshly isolated or cryopreserved hepatocytes (10^6 cells/ml) was achieved by digitonin after a 10min pre-incubation in Williams' E medium as described in legend of Figure 3. Permeabilized cells were then incubated in the presence of 5 mM glutamate + 2.5 mM malate. The mitochondrial respiratory rate (JO_2 , nmol O_2 .min⁻¹. 10^6 cells⁻¹) was measured before and after the successive additions of 1 mM ADP/Tris, 0.5 μ M oligomycin, 50 μ M DNP, 0.15 μ g/ml antimycin and 1 mM TMPD+5 mM 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 mean \pm S.E.M. (n=30 for fresh and cryopreserved samples, n=3 for cryoprotectants samples). * p<0.05 compared to fresh cells.

	State 4	State 3	DNP	TMPD/Asc
Fresh	48.2 ± 5.6	82.8 ± 12.6	95.7 ± 13.1	331.3 ± 15.6
Cryopreserved	46.2 ± 3.4	64.3 ± 7.9	53.8 ± 9.7*	305.6 ± 10.9
Bilobalide	44.5 ± 2.7	61.7 ± 8.9	55.4 ± 6.3*	312.6 ± 8.9
Bilobalide	48.3 ± 4.4	68.3 ± 10.3	57.3. ± 8.6*	321.3 ± 12.5
(+ isolation)				
Bilobalide	47.5 ± 4 .5	67.1 ± 7.0	56.9 ± 7.5*	298.6 ± 12.8
(+isolation, + pre-culture)				
Ascorbic acid 1 mM	48.1 ± 2.5	67.9 ± 8.2	56.3 ± 6.2 *	325.6 ± 13.2
N-acetyl-L-cysteine 5mM	44.3 ± 2.5	63.2 ± 6.7	54.3 ± 6.5*	319.0 ± 13.1
Ciclosporin A 0.1 µM	44.2 ± 3.0	61.3 ± 6.5	51.2 ± 8.5*	298.6 ± 10.1
Sucrose 50 mM	45.3 ± 2 .9	61.7 ± 5.9	49.9 ± 6.7*	281.1 ± 12.3
Sucrose 100 mM	41.3 ± 3.6	69.3 ± 7.5	44.6 ± 11.3 *	271.3 ± 11.3
Sucrose 200 mM	49.5 ± 2 .9	65.4 ± 6.8	51.1 ± 11.3 *	281.3 ± 10.5

Table 13: Oxygen consumption rate in permeabilized hepatocytes, energised by succinate/malate from freshly isolated and cryopreserved/thawed mouse hepatocytes with or without "cryoprotectants"

Permeabilization of freshly isolated or cryopreserved hepatocytes (10^6 cells/ml) was achieved by digitonin after a 10min pre-incubation in Williams' E medium as described in legend of Figure 3. Permeabilized cells were then incubated in the presence of 5 mM succinate + 0.5 mM malate + 1.25 μ M rotenone. The mitochondrial respiratory rate (JO_2 , nmol O_2 .min⁻¹.10⁶ cells⁻¹) was measured before and after the successive additions of 1 mM ADP/Tris, 0.5 μ M oligomycin, 50 μ M DNP, 0.15 μ g/ml antimycin and 1 mM TMPD+5 mM ascorbate. The antimycin- and oligomycin-sensitive JO_2 was calculated and the results expressed in nmol O_2 .min⁻¹.10⁶ hepatocytes⁻¹ as mean \pm S.E.M. (n=30 for fresh and cryopreserved samples, n=3 for cryoprotectants samples). * p<0.05 compared to fresh cells.

4.1.1.4.2 Evaluation of the resistance of hepatocyte sub-populations to cryopreservation/thawing

4.1.1.4.2.1 Introduction

The liver contains hepatocytes with varying ploidy and gene expression, separable by Percoll gradient and centrifugation. Gupta et al. showed that specific fractions are enriched in polyploid (H2 fraction) or diploid (H3 and H4 fractions) rat hepatocytes containing glycogen and glucose-6-phosphatase activity. H4 fractions containing the diploid cells are relatively smaller with greater nuclear/cytoplasmic ratios, less complex cytoplasm, and higher serum albumin or ceruloplasmin biosynthetic rates. H2 fraction cells, with polyploidy cells, were larger with lesser nuclear/cytoplasmic ratio, more complex cytoplasm, and higher CYP activity. Phenotypic marking shows that H4 cells originated in zone one and H2 cells in zones two or three of the liver lobule (97).

We have evaluated the resistance of cryopreservation damages of these sub-populations in a rat model.

Resistance to freeze impairment of mice hepatocytes in proliferation, isolated 48 hours after partial hepatectomy, was also analyzed.

4.1.1.4.2.2 Material and Methods

4.1.1.4.2.2.1 Animals

See Material and Methods (Section 4.1.1.2.1).

We isolated hepatocytes from Sprague-Dawley rat livers for Percoll experiments. Cell return with mice liver was indeed insufficient.

4.1.1.4.2.2.2 Isolation and cryopreservation/thawing of rat hepatocytes

See Material and Methods (Section 4.1.1.2.1).

4.1.1.4.2.2.3 Isolation of hepatocyte sub-populations by Percoll gradient

Percoll (1.129 g/ml; Pharmacia Inc., Uppsala, Sweden) was diluted with PBS, pH 7.4, containing 5 mg/ml bovine serum albumin (Sigma) to 70, 52, 42, and 30% Percoll solutions. The gradients were

freshly made in 15-ml polystyrene tubes by first layering 1 ml 70% Percoll followed by 3 ml 52%, 4 ml 42%, and 5 ml 30% Percoll, from the base upwards, respectively. Hepatocytes (10^7 cells) were suspended in 1 ml Williams' E medium and loaded onto the top of the gradients per tube and centrifuged under 1000*g* for 30 min at 4°C (97). Cell fractions in discrete bands were isolated by pipetting and washed twice in Williams'E medium.

4.1.1.4.2.2.4 Isolation of hepatocytes after partial hepatectomy

Partial hepatectomy in mice was performed and constituted ~50-60% hepatectomy (132). All animals resumed normal activities promptly after recovering from anesthesia, and no mortality or morbidity was encountered. Livers were isolated 48 hours (peak of DNA synthesis) following hepatectomy according to Material and Methods (Section 4.1.1.2.1).

4.1.1.4.2.2.5 Hepatocyte viability evaluation

See Material and Methods (Section 4.1.1.2.1).

4.1.1.4.2.3 Results and discussion

We first confirmed the presence of 4 distinct hepatocyte populations in a rat model. While cryopreserving these hepatocyte sub-populations, we observed no resistance to freezing damage, despite the ATP ratio prior to cryopreservation being higher in the H2 fraction (Table 14).

Hepatocytes isolated following hepatectomy did not show any freeze resistance in terms of intracellular ATP levels (Table 14).

In conclusion, these subtypes of hepatocytes, isolated by Percoll and following hepatectomy did not show any freeze resistance.

Trypan blue (% viability)

Fresh H1	1.9 ± 0.5	50.1 ± 9.2
Cryopreserved H1	0.1 ± 0.0 *	30.5 ± 4.3*
Fresh H2	3.6 ± 1.5	75.6 ± 7.8
Cryopreserved H2	0.1 ± 0.0 *	41.3 ± 3.2*
Fresh H3	2.1 ± 0.5	95.4 ± 3.1
Cryopreserved H3	0.1 ± 0.0 *	51.3 ± 2.1*
Fresh H4	2.6 ± 0.4	93.1 ± 4.2
Cryopreserved H4	0.1 ± 0.0 *	49.8 ± 5.6*
Fresh hepatectomy	1.6 ± 0.3	69.1 ± 7.8
Cryopreserved hepatectomy	0.1 ± 0.0 *	40.5 ± 3.9*

Table 14: Evaluation of cellular viability of freshly isolated and cryopreserved/thawed hepatocyte sub-
populations after Percoll gradient (H1, H2, H3 and H4) or following hepatectomy
Results are expressed as mean \pm S.E.M. (n=5). *p<0.05 compared with fresh cells.</th>

4.1.2 Discussion

The post-thawing quality of C/T hepatocytes remained affected using the current protocols of C/T, and despite using the best isolation protocol, UW supplemented with FCS, 10% DMSO as freezing solution, slow freezing and rapid thawing protocols. We failed to avoid mitochondrial damage (the critical point according to our data) with complex 1 protector, hyperosomotic solutions, anti-oxidant molecules, or permeability transition inhibitors. In this section, the literature describing cell death and cryopreservation will be summarized and discussed. The importance of our results (complex 1 impairment) will be integrated in this literature review. This will lead us to analyze the IIF phenomenon during the cryopreservation process.

4.1.2.1 Cryopreservation and cell death

Necrosis, described following C/T (44) begins with an impairment of the cell's ability to maintain homeostasis, leading to an influx of water and extra-cellular ions. Intracellular organelles, most notably the mitochondria, and the entire cell swell and rupture (cell lysis). Due to the ultimate breakdown of the plasma membrane, the cytoplasmic contents including lysosomal enzymes are released into the extra-cellular fluid. This phenomenon has been tested predominantly by measuring LDH release from the cytoplasm to the extra-cellular medium and reflects cell membrane integrity. With the current C/T protocols, we demonstrated that LDH release and 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2H tetrazolium bromide (MTT) assays are unaffected by C/T and do not adequately assess viability after C/T. We also demonstrated that intracellular ATP concentration, which is an indirect mitochondrial marker, is probably the most sensitive parameter to detect early cellular damages related to cryostorage, showing the importance of the choice of the cytotoxicity test to be used for evaluation of C/T protocols (Section 4.1.1.2.1).

Apoptosis, a programmed cell death, was also described following C/T. Cells undergoing apoptosis show characteristic morphological and biochemical features which include chromatin aggregation, nuclear and cytoplasmic condensation, partition of cytoplasm and nucleus into membrane bound-vesicles (apoptotic bodies), morphologically intact mitochondria and nuclear material. *In vivo*, these

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apoptotic bodies are rapidly recognized and phagocytized by either macrophages or adjacent epithelial cells. Due to this efficient mechanism for apoptotic cells removal no inflammatory response is elicited *in vivo*. *In vitro*, the apoptotic bodies as well as the remaining cell fragments ultimately swell and finally lyse. This terminal phase of *in vitro* cell death has been termed "secondary necrosis". Apoptosis was described at least 12 hours post-thawing as demonstrated by Annexin V staining, in situ TUNEL assay combined with confocal laser scanning microscopy or by deoxyribonucleic acid (DNA) fragmentation (6,44,136).

The mitochondrion is a key player in the initiation of cell death and recent studies have highlighted its role in C/T-induced cellular damage (48). Indeed, disruption of mitochondrial membrane potential ($\Delta\Psi$) was reported following C/T. This mitochondrial damage is followed, within hours after thawing, by cytoplasmic cytochrome c release and caspase-3 activation leading to DNA fragmentation and ultimately apoptosis. Addition of caspase inhibitors (IDN-1965 or ZVAD-fmk) in the medium during cryopreservation and static culture rescued cells from apoptosis and was associated with increased Phase 1 and Phase 2 metabolism (85,136).

As mitochondria are the major source of ROS, induction of apoptosis by oxidative stress was also proposed to be involved in the impairment of hepatocytes after C/T (39,45). Combination of antioxidant medium and caspase inhibitors allowed significant improvement in viability and function in treated rat hepatocytes (45). In the same way, other authors proposed adding S-adenosylmethionine to the cryopreservation medium, to avoid glutathione decrease, during cold preservation or cryopreservation in liquid nitrogen. Glutathione plays a critical role in cell protection against oxidant stress The addition of S-adenosylmethionine attenuated the decrease in both viability and GSH content, observed after thawing (131).

In our study (Section 4.1.1.2.1), we found that C/T, according to a computer-controlled cooling process by using a cryozon or a freezing-protocol which consists of 20 min at -20° C followed by 2 hours at -80°C before storing in liquid nitrogen, decreased mitochondria related cellular respiration and oxygen consumption rate. This effect was evidenced on oligomycin (ATP synthase inhibitor)-

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). In permeabilized hepatocytes, marked impairment of mitochondrial oxidative phosphorylation following C/T was observed under state 4 (basal mitochondrial respiratory rate), state 3 (respiration coupled with ATP synthesis) and uncoupled conditions with substrates for complex 1. Interestingly, the inhibition of state 4 respiration was not present with complex 2 substrates, whereas both reduction of state 3 and abolition of the uncoupling molecule were still present. The respiratory-chain complex 1 is one of the largest and sensible mitochondrial respiratory chain complex, and constitutes also the major source of mitochondrial ROS (94,102). Thus, specific alterations of complex 1 subunit(s) and/or deregulation of ROS production leading to oxidative stress could constitute one of the starting-points for the C/T-induced damage. This may explain the decrease in oxygen consumption rate and $\Delta\Psi$, leading to ATP depletion and ultimately to cytochrome c release and apoptosis.

However we failed to improve any of these affected mitochondrial parameters using the addition of bilobalide to the isolation and/or cryopreservation media (Section 4.1.1.4.1).

We note that the mitochondrial damage, following C/T, ATP depletion, oxygen consumption and complex 1 impairment, were already observed following the step 1 of the cryopreservation process, the -20°C level, and of course thawing (Section 4.1.1.2.2). This suggests that the cryopreservation damage occurred early in the process, around the nucleation point and IIF. Cellular impairment could possibly be explained by the mechanical alteration of mitochondria, induced by water crystallisation during the cryopreservation or thawing procedures.

4.1.2.2 Intra-cellular ice formation

IIF was already evaluated during the cryopreservation process. It was demonstrated that for cryopreservation of cells or tissues, each system has its specific optimal cooling rate, showing a decreased survival at both too low (slow cooling damage) and too high cooling rates (fast cooling damage).

During freezing, the transition phase of water leads to a decrease in the extra-cellular water potential. Since water can pass through the plasma membrane, this will in turn lead to water efflux and cell dehydration. Slow cooling damage has been attributed to such phenomena as the increase in the external and internal solute (salt) concentration, the small size of the channels of unfrozen solution or the mechanical stress of cell shrinkage and destabilisation of membranes and proteins at low water potential. At high cooling rates, the intracellular dehydration (by water efflux) cannot keep pace with the extra-cellular dehydration (by phase transition of water). As a consequence, higher cooling rates result in higher levels of intra-cellular supercooling, high trans-membrane differences in osmotic pressure and solute concentrations, and high rates of water efflux through the membrane. This fast cooling damage seems to be particularly due to IIF. In a rat model, at -50 °C/min and below, none of the hepatocytes underwent IIF; whereas at -150 °C/min and above, IIF was observed throughout the entire hepatocyte population. The temperature at which 50% of hepatocytes showed IIF (50TIIF) was almost constant with an average value of -7.7°C. Different behaviour was seen in isothermal subzero holding temperatures in the presence of extra-cellular ice. 50TIIF from isothermal temperature experiments was approximately -5°C as opposed to -7.7°C for constant cooling rate experiments. These experiments clearly demonstrated both the time and temperature dependence of IIF. On the other hand, in cooling experiments in the absence of extracellular ice, IIF was not observed until approximately -20°C (at which temperature the whole suspension was frozen spontaneously) suggesting the involvement of the external ice in the initiation of IIF. The effect of DMSO on IIF was also quantified. 50TIIF decreased from -7.7 °C in the absence of DMSO to -16.8°C in 2.0 M DMSO for a cooling rate of 400°C/min. However, the cooling rate (between 75 and 400°C/min) did not significantly affect 50TIIF (-8.7°C) in 0.5 M DMSO.

Other authors have attributed membrane damage to the high trans-membrane osmotic pressure difference resulting in a very high rate of trans-membrane water flux. Alternatively, fast cooling damage could result from the fact that the cells are subjected to very sudden changes in size, shape

and ultrastructure due to the rapid efflux of water and the shrinking of the cells (22,53,59,68,128,129). Despite this debate, one can summarize that all the mechanisms of slow and fast cooling damage mentioned above relate to the osmotic changes during freezing and thawing, and the resulting flux of water across the membrane. This was studied by a differential scanning calorimetry (DSC) coupled with a cryomicroscope. DSC can provide information on energy flow associated with state transitions in a specimen during thermal processing. DSC quantitatively records the collective (aggregate) behaviour of a specimen, but does not resolve the details of individual components such as cells. The cryomicroscope, on the other hand, can provide information on the transient morphologies and structures for a small number of individual cells. However, quantitative analysis of cryomicroscopical data usually requires compromising assumptions such as extrapolation of three dimensional cell geometry from two-dimensional images to extract transient volumetric data from micrographs during freezing and/or dehydration. The integration of cryomicroscopy and DSC to perform both measurements simultaneously on a single experimental specimen of interest would provide a more comprehensive and quantitatively robust description of organic system response to thermal stress. The cell size would influence initial cell volume, surface area, and water content, which in turn, influence freezing response. Cryomicroscopic measurements of the cell/mitochondria volume as a function of temperature is obtained through image analysis of the freezing experiments. Authors observed that there is a decrease in cell volume as a function of temperature as cells attempt to efflux water to obtain equilibrium with the increasingly concentrated extra-cellular solution. The IIF, which results from the increase in the undercooling of the cytoplasm combined with the catalysis of ice formation by structures within the cell, will be function of both time and temperature. For sufficiently high cooling rates, there will be probably insufficient time for water transport across the plasma membrane (137).

In order to avoid damage due to IIF, cells must become sufficiently dehydrated before reaching the temperature range at which IIF can be observed. The combination of high temperatures for IIF

combined with rapid decrease in hydraulic permeability as the cell is cooled implies that achieving sufficient dehydration to avoid IIF remains a challenge. Also, the technical problem of thawing must be solved, to avoid the problem of re-crystallization, which is also probably a main source of cell damage in the C/T protocol.

4.1.3 Perspectives

According to the current cryopreservation protocols and based on our results, C/T hepatocytes quality is not satisfactory. In each model, in each study, at least one post-thaw hepatocyte quality parameter is impaired by the C/T process. IIF, as discussed earlier, probably remains the critical damaging factor principally at the structural level. Hereafter, we review new strategies of interest to cryopreserve hepatocytes: new hepatocytes configurations applicable for LCT, new cryoprotectants or new cryopreservation protocols such as vitrification trying to avoid IIF.

4.1.3.1 New hepatocyte culture configurations

4.1.3.1.1 Encapsulation: in vitro, in vivo

Encapsulation, by conferring a mechanical protection, was recently investigated for hepatocyte cryopreservation protocols. From several studies, encapsulation of hepatocytes in specially designed multi-component capsules (alginate, cellulose sulphate and poly(methylene-co-guanidine) hydrochloride) retained their specific functions-transaminase activity, urea synthesis and protein secretion-over the first days of culture. Several of the most detoxifying enzymes were expressed, in alginate-entrapped hepatocytes, at levels close to those found in corresponding unfrozen encapsulated hepatocytes (15). Long-term preservation of drug metabolism and transport activities, up to 120 days of cryopreservation, was recently demonstrated in microencapsulated rat hepatocytes (70). Finally, cold-induced apoptosis in hepatocytes can be significantly reduced following their entrapment within alginate gel beads, as demonstrated by measurement of caspase-3-like activity (83).

To extrapolate the use of C/T encapsulated hepatocytes for LCT consideration, the dedifferentiation problem must be adressed. *In vivo*, the first use of encapsulated hepatocytes was

documented in 1993, in the Gunn rat model, in which the authors demonstrated that the intraperitoneal transplantation of cryopreserved alginate-encapsulated hepatocytes, significantly reduced hyperbilirubinemia as well as freshly isolated encapsulated hepatocytes, up to 28 days following transplantation (36). In a liver failure model, two-stage 95% hepatectomy, with xenogenic hepatocytes and without immunosuppression, another advantage of the encapsulation, authors demonstrated the interest of encapsulated hepatocytes (4). More recently, Mai et al. demonstrated that cryopreserved or fresh encapsulated rodent hepatocytes showed a progressive decrease of albumin secretion over 1 week in culture. In contrast, C/T or fresh encapsulated immortalised human hepatocytes using lentiviral vectors showed minimal, but stable albumin secretion. Transplantation of C/T or fresh encapsulated rat hepatocytes significantly increased survival rate to 66% and 80% in ALF model (acetaminophen administration and 30% hepatectomy). Transplantation of C/T or fresh encapsulated immmortalized hepatocytes improved survival to 50% and 55%, respectively. Histopathology revealed that encapsulated hepatocytes were viable, but in a limited period, up to 2 weeks post-transplantation. This time-limited effect explains that last publications of transplantation on encapsulated hepatocytes only concern ALF models (3,84). In conclusion, cryopreservation of encapsulated hepatocytes is a promising tool for the establishment of banks for the supply and storage of hepatocytes, but the efficacy of transplantation remains time-limited. This kind of cell configuration also limits the LCT indications (type of liver disease).

Therefore, new projects must in the future evaluate the interest of co-encapsulation of hepatocytes with mesenchymal bone marrow cells or pancreatic islets, as a new type of feeder cells.

4.1.3.1.2 Hepatocyte spheroids

Hepatocyte spheroids were successfully cryopreserved in UW solution and no significant differences with freshly cultured hepatocyte spheroids, were noticed in terms of cell viability and functionality. UW solution was described to be superior to other tested cryopreservation solutions (Williams'E medium, fetal bovine serum or mixture of the several components) (75). A study by

Korniski et al. has been conducted to examine basic transport characteristics of pig hepatocytes cultured as spheroids. The spheroids were disaggregated and low-temperature cryomicroscopy experiments were performed to examine the transport and IIF characteristics. The results infer a decrease in the temperature range over which IIF is observed compared to freshly isolated pig hepatocytes. The technique of freeze substitution was used to examine the structure inside the spheroid during freezing and shows the location of ice in freezing hepatocyte spheroids and increased amounts of intercellular ice. It confirms that cells cultured as spheroids do not transport water in the same manner as that of isolated cells (71). Further studies are needed to assess the optimal methods of culture and of cryopreservation environments including the freezing rate or cryoprotectant from damage during freezing.

4.1.3.2 New cryoprotectants

4.1.3.2.1 Pre-incubation

Maintenance of ATP levels allows the recovery of hepatocytes from the isolation "trauma" and has been shown to be crucial during cryopreservation. Hence, ATP cellular boosters or antioxidants have been proposed to supplement pre-incubation medium and to potentialize the beneficial effects of pre-culture. Two hours hyperosmotic (100 - 300 mM) glucose pre-incubation improved the viability and attachment efficiency of rat hepatocytes and improved the viability and reduced LDH leakage of human hepatocytes. In parallel, fructose pre-incubation (100 - 300 mM) improved both the viability and attachment efficiency of rat hepatocytes but only the attachment efficiency of human hepatocytes. Pre-incubation with anti-oxidant, such as alpha-lipoic acid (0.5 - 5 mM), improved the viability and attachment efficiency of both rat and human hepatocytes (125,126). The beneficial effects of this pre-treatment (at lower concentration: 15 mM glucose for 30 min at 37 °C) in human hepatocytes were confirmed by Silva et al. (106). They found that the response of CYP enzymes to typical inducers was significantly improved in the pre-incubated hepatocytes. If pre-incubated C/T human hepatocytes had similar viability and urea production than in freshly isolated hepatocytes, they also showed a significant higher plating efficiency than C/T hepatocytes without

pre-incubation. Quality of C/T cells after this new pre-incubation protocol needs to be evaluated in terms of mitochondrial functions.

4.1.3.2.2 Non permeating cryoprotectants

Oligosaccharides (trehalose and related oligosaccharides) in addition to DMSO give a significant higher viability after thawing in primary rat or human hepatocytes cooled in a controlled rate freezer, as estimated by trypan blue exclusion. Use of oligosaccharides with higher molecular weights resulted in greatest improvement in viability. Moreover, attachment and survival rates in plastic dishes of rat hepatocytes were approximately 1.2-1.8-fold greater after freezing in the presence of di-, tri-, and tetrasaccharides. Improvement of plating was not confirmed with human hepatocytes (87). Furthermore, the use of trehalose and DMSO for cryopreserving human hepatocytes resulted in a significantly increased total protein level, higher secretion of albumin and lower aspartate aminotransferase level in the attached cells after thawing (69). These works were inspired from data demonstrating the influence of trehalose on cell quality on bull sperm (14,133).

Finally, a novel method for the cryopreservation of hepatocytes, using a non-metabolizable glucose derivative in an attempt to mimic the natural cryoprotective adaptations observed in freeze-tolerant frogs, was recently proposed. Primary rat hepatocytes were loaded with 3-O-methyl glucose (3OMG) through endogenous glucose transporters without evident toxicity and cryopreserved according to a controlled rate freezer program down to -80 °C before storing in liquid nitrogen. In this study, hepatocytes cryopreserved with a relatively small amount of intracellular 3OMG (<0.2 M) showed high post-thaw viability and maintained long-term hepatospecific functions, including synthesis, metabolism, and detoxification. Metabolite uptake and secretion rates were also largely preserved in the C/T hepatocytes (120). In the near future, mitochondrial functions, including complex 1 activity, must be evaluated after C/T with a freezing medium including non permeating cryoprotectants, such as trehalose or non metabolizable glucose. Indeed, protection delivered by non-permeating cryoprotectants, needs to be evaluated in terms of cell death.

4.1.3.2.3 Wheat extracts

A recent report proposed the use of wheat protein extracts for long-term storage and recovery of large quantities of healthy cells that maintain high hepatospecific functions (52). In primary culture, the morphology of hepatocytes cryopreserved with wheat protein extracts was similar to that of freshly isolated cells. Furthermore, hepatospecific functions such as albumin secretion and biotransformation of ammonium to urea were well maintained during 4 days in culture. Inductions of CYP1A1 and CYP2B in hepatocytes C/T with wheat extracts were also similar to those in freshly isolated hepatocytes. However, viability and plating of C/T hepatocytes remained lower as compared to freshly isolated cells.

4.1.3.3 New cryopreservation strategies: vitrification

Since the major problem with the current protocols remains IIF, alternatives such as vitrifying hepatocytes are an interesting strategy to reach a best post-thawing cell quality. Vitrification (from the Latin, vitreus, glassy) is essentially the solidification of a supercooled liquid by adjusting the composition and cooling rate such that the crystal phase is avoided. The process involves a progressive and marked increase in viscosity during cooling and prevention of ice nucleation and growth. The system is stabilized in the glassy state as translational molecular motion is essentially halted. Vitrification eliminates the biologically damaging effects associated with freezing. No appreciable degradation occurs over time in living matter trapped within a vitreous matrix, and vitrification is potentially applicable to all biologic systems.Vitrification of precision cut-slices, tissue engineered pancreatic substitute, jugular veins/vessels constructs, embryonic kidneys was already performed allowing the absence of ice into the vitrified samples and an excellent post/thawing quality and/or viability (11,13,27,40,112,113).

Classically, tissues (vessels constructs, embryonic kidneys are vitrified at cooling rates of $>40^{\circ}$ C/min in a specific solution, which consisted of 3.10 mM/L DMSO, 3.10 mM/L formamide and 2.21 mM/L 1,2-propanediol in EuroCollins solution (VS55) or a polyethylene formulation consisting of 5M propanediol, 1M DMSO and 12% polyethyleneglycol 400 (11,40). Best viability

results were obtained with the VS55 solution. To obtain cooling rates of >40°C/min, tissues contained in vials, were cooled to -100° C in a isopentane bath (conductive cooling, freezing point -160° C) placed in a -135° C freezer, removed from the 2-methylbutane bath and vitrified to -120° C in the -135° C freezer (convective cooling).

Re-warming was performed under controlled conditions, and the chemicals removed in a stepwise manner. However de-vitrification may occur during warming from the vitrified state. To prevent de-vitrification, the vitrified material must be warmed uniformly as quickly as possible [slowly re-warmed to -100°C using convection followed by rapid re-warming achieved by placing the vial in a DMSO/H2O mixture at room temperature (225°C/min)] so that ice does not have the opportunity to form in significant quantities.

Vitrification of encapsulated hepatocytes in M or G-collagen was recently proposed as an alternative to classical freezing protocol (72). Wu et al. proposed a rapid stepwise introduction of microencapsulated hepatocytes to vitrification solution (40 volume/volume % ethyleneglycol 0.6M sucrose in medium) and their direct immersion in liquid nitrogen. Using this technique, they obtained 100% retention of hepatocyte function, correlated to an excellent viability and no detectable microcapsule damage (135). Further investigations are however needed to confirm the potential of vitrification for pharmacological studies as for LCT protocols.

4.1.4 Conclusions

Using current protocols, C/T of hepatocytes induces cell alteration, mainly at the level of the mitochondria, with marked impairment of the mitochondrial respiratory chain complex 1 activity. Cytochrome c release will lead to cell death within hours by apoptosis. *In vitro* functions of C/T hepatocytes remain poorer than those of freshly isolated hepatocytes, while the efficacy *in vivo* seems to be time-limited both in animal models and in humans.

The IIF remains probably the major factor that affects the quality of the cells.

Protection delivered by non permeating cryoprotectants in terms of cell death and mitochondrial functions should be evaluated in future.

New perspectives such as vitrification, to avoid the crystalline state, coupled or not with encapsulation should be investigated in the future, while considering the problem of hepatocyte dedifferentiation during the long term in this configuration. The current developments of stem cells, that are able to self renew, may allow resistance to freezing damage and alternative use for LCT.

4.2 Study of the pro-coagulant activity of the cell preparation

Low engraftment of transplanted hepatocytes is a major limit of LCT. Indeed, the majority of transplanted hepatocytes (70-80%) are cleared early by phagocytes and macrophages in the hepatic vascular spaces. Preventing this early cell loss requires not only the improvement of the quality of transplanted hepatocytes but also the understanding of the role of hepatocytes and other resident liver cells in the induction of immunologic events. It was recently demonstrated that pancreatic islet transplantation, a therapeutic modality for type 1 diabetes, induces activation of coagulation cascade. Tissue factor synthesized by beta cells and duct cells contaminating clinical islet preparations is directly implicated in this pro-coagulant activity (8,10). This may compromise the outcome of islet transplantation by early graft loss (65,88). We therefore determined if hepatocytes, freshly isolated or C/T, also express tissue factor and are responsible of a pro-coagulant activity. Since N-acetyl-L-cysteine derivative inhibits tissue factor-dependent pro-coagulant activity of islet preparations (9), we also investigated the potential of N-acetyl-L-cysteine to inhibit *in vitro* pro-coagulant activity associated with hepatocyte suspensions.

4.2.1 Results

4.2.1.1 Tissue factor-dependent pro-coagulant activity of isolated human hepatocytes:

relevance to liver cell transplantation

Increasing the quality and the replicative advantage of the transplanted cells is likely to improve the yield of engraftment and subsequent repopulation of the recipient liver. Tissue factor dependent activation of coagulation was found to contribute to low rate of beta cell engraftment in pancreatic islets transplantation. In this Section (Article 4), we demonstrated that human hepatocytes either fresh or C/T exert *in vitro* and *in vivo* tissue factor-dependent pro-coagulant activity. Tissue factor expression on hepatocyte preparations was assessed by flow cytometry, reverse-transcription polymerase chain reaction and immunofluorescence. Pro-coagulant activity depending on tissue factor was evaluated in human plasma and in whole blood systems.

The thiol-containing drug N-acetyl-L-cysteine interferes with the tissue factor-dependent procoagulant activity of isolated hepatocytes. This could represent candidate for a pharmacological modulation of the coagulation process. Article 4: Tissue factor-dependent pro-coagulant activity of isolated human hepatocytes: relevance to liver cell transplantation. Liver Transplantation, 2007 Apr; 13(4):599-606.



Tissue Factor-Dependent Procoagulant Activity of Isolated Human Hepatocytes: Relevance to Liver Cell Transplantation

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Liver cell transplantation (LCT) aims to correct inborn liver function defects by infusing metabolically active cells into the diseased liver. Further improvement in LCT might depend on the prevention of early loss of transplanted cells. As tissue factor (TF)-dependent activation of coagulation was found to contribute to a low rate of beta cell engraftment in islet transplantation, we investigated the potential procoagulant activity (PCA) of hepatocyte preparations. TF expression on hepatocyte preparations was assessed by flow cytometry, reverse-transcription polymerase chain reaction and immunofluorescence. PCA depending on TF was evaluated in human plasma and in whole blood systems. Coagulation parameters were followed by routine techniques in a LCT recipient Crigler-Najjar patient. We determined that hepatocytes express soluble and membrane-bound forms of TF. We showed that hepatocytes exert a TF-dependent PCA. In parallel, delayed increase in D-dimer levels was observed following the hepatocyte infusions in the Crigler-Najjar patient. Furthermore, in vitro experiments demonstrated that TF-dependent PCA of hepatocytes is inhibited by N-acetyl-L-cysteine. In conclusion, hepatocytes exert TF-dependent PCA, which may contribute to early loss of infused cells. Addition of N-acetyl-L-cysteine to the suspensions of hepatocytes might be beneficial in LCT by inhibiting activation of coagulation. *Liver Transpl 13:599-606, 2007.* © 2007 AASLD.

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In various human inborn metabolic diseases, liver cell transplantation (LCT) can provide medium-term partial metabolic control.¹⁻⁵ LCT also aims to bring immediate temporary functional support for the treatment of patients affected by fulminant hepatic failure, although demonstration of its efficacy remains difficult in this condition.⁶ Increasing the quality and the replicative advantage of the transplanted cells is likely to improve the percentage of engraftment and subsequent repopulation of the recipient liver by the infused cell population.

Pancreatic islet transplantation, a therapeutic modality for type 1 diabetes, induces activation of coagulation cascade. Tissue factor (TF) synthesized by beta cells and duct cells contaminating clinical islet preparations is directly implicated in this procoagulant activity (PCA).^{7,8} This may compromise the outcome of islet transplantation by early graft loss.^{9,10} Therefore, the first aim of this study was to determine if isolated hepatocytes, fresh or cryopreserved, also express TF. After demonstrating the presence of TF in hepatocytes, we analyzed the PCA of hepatocyte suspension in several in vitro coagulation assays. Following in vitro demonstration of TF-dependent PCA, we further explored coagulation parameters of a 9-month-old Crigler-Najjar patient before and after cell infusions. Since we recently observed that N-acetyl-L-cysteine (NAC) derivative inhibits TF-dependent PCA of islet preparations,¹¹ we

Abbreviations: LCT, liver cell transplantation; TF, tissue factor; PCA, procoagulant activity; RT-PCR, reverse-transcription polymerase chain reaction; NAC, N-acetyl-L-cysteine; mAb, monoclonal antibody; mRNA, messenger ribonucleic acid; F.E.U., fibrinogen equivalent unit; as-TF: alternatively spliced variant of TF; Ig, immunoglobulin. Supported by Fonds National de la Recherche Scientifique and the French Community of Belgium and Childfund. Xavier Stéphenne and Olivier Vosters contributed equally to this work. Address reprint requests to Etienne M. Sokal, Paediatric Hepatology and Cell Therapy, Université Catholique de Louvain, Cliniques St Luc, 10 av. Hippocrate, B-1200 Bruxelles, Belgium. Telephone: 32 2 764 13 87; FAX: 32 2 764 89 09; E-mail: Sokal@pedi.ucl.ac.be

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also investigated the potential of NAC to inhibit in vitro PCA associated with hepatocyte suspensions.

MATERIALS AND METHODS

The protocol, including all experiments on human and animal samples, the blood investigation in the patient, and the LCT protocol were approved by the institution ethical review board.

Cell Preparations

Liver isolation and hepatocyte cryopreservation/thawing procedures were previously published in detail.^{4,5} After isolation or thawing, cells were suspended in a stable solution of plasmatic proteins (85% of albumin) containing bicarbonate (1 mmol/L), glucose (11 mmol/ L), and 10 U/mL heparin (infusion medium). When not specified, experiments were performed with human cryopreserved/thawed hepatocytes (Trypan blue viability around 80%).

The human adenocarcinoma cell line CAPAN-2 was used as a positive TF control in the several experiments as previously described.⁸

TF Expression of Human Hepatocyte Suspension

Immunofluorescence studies were performed to evaluate the presence of TF. For this, fresh hepatocytes were placed on cover slips and fixed by paraformaldehyde 4% (Merck, Darmstadt, Germany) for 20 minutes. Then, these cells were incubated with Triton X-100 (Sigma, Bornem, Belgium) 1% in Tris base sodium buffer (50 mmol/L Tris-HCl pH 7.4 and 150 mmol/L NaCl) (Organics [VWR], Leuven, Belgium) for 15 minutes and then with milk 3% in Tris base sodium buffer for 1 hour.⁵ The primary antibodies, monoclonal mouse anti-human cytokeratin-18 antibody (Progen, Heidelberg, Germany) and polyclonal rabbit anti-TF monoclonal antibody (mAb) (immunoglobulin [Ig]G1 n4509; American Diagnostica, Andresy, France) were diluted (1/100 and 1/50, respectively) in Tris base sodium and incubated with cells for 1 hour. The secondary antibodies used were cyanine 3-conjugated anti-mouse IgG (Jackson Immunoresearch Laboratories, West Grove, PA) and fluorescein isothiocyanate conjugated anti-rabbit IgG (Sigma). The nuclei were revealed by 4', 6-diamidino-2-phenylindole (DAPI; Sigma) staining. Negative experimental controls were performed (absence of primary or secondary antibodies).

Presence of the classical membrane-bound (TF) and the recently new described soluble (alternatively spliced TF; as-TF) forms was analyzed by reverse-transcription polymerase chain reaction (RT-PCR). Messenger ribonucleic acid (mRNA) was extracted from 0.5×10^6 hepatocytes using the Tripure isolation reagent kit (Roche Applied Science, Brussels, Belgium) following the manufacturer's instructions. One-step RT-PCR was performed on a Thermocycler instrument (Applied Biosystems, Lennik, Belgium) with primers synthesized at Invitrogen. RT-PCR for TF or glyceraldehyde 3-phosphate dehydrogenase was realized with the following primers:

TF sense primer, 5'-TGAATGTGACCGTAGAAGATGA-3';

TF antisense primer, 5'-GGAGTTCTCCTTCCAGC-TCT-3'; 8

Glyceraldehyde 3-phosphate dehydrogenase: sense primer, 5'-CGGACTCAACGGATTTGGTCGTAT-3';

Glyceraldehyde 3-phosphate dehydrogenase: antisense primer, 5'-AGCCTTCTCCATGGTGGT-3'.

Products were separated by electrophoresis on 1% agarose gel and visualized with ethidium bromide under ultraviolet lamp.

We also realized a real-time RT-PCR for TF, as-TF, and β -actin on a Lightcycler instrument (Roche Applied Science) with primers synthesized at Biosource Europe, Nivelles.

Belgium as previously described.⁸ mRNA was extracted from 0.5×10^6 hepatocytes with the MagNA Pure mRNA extraction kit on the MagNA Pure instrument (Roche Applied Science) following the manufacturer's instructions. Primers were as follows:

TF sense primer: 5'-GGGAATTCAGAGAAATATTCTA-CATCA-3';

TF antisense primer: 5'-TAGCCAGGATGATGACAA-GGA-3';

as-TF sense primer: 5'-TCTTCAAGTTCAGGAAAG-AAATATTCT-3';

as-TF antisense primer: 5'-CCAGGATGATGACAAG-GATGA-3';

probe: 5'-TGGAGCTGTGGTATTTGTGGTCA-3'

 $\hat{\beta}$ -actin sense primer: 5'-GGTCAGAAGGATTCCT-ATG-3';

 $\beta\text{-actin}$ antisense primer: 5'-GGTCTCAAACATGA-TCTGGG-3'

The presence of membrane-bound TF was also confirmed by flow cytometry analysis. Therefore, cells were washed in phosphate-buffered saline supplemented with 1% bovine serum albumin and 10% pooled human serum and incubated for 20 minutes at $+4^{\circ}$ C with the fluorescein isothiocyanate-conjugated IgG1 mAb against TF no. 4508CJ (American Diagnostica) or the corresponding isotype-matched control mAb (Dako, Heverlee, Belgium). Cell fluorescence was analyzed using a Cyan flow cytometer (Dako).

TF-Dependent PCA of Human Hepatocyte Suspension

PCA Assay

PCA was determined in duplicate by a single-stage clotting assay on cell suspensions in phosphate-buffered saline. A total of 100 μ L of each sample was incubated at +37°C for 1 minute with 100 μ L of normal citrated plasma before the initiation of clotting by the addition of 100 μ L of 25 mmol/L CaCl₂. Clotting time was recorded with a KC10 apparatus (Amelung, Lemgo, Germany) and PCA in mU/mL was determined by reference to a standard curve generated by serial dilutions of a commercial rabbit thromboplastin (Excel SA; Organon Teknica, Turnhout, Belgium). The amount of thrombo-

plastin that yielded a clotting time of 12.4 seconds was assigned a value of 1 unit. To determine the role of the TF/factor VII pathway in the PCA, additional experiments were performed using factor VII-deficient plasma (Dade Behring, Marburg, Germany). The number of cells was determined by simple counting.

Tubing loop model

A whole-blood experiment protocol was adapted from a model previously described.8,10 Loops made of polyvinylchloride tubing (inner diameter 6.3 mm, length 390 mm) and treated with a Corline heparin surface were purchased from Corline (Uppsala, Sweden). Loops were supplemented with cell samples suspended in phosphatebuffered saline before blood addition. To ascertain the role of TF in this model, cells $(0.5 \times 10^6 \text{ hepatocytes})$ were preincubated at room temperature for 10 minutes with either 0.2 mg/mL mAb anti-human TF IgG1 (American Diagnostica) or 0.2 mg/mL mAb mouse IgG1 (clone 11711.11; RnD Systems, Abingdon, United Kingdom) before extensive washing in phosphate-buffered saline and tubing loop assay. Five mL of non-anti-coagulated blood from healthy volunteers was then added to each loop. To generate a blood flow of about 45 mL/minute, loop devices were placed on a platform rocker inside a +37°C incubator. Blood samples were collected into ethylene diamine tetraacetic acid (4.1 mmol/L final concentration) and citrate (12.9 mmol/L final concentration) tubes before and 30 minutes after start. Platelets were counted on a CellDyn 4000 automate (Abbott Laboratories, Abbott Park, IL) and D-dimers were evaluated by immunoturbidimetric assay (Liatest D-DI; Diagnostica Stago, Asnières sur Seine, France).

Viscometry measurement

ReoRox4 is a free oscillation rheometer, a device that enables to monitor blood viscosity over time in small blood volumes.¹² It is based on the recording of magnetically-induced oscillations; during the coagulation process, increase in viscosity (as a consequence of coagulation) results in a transient increase of oscillation damping. Human citrated blood samples were recalcified extemporaneously with a solution of 25 mmol/L Ca²⁺. Reaction was started by adding 400 μ L human citrated blood to cups containing 210 μ L of Ca²⁺ solution and increasing the number of hepatocytes suspended in 40 μ L of phosphate-buffered saline.

Where specified, graded doses (1-25 mmol/L) of NAC (Lysomucil; Zambon, Brussels, Belgium) were added.

Follow-Up of Coagulation Parameters in a LCT Recipient Child

A 9-month-old girl (7.5 kg) with Crigler-Najjar disease was referred to our unit for LCT. A Broviac catheter device was inserted surgically in the portal system, allowing repetitive infusions as reported.¹³ The child received 2.6 billion cells (2.2 billion cryopreserved/ thawed cells) in 14 successive infusions (2 infusions per day) over 2 weeks, in 3 steps of infusions (3 consecutive days for the first step, 2 consecutive days for the 2 others). Each cell-infused suspension contained 60 to a maximum of 250×10^{6} cells, suspended in 25 mL of the infusion medium, given at a rate of 50 mL/minute. We followed the coagulation parameters, including fibrinogen, international normalized ratio of prothrombin (prothrombin time), activated partial thromboplastin time, D-dimers on a CA7000 automated coagulation analyzer (Sysmex, Barchon, Belgium), and platelets on a Advia 120 analyzer (Bayer, Brussels, Belgium); before infusion, after 15 minutes of infusion, and at the end of infusion in peripheral blood. We also followed those parameters in portal blood at the end of infusions.

Statistics

Results are expressed as mean \pm standard error of the mean or mean \pm standard deviation, and statistically significant (*P < 0.05, **P < 0.01, ***P < 0.001) differences were assessed by Wilcoxon or Mann-Whitney tests.

RESULTS

Cryopreserved Hepatocytes Express Functional TF

TF expression was first documented on fresh hepatocytes by immunofluorescence. As shown in Figure 1A, we found that all cells express TF constitutively (uniform cytoplasmic staining). In parallel, the coexpression of TF and cytokeratin-18 of these cells confirmed their hepatocyte lineage. We also assessed the expression of TF at the mRNA level on hepatocytes using RT-PCR (Fig. 1B). Interestingly, both the membrane form and the alternatively-spliced variant of TF mRNA were expressed. In additional experiments, we used real-time RT-PCR to quantify TF and as-TF mRNA levels in hepatocytes. As shown in Figure 1C, the as-TF variant was predominantly expressed with an ~2-fold magnitude. Furthermore, flow cytometry analysis of hepatocytes confirmed a positive and specific staining for TF (Fig. 1D).

We then assessed PCA of hepatocytes in human plasma. As shown in Figure 2A, we observed that hepatocyte preparations exert significant PCA that was dependent on the number of cells engaged. The PCA of the hepatocyte suspensions was factor VII-dependent since it was not observed in factor VII-depleted plasma and demonstrated the involvement of TF (Fig. 2B).

PCA of Hepatocytes in Whole Blood Preclinical Model

To further investigate the PCA of hepatocytes in a model closer to the in vivo situation, we adapted the tubing loop system that was used to demonstrate the thrombotic reaction induced by islet preparations⁷ and duct cells.⁸ This model is based on the injection of non-anticoagulated blood in plastic loops in which the inner surface was coated with heparin to prevent contact-

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Figure 1. TF expression on hepatocytes. (A) Immunofluorescence for (1) TF (green) and (2) cytokeratin-18 (red) was performed on fresh hepatocytes placed on cover slips and fixed by paraformaldehyde. (3) A merging image of CK-18 and TF staining is also presented (magnification $20\times$). The nuclei were revealed by (DAPI) (blue) staining. (B) mRNA extracted from 2 cell preparations was analyzed by RT-PCR for glyceraldehyde 3-phosphate dehydrogenase (1-2) and for TF (3-4). The conditions 1 and 3, and 2 and 4 were obtained from fresh and cryopreserved/thawed cells, respectively. One representative experiment out of 3 is shown. Arrows indicate the size of either classical mRNA (407 bp) encoding the membrane form (TF) or the as-TF (247 bp) encoding the soluble form. (C) Guantification of TF and as-TF mRNA using real-time RT-PCR. Results are expressed per 10^{6} mRNA copies of β -actin. Data represent mean \pm standard error of the mean of 5 independent experiments. (D) Hepatocytes were analyzed by flow cytometry after staining by either anti-TF mAb (gray histogram), corresponding control isotype mAb (white histogram, thick line) or unstained cells (white histogram, dotted line). One representative experiment out of 3 is shown.

dependent blood coagulation, thus allowing the investigation of coagulation in the presence of platelets. Blood coagulation was assessed by macroscopic examination for the presence of clots and by monitoring platelet counts and D-dimer levels, which decrease or increase as a consequence of coagulation activation, respectively. We show that 5×10^5 hepatocytes added to 5 mL of blood were sufficient to induce clot formation within 30 minutes. This was associated with a dramatic drop in platelet counts (Fig. 3A) and increased D-dimer levels (Fig. 3B). To demonstrate the role of TF, pretreatment of hepatocytes with an anti-TF mAb was performed in these settings; this led to the prevention of the formation of visible clots, prevention of the drop in platelet counts, and prevention of the increase in Ddimer levels. This was not observed with an isotypematched control mAb (Table 1).

The PCA of hepatocytes in whole blood was also assessed by free oscillating rheometry, another global coagulation measurement technique. As shown in Figure 4, we were able to confirm that the addition of increasing number of hepatocytes to citrated blood in the presence of Ca⁺ resulted in the activation of coagulation. We observed that as few as 10^4 cells were sufficient to promote a statistically significant induction of human blood coagulation.

D-Dimers Increased Following LCT

To demonstrate PCA of infused hepatocytes in vivo, we also evaluated coagulation parameters in an LCT recipient patient. When the Broviac was surgically placed in the portal system and before infusions, D-dimer levels (ng/mL fibrinogen equivalent unit [F.E.U.]) were mea-



Figure 2. TF-dependent PCA of hepatocytes in plasma. (A) PCA was assessed in plasma after addition of hepatocytes (6.25, 12.50, 25.00 × 10⁶/mL). Data represent mean ± standard error of the mean of 4 independent experiments. *P < 0.05 compared with normal citrated plasma. (B) PCA of hepatocytes in presence of factor VII-deficient plasma (2) was determined and compared to PCA assessed in human plasma (1). Data represent mean ± standard error of the mean of 4 independent experiments. *P < 0.05 compared with normal citrated plasma.

sured in Crigler-Najjar patient peripheral blood and were considered as normal (335 ng/mL F.E.U., normal values: <500 ng/mL F.E.U.). The D-dimer values remained within normal laboratory range during the first of the 14 infusions in peripheral and portal blood (378 ng/mL F.E.U. in peripheral blood, 344 ng/mL F.E.U. in portal blood). Interestingly, we observed an increase in D-dimer levels the day following the 2 first infusions, before infusions of the second day (first step) (865 ng/mL F.E.U. in peripheral blood). No further increase in D-dimer values was observed during the infusions of the second day (873 ng/mL F.E.U. in peripheral blood, and 855 ng/mL F.E.U. in portal blood.) The increase in D-dimer levels was also confirmed on blood samples



Figure 3. PCA of hepatocytes in whole blood (tubing loop model). Hepatocytes were added to 5 mL whole blood incubated in tubing loops under agitation at +37°C. Platelet count (A) and D-dimer levels (B) were monitored after 30 minutes of incubation in the absence or presence of increasing concentrations of hepatocytes (10^5 , 5×10^5 , and 10^6). Data represent mean \pm standard deviation of 3 to 5 independent experiments. *P < 0.05 compared with platelets count in absence of cells.

taken the day after the second step infusions, at which time D-dimer values up to 1,374 ng/mL F.E.U. were measured. One week after all the infusions, the D-dimer levels returned within normal values (397 ng/mL F.E.U.). All the other coagulation parameters remained within normal laboratory ranges during and after the infusions, in peripheral or portal blood (data not shown).

NAC Inhibits TF-Related PCA of Hepatocytes Without Inducing Toxicity

According to the modulation of the hemostatic parameters described in healthy subjects after intravenous infusion with NAC,¹⁴ this compound was tested for its capacity to inhibit the PCA induced by hepatocytes. We first investigated the capacity of graded doses of NAC to impair the PCA in human plasma. As shown in Figure 5A, we observed a dose-dependent inhibition of hepatocyte-induced PCA with an effect already statistically significant at 10 mmol/L. We then assessed the capacity of graded doses of NAC to impair the PCA in the

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HEPATOCYTES EXERT A TF PROCOAGULANT ACTIVITY 603

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Cells	Time (minutes)	mAb	Platelet count (10 ² /µL)		D-dimers (ng/mL F.E.U.)	
			Experiment 1	Experiment 2	Experiment 1	Experiment 2
0	0	No	294.5	294.5	85.0	85.0
0	30	No	252.0	252.0	105.0	105.0
5×10^{5}	30	No	36.3	1.6	1265.0	870.0
5×10^{5}	30	Anti-TF	241.2	385.0	305.0	415.0
5×10^{5}	30	Isotype control	0.7	2.8	965.0	980.0

NOTE: Hepatocytes (5 \times 10⁵), pretreated for 10 minutes at room temperature with anti-TF mAb or isotype-matched control mAb, were added to 5 mL of whole blood and incubated in tubing loops under agitation at +37°C. Platelet count and D-dimer levels were monitored prior to the assay and after 30 minutes incubation in the presence or absence of cells. Results of 2 experiments performed on 2 independent blood samples are shown. Data represent the mean value for platelet count and D-dimer levels.



Figure 4. PCA of hepatocytes in whole blood (ReoRox model). Viscosity was assessed using free oscillating rheometry (Reo-Rox4) in 600 µL of recalcified human citrated peripheral blood after addition of hepatocytes (10^4 , 10^5 , 5×10^5 , and 10^6). Data represent mean \pm standard error of the mean of 4 to 8 independent experiments. *P < 0.05, **P < 0.01, ***P < 0.001 compared with control (absence of hepatocytes). Coagulation induction fold was calculated by dividing the coagulation time of the blood in absence of hepatocytes by coagulation time of the blood with the determined number of cells.

human whole blood model. NAC dose-dependently prevented the drop in platelet count induced by hepatocytes and the increase in D-dimer levels. The effect of NAC was already significant at the dose of 10 mmol/L (Fig. 5B).

These inhibitions were not due to a toxic effect of the compound as no toxicity related to several concentrations of NAC was evidenced in murine hepatocytes (n = 3).

DISCUSSION

The important finding of this study is the demonstration of hepatocyte suspension TF-dependent PCA. This in vitro observation has a practical consequence in vivo, since we observed a modification of the coagulation parameters, delayed D-dimer increase, in a LCT recipient patient. Based on its ability to inhibit TF-dependent PCA of hepatocyte suspension, we may further consider, to ameliorate LCT engraftment, addition of NAC to the hepatocyte suspension.

Indeed, low cell engraftment rate represents a major limitation of LCT, resulting in limited correction of the metabolic inborn defect.⁵ Activation of the coagulation cascade was evidenced and associated with negative clinical outcome after pancreatic islet transplantation. It has been demonstrated that islet preparations, betacells, and contaminating duct cells exert a TF-dependent PCA. Activation of coagulation could not only lead to thrombotic events, but also elicit inflammatory reactions involving upregulation of adhesion molecule expression and chemokine production.9.15 We therefore hypothesize that a similar mechanism may occur following LCT. In rat liver transplantation, PCA is postulated to be important in acute rejection (immune cell adherence, vascular thrombosis, and delayed-type hypersensitivity) of allograft.¹⁶ In an animal model, fibrin deposition in the hepatic sinusoids, associated to a hypercoagulative state, is also demonstrated after liver transplantation.17 In humans, modification of prothrombin plasma level might be considered to evaluate rejection after liver transplantation.¹⁸ Furthermore, several LCT studies demonstrate, on animal models, that allogenic hepatocytes are highly immunogenic and stimulate strong cell-mediated immune responses.¹⁹⁻²¹

In the 1990s, 2 independent studies showed either weak or no TF expression by hepatocytes using immunohistochemistry on human liver frozen sections, although TF is known to be present in the HepG2 hepatoblastoma cell line.²²⁻²⁴

Therefore, we have first determined if isolated hepatocytes express TF. The majority of our experiments were performed on cryopreserved/thawed hepatocytes, which represent the main cell source in LCT. Both membrane-bound forms and soluble TF forms were found in isolated hepatocytes, but in low concentration as compared to islet preparations or the CAPAN cell line.⁸ We also showed that expression of TF in hepatocytes induces coagulation in vitro. With the plasma PCA measuring technique, we determined that the PCA activity of the hepatocyte suspension was factor VII-de-



Figure 5. Modulation of TF-dependent PCA of hepatocytes by NAC. (A) PCA of hepatocyte suspension in human plasma. Graded doses of NAC were added to 90 µL of hepatocyte suspension (0.5×10^6 /mL) before PCA was measured. Data represent mean \pm standard deviation of 4 independent experiments. *P < 0.05 compared with absence of NAC. (B,C) 5 × 10^5 hepatocytes were added to 5 mL whole blood incubated in tubing loops under agitation at $+37^\circ$ C. Graded concentrations of NAC were added to the loops extemporaneously. Platelet count (B) and D-dimer (C) levels were monitored after 30 minutes of incubation. Data represent mean \pm standard error of the mean of 5 independent experiments. *P < 0.05 compared with absence of NAC.

pendent and thus related to TF. The tubing loop technique, which mimics whole blood coagulation, allowed us to demonstrate that human hepatocytes do exert a PCA in physiologic context. Finally, we confirmed these results on whole citrated blood and in presence of Ca⁺ by viscometry measurement. Taken together, these in vitro results can possibly be correlated to the portal flow modifications, the decreased number of platelets, and/or the portal presence of hepatocyte-containing thrombi described by Muraca et al.²⁵ after intraportal hepatocyte transplantation in the pig. We also observed a delayed D-dimer increase following hepatocyte infusions in a Crigler-Najjar patient. Interestingly, the other coagulation parameters followed were not affected by the hepatocyte infusions, suggesting that the PCA in vivo can be reduced to an infraclinical level or that cell-dependent coagulation occurs mainly in small liver sinusoids, being therefore undetectable in the high peripheral and portal flows. Similar observations, isolated increase in D-dimers, were noted during the painful coagulation crisis of venous skin malformations or after islet transplantation.^{26,27}

NAC is often used in adult and pediatric treatment of paracetamol intoxication and is considered as safe in human and particularly in children.²⁸ Furthermore, acetaminophen cell injury in cultured mouse hepatocytes is prevented by NAC, probably through a mitochondrial permeability transition inhibition.^{29,30} We also demonstrated in this study that several concentrations of NAC, lower in concentration than that used in paracetamol intoxications, were not toxic for cultured hepatic cells. Recent works also demonstrated that NAC corrects the TF-PCA on TF-expressing pancreatic islets and duct cells.¹¹ NAC is also proposed to ameliorate the early function of the hepatic graft, being able to limit the rate of acute rejection.³¹ Clinical studies of NAC in liver transplants show better liver microcirculation without affecting early function.^{32,33} Finally, NAC is known to modulate the vitamin-K-dependent hemostatic proteins and, therefore coagulation, in paracetamol overdose patients but also in healthy subjects.14 In our study, we highlighted the anticoagulant effect of NAC, as this drug was able to prevent the TF-dependent PCA in vitro.

In conclusion, we demonstrate the TF-dependent PCA of a hepatocyte suspension. As in pancreatic islet transplantation, we may postulate that hepatocyte-related PCA may interfere with hepatocyte engraftment. This phenomenon is probably clinically expressed, after hepatocyte infusions, as we observed a delayed D-dimer increase following LCT in a patient. Further investigations are needed to evaluate the consequences of hepatocytes TF-dependent PCA on cell engraftment and rejection in vivo and to determine the potential clinical benefit of NAC.

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4.2.2 Discussion and perspectives

As observed for islet pancreatic cells, isolated human hepatocyte population expresses tissue factor and induces an *in vitro* dependent pro-coagulant activity, inhibited by N-acetyl-L-cysteine. This has clinical implications since we observed a delayed D-dimer increase in a hepatocyte transplanted patient. Such phenomenon may impair hepatocyte engraftment and promote early rejection. This work represents a starting point for a LCT rejection study. Indeed, LCT efficiency remains time limited and rejection pathways for hepatocyte suspensions may differ from those involved in solid organ or islet allografts transplantation. Evidence of the tissue factor induced pro-coagulant activity and its consequences on cell engraftment, notably by studying inflammatory reaction, must be evaluated *in vivo*. Hepatocyte encapsulation may represent, in addition to freeze damage resistance and absence of tissue factor pro-coagulant activity, an alternative to protect the cells against immunologic response. The understanding of the specific immunogenicity of isolated hepatocytes may be helpful for the development of future suitable strategies (generation of specific immunosuppressants or co-transplantation of hepatocytes with other tolerigenic cell populations). **5.** General Conclusion

5. General Conclusion

LCT with C/T hepatocytes provides short to medium term metabolic improvement and is an efficient bridge for unstable patients to orthotopic liver transplantation. Improvement of engraftment and durability of its effect remain crucial to consider LCT as a full therapeutic option. We provide in this work several ways to enhance cell engraftment, acting on cell suspension. Quality of infused cells is essential for clinical success while cryopreservation remains widely necessary. However, C/T causes significant damage to the cells and may interfere with LCT success. Cryopreservation induces a dramatic drop of ATP levels in hepatocytes. The oxygen consumption rate of C/T hepatocytes is also significantly lower compared to freshly isolated cells, in parallel with a reduction of $\Delta \Psi$. 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. IIF occurs probably during the cryopreservation and/or thawing process and induces mitochondrial damages at the level of respiratory chain complex 1 that cannot be avoided using the current C/T protocols. New perspectives such as vitrification, coupled or not with encapsulation, are potential explorative ways to prevent IIF.

Isolated hepatocytes, whether or not C/T, are also responsible for a tissue factor pro-coagulant activity that may interfere with cell engraftment, by the induction of an aspecific inflammatory reaction. Modulation of this phenomenon with N-acetyl-L-cysteine makes this drug valuable for additional *in vivo* studies.

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6. List of publications

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Annex

Annex: Radiotherapy, a Liver Cell Transplantation recipient pre-conditioning

Another way to improve engrafment and subsequent repopulation is to prepare the recipient liver. Radiotherapy, by inhibiting cell cycle, may provide selective advantage to the transplanted cells as compared to the diseased host cells. Inactivation of passenger lymphocytes, which helps to prevent early steps of rejection of the infused cells is also to be considered. This was evaluated earlier on animal models with high dose radiotherapy giving place and a replicative advantage to the transplanted cells (49,123). Inocuity of radiotherapy needs to be evaluated before eventual clinical use. We therefore retrospectivally reviewed safety and efficacy of low dose radiotherapy as rejection rescue treatment of orthotopic liver transplanted patients. Low dose radiotherapy was not yet evaluated as recipient liver pre-treatment in LCT technique in vivo models.

Liver allograft radiotherapy to treat rejection in children: efficacy in orthotopic liver transplantation and long-term safety

In this annex (article 5), we demonstrated by studying a retrospective cohort of orthotopic liver transplanted patients that low dose radiotherapy appeared efficient and safe as an acute rejection's rescue treatment. However, this is a retrospective evaluation with all the limitations related to this kind of study. No data are available in the LCT literature with low dose radiotherapy as a pre-conditioning recipient treatment. Furthemore, in the animal models used, radiotherapy is combined with another preparation of the recipient liver, partial hepatectomy or liver Fas-induced apoptosis, to obtain a beneficial effect. However, they are difficult to extrapolate to human application. Less invasive procedures are expected. Finally, radiotherapy has no good reputation in the general population and the use of radiotherapy will be difficult to introduce in clinical trials.

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Liver allograft radiotherapy to treat rejection in children: efficacy in orthotopic liver transplantation and long-term safety

Stephenne X, Najimi M, Janssen M, Reding R, de Ville de Goyet J, Sokal EM. Liver allograft radiotherapy to treat rejection in children: efficacy in orthotopic liver transplantation and long-term safety.

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Abstract: Background: We studied, retrospectively, the efficacy to control rejection and long-term safety of liver allograft radiotherapy (RT) performed in 14 children. Long-term safety data were collected with the prospect of possible use of RT in liver cell transplantation (LCT). Methods: Immune suppression included cyclosporine, azathioprine and prednisone. In case of intractable rejection, low-dose allograft RT was administered daily for 3 days, and short-term efficacy was evaluated by liver enzyme assays and histology. The long-term outcome was compared with that of 122 patients undergone transplantation and who had similar treatment, but no RT. Results: Survival at 15 years was 71.4% vs 69.7% in the comparison group. In the RT group, rejection control was complete in six of 14 children and partial in two, all being alive and well 14-18 years later. Ten of 14 children had follow-up biopsy. Six children had normal histology and four had mild unspecific fibrosis. The long-term follow-up biopsy in the comparison group showed fibrosis in 42 of 85 children. The incidence of complications was similar in both groups. Conclusions: This series shows that, such a RT regimen appeared to be efficient and safe as a rescue treatment for acute rejection. Provided that further investigations in animal models show a certain benefit of low-dose irradiation around LCT, such a regimen could be proposed in human liver cell transplant programmes.

Calcineurin inhibitors have paved the way towards the era of routine solid-organ transplantation, but intractable rejection with subsequent graft loss remains common, requiring additional rescue treatments such as OKT3, anti-thymocyte globulins (ATGs), anti-IL2 receptor antibodies and later switching to newer drugs such as tacrolimus, mycophenolate mofetyl, and sirolimus (1–3).

Graft-targeted radiotherapy (RT) has been reported and has been shown to be efficient in adult renal transplantation, but not in liver transplantation or in paediatric solid-organ transplantation. Safety concerns about liver graft RT may be unjustified and have not been properly evaluated, especially in the context of the heavy morbidity of multiple drug immune suppression used in the case of rejection. We therefore retrospectively reviewed the immediate efficacy of graft RT in liver-transplanted children. We also evaluated its safety by comparing their longterm follow-up with that of patients who had Xavier Stephenne¹, Mustapha Najimi¹, Magda Janssen², Raymond Reding², Jean de Ville de Goyet² and Etienne M. Sokal¹

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orthotopic liver transplantation (OLT) during the same period.

Although RT is not currently used in paediatric solid-organ transplantation, this experience in 14 children had to be reported, also because emerging techniques such as liver cell transplantation (LCT) may require pre-LCT-RT to facilitate engraftment and long-term success (4–8).

The underlying idea is that, RT will confer selective advantage to the transplanted cells as compared with the diseased host cells by inhibiting the cell cycle. An additional benefit could be the inactivation of passenger lymphocytes, helping to prevent early steps of rejection of the infused cells.

Patients and methods

Subjects

Between 1986 and 1990, 14 liver-transplanted recipients among a total cohort of 136 children

who had undergone transplantation during the same period received RT as a rescue treatment to control rejection after OLT.

The median age of subjects at the time of OLT in this group was 3.12 years (range: 0.99-12.88 years) as compared with 2.39 years (range: 0.70-14.44 years) in the comparison group (n = 122). In five of 14 (36%) subjects, the pretransplant diagnosis was biliary atresia, and in nine of 14 (64%) subjects, the pretransplant diagnoses were miscellaneous diseases including tyrosinaemia (n = 2), Wilson's disease (n = 1), Crigler Najjar disease (n = 1), type I glycogenosis (n = 1), progressive familial intrahepatic cholestasis (n = 2), cryptogenic cirrhosis (n = 1) and acute liver failure (n = 1). In the comparison group, 91 of 122 (75%) subjects had transplantation for biliary atresia, while most other indications were chronic liver diseases and acute liver failure in four cases.

Immunosuppressive treatment

At the time of transplantation, patients in the two groups received a triple immune suppression therapy consisting of cyclosporine (to reach the plasma trough level of 100 ng/ml), azathioprine (1.5 mg/kg) and prednisone (0.5 mg/kg).

Treatment of rejection initially included high doses of methylprednisolone (1g/1.73 m² body surface area) (9). All patients in the RT group received steroids bolus. In addition, 13 of 14 patients in the RT group received one or more courses of muromonab-CD3 (OKT3, Ortho Diagnostic Systems, Inc., Raritan, NJ), a mouse monoclonal antibody against the CD3 complex of T lymphocytes, for a median of 10 days (range: 1-26) as compared with 22 of 122 patients in the comparison group; 12 of 14 patients also received ATGs (Fresenius, AG Hoeschst, Bad Homburg, Belgium), a polyclonal preparation against T lymphocytes (3), for a median of 8 days (range: 1–17) when liver enzyme levels were elevated and biopsies showed rejection (D2-D3) (43 of 122 in the comparison group). Three of 14 patients had already undergone re-transplantation before RT, two for chronic rejection, one for graft venous occlusion.

After failure of more intense immunosuppressive treatment (steroids bolus, OKT3 and ATG) in 14 patients, it was decided to use liver RT as a salvage treatment for untractable rejection (D2– D3). RT use was based on the experience in renal transplantation, as most of the immunosuppressive medications. Hundred and fifty centi-Gray (cGy) was administered for 3 consecutive days (Cobalt, Mevatron, Saturne- Co60, X8, X18) within 45 days from transplant surgery. The

Liver radiotherapy to control rejection

children were not sedated or anaesthetised. Four of 14 patients received RT within 15 days following OLT, five of 14 within 15 days and 30 days following OLT and the last five patients received it between 30 and 45 days post-OLT. No curie therapy was administered. As tacrolimus (Fujisawa, Berlin, Germany) became available, all patients in both groups, were switched to this drug as rescue treatment (2, 10).

Efficacy to control rejection was evaluated by serum enzyme measurements and histology. Treatment was considered efficient if no further rejection treatment was required following RT and if there was no subsequent graft loss.

Long-term follow-up

Long-term safety was compared with that of the time-matched cohort. For the two groups, patient survival and long-term complications were reviewed. The data analysed included causes of death, incidences of re-transplantation, posttransplant lymphoproliferative disease (PTLD) and Epstein–Barr virus (EBV)-related lymphoma, adenovirus infection and posttransplant hepatitis C infection. Liver biopsies were performed during routine posttransplant follow-up visits, and were evaluated by the same pathologist for inflammation and fibrosis using Batts and Ludwig's score (one grading system used for assessing inflammation and fibrosis) (11), with particular attention to possible RT-induced sequellae.

Statistical analysis

The data were analysed using Fisher's exact test. Patient survival was calculated by the Kaplan– Meier method.

Note: It is important to mention that the heavy immune suppression schema of this early transplant era is no more in use today.

Results

Efficacy on rejection

Rejection control categorisation was based on results of hepatic enzymes levels and histology. Following RT, full rejection control was achieved in six patients, partial control in two, while two had ongoing rejection. All subjects are alive and well at the 2004 follow-up; one subject was suffering from chronic hepatitis C. One of the subjects with partial rejection control had experienced acute limited adenovirus infection, and one has received chemotherapy for EBV-related Hodgkin's disease 96 months after RT. The two patients with partial control of rejection were

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later switched to tacrolimus. The two remaining survivors with ongoing rejection received additional OKT3, and were later switched to tacrolimus. RT was judged as the ultimate treatment to control rejection in six patients, and as a partial control and bridge to newer drugs without re-transplantation in the remaining four.

Early acute adverse events

The RT administration was well tolerated without any skin rash, nausea or liver enzyme elevation.

Survival and death

Long-term 15-year survival in the RT group is 71.4% (seven of 14) as compared with 69.7% (85 of 122) in the comparison group (P = 0.891), within the range of transplantation survival at that time (Fig. 1).

In the RT group, four patients died: one of adenovirus infection 2 months post-OLT, one of autoimmune hepatitis recurrence (1 month post-OLT), and two following re-transplantation because of peroperative complications (third graft).

In the comparison group, 37 patients died: 15 (40.5%) from bacterial, fungal or viral infections, 15 (40.5%) from peroperative complications, two (5.4%) from tumoral diseases, two from cerebral oedema (5.4%), one (2.7%) from intracranial haemorrhage and two (5.4%) from other causes.

Long-term adverse events

Posttransplant lymphoproliferative disease

One of 14 patients in the RT group developed EBV-related cervico-mediastinal Hodgkin's disease 96 months post-OLT. Successfully treated with chemotherapy, the patient is alive and well 14 years later (12). Four of 122 (3.3%) patients in



 $\mathit{Fig.\,I}.$ Comparative survival of the two groups with or without radiotherapy.

the comparison group developed EBV-related PTLD, 9–48 months post-OLT. Three responded to reduction of immune suppression being alive and well on follow-up, while one died of multiple organ failure. At the 15 year follow-up, three are alive and well. The incidence of EBV-related lymphoproliferative disorders (3.3%) is in agreement with incidences reported in other studies (13, 14). The difference in incidence of PTLD between the RT and the comparison group was not significant (P = 0.424). No other malignancy was observed in either of the groups at the time of the 2004 follow-up.

Adenovirus infection

Five patients in the total cohort (136) experienced acute adenovirus hepatitis after OLT: two of 14 (14.2%) in the RT group and three of 122 (2.5%) in the comparison group (P = 0.083) (9).

All had received the classical immunosuppressive treatment including cyclosporine, azathioprine and prednisone. Despite this treatment, each presented a first episode of rejection. The two of 14 with steroid-resistant rejection received additional immune suppression, including an initial 10-day course of ATG and OKT3 monoclonal antibody. The same treatment was administered to two patients of the comparison group; the remaining acute adenovirus case in the comparison group did not need this particular treatment. One patient in each group died of acute hepatic failure, both with the most elevated liver enzymes of the subjects discussed. Others recovered after cessation of immune suppression. Adenovirus infection was related to excess of immune suppression.

Hepatitis C

This cohort had OLT before the implementation of systematic screening of donors for hepatitis C virus, and 13 of 136 (9.6%) developed chronic hepatitis C (15). Twelve of 122 (9.8%) were hepatitis C positive in the comparison group as compared with one of 14 (7.1%) in the RT group (P = 0.855).

Re-transplantation

Re-transplantation was performed in three of 14 (21.4%) in the RT group vs 26 of 122 (21.3%) in the comparison group (P = 1.000). The mortality rate among re-transplantation subjects in the RT group was 66.7% (two of three) as compared with 42.3% (11 of 26) in the comparison group. Re-transplantation occurs before RT treatment.

Histology

Ten patients in the RT group survived and had long-term follow-up biopsy, up to 152 months after RT (range: 25-152 months). Six of 10 had normal histology and four had mild fibrosis, one related to hepatitis C. The biopsies of these four patients were classified according to Ludwig, P2L2S1, P1L2S2, P2L2S2, P2L2S2, with minimal piecemeal necrosis in one case and mild piecemeal necrosis in the three other cases. Lobular inflammation and necrosis with little hepato-cellular damage were observed in four cases, while fibrosis was demonstrated in the portal space in the biopsy of one patient, and was extended to the peri-portal zone in the three other biopsies. The long-term follow-up biopsy in the comparison group showed mild (S2) to moderate (S3) fibrosis in 41 of 85 (48.20%) (P = 0.748), 12 of them in relation to hepatitis C. Severe fibrosis (S4) was observed in one of 85 patients. There was no significant difference in the incidence of mild to moderate fibrosis between the two groups.

No other RT-related specific histological signs were observed (Tables 1 and 2).

Discussion

The present experience in 14 children who received RT to treat intractable graft rejection is unique, and can be considered as a pilot study to control rejection and demonstrate the long-term safety of RT. Nevertheless, this is a retrospective study, with a limited number of patients, with all the limitations associated with this kind of analysis.

RT has not been reported so far for liver graft rejection treatment, probably because of increasing availability of new anti-rejection drugs and cytokines. RT was proposed as a last-resort treatment of rejection in our patients, based on the data available on renal transplantation. The 14 patients were compared with 122 patients who

Table 1. Clinical characteristics of 14 patients who received radiotherapy

Table 2. Safety (infections, re-transplantation and liver fibrosis) was compared with that of the time-matched cohort

	RT group (%)	Comparison group (%)	P value
PTLD	7.1	3.3	0.424
Adenovirus	14.2	2.5	0.083
Hepatitis C	7.1	9.8	0.855
Re-transplantation	21.4	21.3	1.000
Mild to moderate fibrosis	40	48.2	0.748

RT, radiotherapy; PTLD, posttransplant lymphoproliferative disease.

had undergone transplantation during the same period under the same immunosuppressive treatment. RT was followed by full or partial control of rejection in 80% of survivors. Nevertheless, it is difficult to prove the independent beneficial effect of RT, because this rescue treatment was administered only after the failure of other immunosuppressive drugs.

In parallel, no long-term side effects were observed, and adverse events in this group were not different in nature or more frequent than in the comparison group.

Long-term histological evaluation of the liver in the RT group did not show any different pattern as compared with the period-matched group. In addition, there was no increased incidence of infectious or degenerative diseases including PTLD or lymphomas, known to be facilitated by RT. Between 1986 and 1990, heavy immune suppression was used and this was responsible for severe infectious and PTLD problems, which we extensively reported (12, 16–18). Over the years, immune suppression protocols have been alleviated to move towards tacrolimus mono-therapy in all these children (19).

In accordance with our data, one case report of liver targeted high-dose (24 Gy delivered in

Graft no.	RT	0 KT3	ATG	PTLD	Adenovirus	Hep C	Retx	Liver fibrosis*	Death
304	Yes	Yes	Yes					P1L1S1	
274	Yes	Yes	Yes					ND	Yes
63	Yes		Yes					P1L1S1	
303	Yes	Yes	Yes			Yes		P1L1S1	
269	Yes	Yes	Yes				Yes	ND	Yes
232	Yes	Yes	Yes					P2L2S1	
259	Yes	Yes	Yes	Yes				P1L1S1	
335	Yes	Yes	Yes		Yes			ND	Yes
275	Yes	Yes	Yes					P1L2S2	
143	Yes	Yes					Yes	ND	Yes
331	Yes	Yes					Yes	P1L1S1	
338	Yes	Yes	Yes					P2L2S2	
254	Yes	Yes	Yes		Yes			P2L2S2	
39	Yes	Yes	Yes					P1L1S1	

ND, not done; RT, radiotherapy; ATG, anti-thymocyte globulins; PTLD, posttransplant lymphoproliferative disease. *Batts and Ludwig histological score of the biopsies (9).

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fractions of 2 Gy) RT proposed as treatment of post-OLT portal hepatic region PTLD was reported. This patient did not show any radioinduced damage during the 19-month follow-up (20).

High-dose RT may induce different kind of cancers especially when associated with concomitant use of cytostatics drugs (21) (virtual risk: 10% of radioinduced cancers after 1 Gy of total irradiation). Five Gy in full-body irradiation represents 50% lethal dose (the dose that may cause death in 50% of cases during the 15 days following RT). The liver is not a radiosensitive organ, as are the lungs and the bone marrow, but every organ may develop RT-induced damages such as fibrosis. X-ray-induced hepatitis is expected after 25 Gy of RT directed to the liver, while loss of liver function occurs from 45 to 50 Gy, far higher than the doses used in the present series.

In renal transplantation, RT was shown to be effective against rejection in 60 of 72 patients with no radiation-related toxicity (local irradiation: 8.0 Gy), while OKT3 and pulse of steroids were ineffective. Thirty-five of the 60 patients had no more episodes of rejection following RT (22). Other trials failed, however, to confirm any benefit from local graft irradiation as an adjuvant treatment of rejection (23). The authors have concluded that RT in kidney graft rejection is best indicated as a last-resort treatment of rejection, using a response-predicting score (24-26). Endolymphatic irradiation was also performed in some centres in preparation for renal transplantation. Patients in the treated group received lipoiodine containing ¹³¹I with specific activity ranging between 4 and 6 mCi/ml. After a 26-year follow-up, the authors concluded that this technique was safe with no increase in tumour incidence and could be indicated for cadaveric renal recipients (27). In renal transplantation as in liver transplantation, lymphoproliferative diseases and *de novo* malignancies are related to immune suppression in general, and not specifically to RT (28).

Besides the potential to control rejection in OLT, pretransplant RT is of potential benefit in LCT. Indeed, LCT in animal models without selective advantage to the transplanted cells is followed by a limited engraftment. RT is known to inhibit the cell cycle and in this way may confer an advantage to the transplanted cells if RT is applied on host liver. Known data on RT and LCT were obtained using high-dose RT, not compatible with human care. Guha et al. evaluated the effect of high-dose RT (50 Gy) on the host liver before LCT in animals. Partial hepa-

tectomy (PH), RT and then LCT into the spleen of rats led to an increased recipient survival as compared with PH or RT alone. Rats with PH and RT developed RT-induced liver damage (portal fibrosis and bile duct proliferation) and steatosis, while additional LCT could prevent onset of portal fibrosis, bile duct proliferation or steatosis (8). The same team achieved repopulation of Gunn rat liver subjected to PH/ RT (50 Gy) using wild-type rat hepatocytes (29). RT associated with Fas Ligand-induced apoptosis before LCT led to re-population of the Gunn rat livers by the infused hepatocytes (30).

Thus, in these animal studies, **RT** was required to block the cell cycle, while liver re-population was facilitated by a mitotic stimulus (**PH**).

Another beneficial aspect of RT would be the inactivation of the passenger lymphocytes to prevent early steps of rejection of transplanted liver cells.

Because the available data obtained on animal models have only used high-dose of RT, further animal studies are required to confirm the benefit of low-dose RT regimen in LCT before extrapolating any data at the human level.

We conclude that clinical use of low-dose RT was possibly efficient in controlling resistant rejection and was safe for the patients, although prospective studies with larger numbers may be required to confirm these data. This information remains an important background if the use of RT is considered in human LCT programmes.

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