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Effect of two storage solutions on proteolysis in isolated rat livers

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Abstract

One process identified as detrimental during liver preservation is proteolysis. The aim of this study was to test two preservation media, the University of Wisconsin (UW) solution and a simplified variant so-called Cochin (Co) solution with respect to their potential antiproteolytic effect. A model of isolated rat liver perfused after 24-h cold storage was used. Graft viability was assessed on biochemical and functional parameters. Proteolysis was assessed on amino acid metabolism. Survival of rats following orthotopic transplantation of 18-h cold-stored livers was determined. Proteolysis, assessed on valine release in the preservation solution, was limited in UW (0.12 ± 0.01 versus 0.18 ± 0.03 μmol/g liver, $p < 0.05$). The protective effect of UW extended to reperfusion after storage and valine uptake was maintained in UW-stored livers but decreased in Co-stored organs (-12.39 ± 5.93 versus -1.84 ± 5.51 nmol/min/g liver). Enzyme release, portal flow rate and bile flow rate were similar in both groups. However, survival after orthotopic transplantation confirmed the superiority of UW over Co. These results demonstrated the antiproteolytic effect of UW solution in cold-stored rat livers. Amongst components omitted in the simplified UW solution, hydroxyethylstarch was identified as the more probable support of this effect.

Keywords: Cold storage; Liver; Perfusion; Proteolysis; Transplantation

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1. Introduction

In the context of liver transplantation, the graft viability is largely dependent on cold storage quality (Clavien et al., 1992). Several studies have shown that proteolysis is an important mechanism of hepatic damages. Indeed, Calmus et al. (1995) have underlined the existence of a proteolysis phenomenon during cold preservation of human liver grafts, correlating with the dysfunction after transplantation. In rats, Ferguson et al. (1993) have observed a progressive increase in total cytosolic protease activity in cold-stored livers. According to Clavien et al. (1993), soluble mediators are released during the hypothermic period and are responsible for a significant part of cold preservation injury. In this latter study, the possibility of preventing this injury by adding antiproteases to the preservation solution confirms that proteolytic reactions are involved in this type of injury. Although various protease inhibitors have been tested by several authors (Takei et al., 1990; Den Butter et al., 1991; Geeraerts et al., 1991; Oldhafer et al., 1991; Dickson et al., 1992), the effect of the cold storage solution itself on proteolytic reactions remains unexplored. Nowadays, most of the studies are performed with University of Wisconsin (UW) solution (Kalayoglu et al., 1988) which constitutes a reference. Beside this solution, some simplified variants are used in human liver transplantation essentially for their lower cost. That is the case in France for Cochin (Co) solution lacking the hydroxyethylstarch, adenosine and allopurinol (Boillot et al., 1991). UW and Co solutions have been used for relatively short-term storage of human liver grafts without clinical differences (Boillot et al., 1991). The present study was undertaken to determine the effect of the composition of the preservation solutions on proteolysis in cold-stored liver grafts with special respect to a potential antiproteolytic role of some of their components. A model of isolated rat liver perfused after 24-h preservation was used in order to determine the graft viability and to assess the proteolysis. Orthotopic transplantation allowed us to determine the effect of the preservation solution on survival.

2. Materials and methods

Composition of UW solution (Du Pont PHARMA S.A., Paris, France), and Co solution (Pharmacie Centrale des Hôpitaux de Paris, France) is shown in Table 1.

2.1. Isolated perfused rat liver

Male Sprague–Dawley rats (Charles River, Cléon, France, 225 ± 6 g body wt.) were fasted overnight before experiment in order to standardize their nutritional status. They had free access to a glucose solution (15 g/l). Animals were anaesthetized with sodium pentobarbital (60 mg/kg, intraperitoneally) and the livers were prepared according to Miller's technique (Miller, 1973). Briefly, the bile duct was cannulated; then, after intravenous injection of 500 units of heparin, a plastic tube was inserted into the portal vein which was clamped distally. Livers were perfused in situ with lactated Ringer's solution at $+4$°C, followed by the preservation solution (either UW or Co) at $+4$°C. They were excised, immersed in 50 ml of preservation solution and stored for 24 h at $+4$°C.

Table 1
Composition of UW and Co solutions

Components	UW	Co
Hydroxyethylstarch (g/l)	50	—
Lactobionic acid (mM)	100	100
Potassium phosphate monobasic (mM)	25	25
Magnesium sulphate heptahydrate (mM)	5	5
Raffinose pentahydrate (mM)	30	30
Adenosine (mM)	5	—
Allopurinol (mM)	1	—
Glutathione (mM)	3	3
Potassium hydroxyde (mM)	100	100
Sodium hydroxyde	Up to pH 7.4	Up to pH 7.4
Water for injection	Up to 1000 ml	Up to 1000 ml

Four series of experiments were performed. Ten livers (7.31 ± 0.38 g) were rinsed and stored for 24 h at $+4$°C in UW solution (UWS, $n = 5$) or in Co solution (CoS, $n = 5$). Ten livers (7.28 ± 0.30 g) were only rinsed and were not stored (control groups: UWC and CoC, $n = 5$ in each group). Preservation solution samples from UWS and CoS groups were taken off after 24 h of storage for biochemical analysis.

After storage, livers were rinsed with lactated Ringer's solution at $+4$°C in order to remove the preservation solution. Then, Krebs–Ringer–albumin buffer at $+37$°C was infused in order to remove the previous solution and to warm up the livers. Unstored control livers were submitted to the same procedure. All the livers were then perfused through the portal vein at a constant pressure with a recirculating system in a thermostatically controlled cabinet ($+37$°C) for 90 min. The perfusate was Krebs–Ringer buffer with bovine serum albumin. According to the protocol determined by De Bandt et al. (1990), this medium was supplemented at the start of the perfusion with eight antiproteolytic amino acids (alanine, glutamine, histidine, leucine, methionine, phenylalanine, proline, tryptophan) at twice their physiological concentration in normal rats; after 30 min of perfusion, a balanced mixture of amino acids was added to the perfusate in order to allow the measurement of amino acid exchanges under physiological conditions (De Bandt et al., 1990). The pH of the perfusate was maintained at 7.40 ± 0.05 by adjusting the O_2/CO_2 ratio. Perfusate samples (1 ml) were taken for biochemical analysis at the portal inflow and at the venous effluent at time 3, 10, 20, 30, 35, 60 and 90 min. Hepatic flow rate and pH were also measured at each time. Bile was collected through the cannulated bile duct at time 30, 60 and 90 min.

2.2. Orthotopic liver transplantation

Liver transplantations were performed according to the technique of Kamada and Calne (1979) under ether anaesthesia. Male Lewis rats (Charles River, Cléon, France, 223 ± 3 g body wt.) were used as donors and recipients. They were fasted overnight before experiments and had free access to a glucose solution (15 g/l).

For donor operation, the bile duct cannulation was performed using a polyethylene tube. After section of hepatic ligaments, 500 units of heparin were injected i.v. through the inferior vena cava. The liver was perfused in situ by infusion through the portal vein of lactated Ringer's solution at $+4$°C, followed by the preservation solution at $+4$°C. The liver was then removed and immersed in the preservation solution at $+4$°C. Two polyethylene cuffs were disposed on the portal vein and on the infrahepatic vena cava. Livers were stored for 18 h at $+4$°C either in UW ($n = 7$) or in Co ($n = 4$).

The recipient liver was removed and the preserved graft was placed orthotopically. The suprahepatic vena cava was anastomosed end-to-end. The liver was then rinsed with lactated Ringer's solution at $+4$°C. Cuffs of donor portal vein and infrahepatic vena cava were inserted into the respective recipient vessels. The bile duct was anastomosed. Survival time of transplanted animals was assessed up to 4 weeks. Blood samples were taken from recipients 1 h after transplantation, then at days 1, 2, 7, 14 and 28, in order to measure enzymatic plasmatic activities. The surviving rats were sacrificed on 28th day.

Isolated perfused liver and orthotopic transplantation in the rat were performed in compliance with the guidelines for animal care of our institution. L.H. and L.C. are officially authorized (# 01386 and # 005226) by the French Ministry of Agriculture and Forestry to use these experimental models.

2.3. Analytical methods

Enzymatic activities (AST, ALT, LDH, CK) were routinely measured using a CX 4 multiparameter analyzer (Beckman, USA). Measurements were made in the preservation solutions after 24-h cold storage of the graft and in the Krebs–Ringer–albumin buffer. Samples were processed in h following the experiments. For analysis of amino acids, the samples were deproteinized immediately using sulfosalicylic acid (50 mg/ml). Individual free amino acid concentrations were measured by ion-exchange chromatography (model 6300, Beckman, USA).

2.4. Calculations and statistics

Rates of amino acid uptake or release (R) by the isolated perfused liver between 35 and 90 min of perfusion were calculated according to the formula:

$$R = \frac{C_{90} - C_{35}}{90 - 35} \times \frac{V}{P}$$

where V is the volume of the perfusate (150 ml), P is the liver wet weight. C_{35} and C_{90} are the portal concentrations of a given amino acid at times 35 and 90 min, respectively. Results are expressed as nmol/min/g of liver.

All results are given as mean ± S.E.M. Analysis of data was performed using the Mann–Whitney test. Differences between groups were considered as significant at $p < 0.05$.

3. Results

3.1. Assessment of proteolysis and viability of cold-stored livers

3.1.1. Measurements in preservation solutions

After 24 h of cold storage, activities of AST and ALT were negligible (< 0.2 U/g liver/24 h). LDH activities were 1.44 ± 0.20 in Co and 1.05 ± 0.17 U/g liver/24 h in UW, and CK activities were 5.76 ± 1.91 in Co and 2.93 ± 0.89 U/g liver/24 h in UW. These values were not significantly different. Some amino acids were released during cold storage in the preservation solutions, especially glutamate, glutamine, glycine, alanine, valine and leucine (Table 2). Their amounts were similar in both solutions, except for valine levels which were significantly increased in Co.

3.1.2. During perfusion

The hepatic flow rate is represented on Fig. 1. Control livers showed a nearly constant portal flow rate during the whole perfusion time. In stored livers, portal flow rate was significantly decreased at the beginning of the perfusion, no matter the preservation solution. From the 10th minute of perfusion, flow rates of stored livers were not statistically different from those of control livers. In the same way, bile flow rate (Fig. 2) of control livers was approximately constant over the 90 min of perfusion, whereas stored livers showed a less bile production during the first 30 min of perfusion.

Table 2
Release of amino acids in preservation solutions during 24-h cold storage

	Amino acid release (μmol/g liver/24 h)	
	By livers stored in UW	By livers stored in Co
Glutamate	1.15 ± 0.07	0.99 ± 0.18
Glutamine	1.08 ± 0.18	1.22 ± 0.04
Glycine	2.23 ± 0.10	2.01 ± 0.13
Alanine	1.41 ± 0.10	1.79 ± 0.25
Valine	0.12 ± 0.01	0.18 ± 0.03*
Leucine	0.11 ± 0.01	0.13 ± 0.01

* $p < 0.05$ versus livers stored in UW.

Between the 35th and 90th minute of perfusion of the livers, an uptake of amino acids was observed (Table 3). Control livers and stored livers had the same behavior with regard to amino acid fluxes. However, the uptake of the two branched-chain amino acids, valine and leucine, was decreased after cold storage, markedly in the Co group. In the UW group, the decrease was moderate and did not reach significance. In the Co group, the decrease was dramatic and significant.

3.2. Survival after transplantation

Survival rate of rats transplanted with livers preserved either in UW solution or in Co solution is shown in Fig. 3. The 14-day survival was five animals over seven for the UW group, and one animal over four for the Co group. Due to the poor survival rate obtained in the latter group, and considering the constraining procedure for transplantation, no further experiments were carried out with Co solution. AST and ALT activities measured in the plasma of rats transplanted with grafts preserved in UW solution are shown in Fig. 4. The profile of enzymatic activities was characterized by a plasmatic peak on the first day after transplantation.

4. Discussion

The major finding of this study relates to the ability of UW solution to protect cold-stored livers from proteolysis. This effect was observed by comparison with the simplified variant of UW solution, so-called Cochin solution. We chose to assess proteolysis on amino acid release in the preservation solution after storage of the graft. This was previously used by Calmus et al. (1995), who correlated amino acid release from human liver grafts with the cold ischemia time and with graft dysfunction. Theoretically, hepatic release of amino acids could result either from a cytolytic process or from a proteolytic one. But cytolysis, reflected by enzyme release, was moderate and similar in UW and Co solutions, an observation which supports the involvement of proteolysis. In the work of Calmus et al. (1995), 20 amino acids were measured in UW solution and the concentration of six amino acids including leucine strongly correlated with the storage duration. In our study, although leucine release was higher in Co than in UW, the difference did not reach significance. In the present study, valine variations were more sensitive. The reason for this discrepancy remains unclear. However, one can underline the fact that valine, as well as leucine, are both branched-chain amino acids whose metabolism is very close. Hence, both are poorly metabolized in the liver (Harper et al., 1984), so that their release reflects a net protein catabolism (Mortimore and Poso, 1987; Ward, 1988). Furthermore, our experimental conditions were appreciably different from those in the paper of Calmus et al.: the authors worked on human livers while we used rat livers

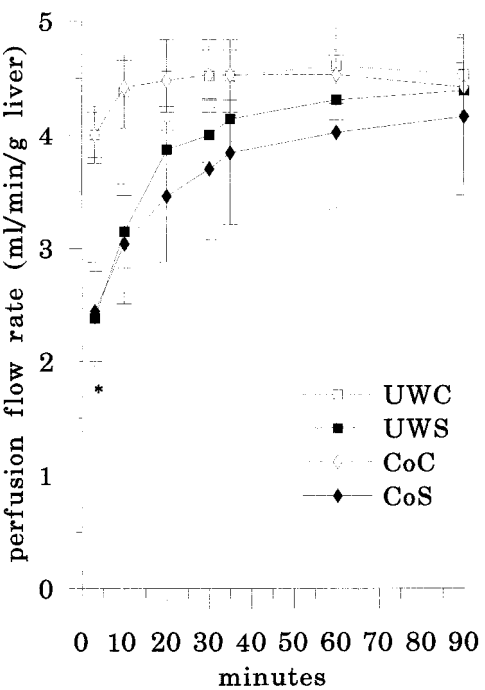

Fig. 1. Portal flow rate in perfused rat livers. Portal flow rate was measured for 90 min on isolated perfused rat liver. Portal flow rate was nearly constant for non-stored control livers (UWC and CoC) and was initially decreased in livers stored for 24 h in UW solution (UWS) or in Co solution (CoS). Data are mean ± S.E.M., $*p < 0.05$ versus respective control groups C.

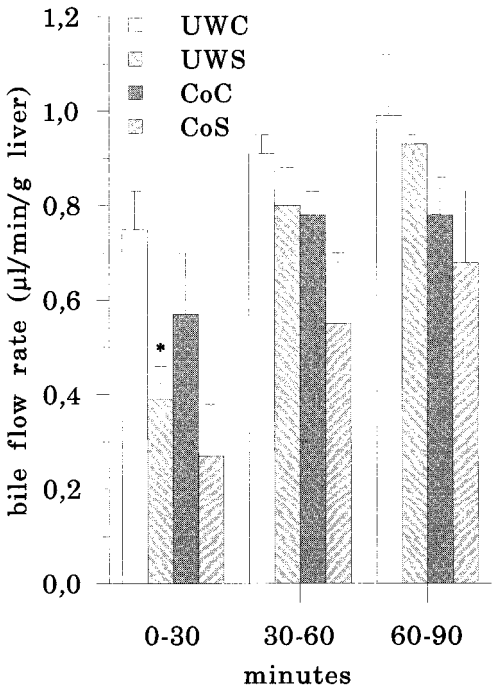

Fig. 2. Bile flow rate in perfused rat livers. Bile flow rate of isolated perfused rat livers was calculated between 0 and 30, 30 and 60, 60 and 90 min of perfusion. After 24 h of cold storage in UW solution (UWS), or in Co solution (CoS), bile production was decreased during the first 30 min, compared to bile production of non-stored control livers (UWC and CoC). Data are mean ± S.E.M., $*p < 0.05$ UWS versus UWC.

Table 3
Amino acid fluxes between 35 and 90 min of perfusion

	Amino acids fluxes (nmol/min/g liver)[a]			
	In UW control livers	In UW-stored livers	In Co control livers	In Co-stored livers
Glutamine	−44.42 ± 6.74	−18.24 ± 17.02	−26.92 ± 4.60	−21.18 ± 9.29
Glycine	−12.92 ± 3.22	−21.10 ± 6.23	−14.21 ± 4.59	−16.57 ± 7.91
Alanine	−112.70 ± 13.48	−103.84 ± 11.23	−97.81 ± 5.04	−95.95 ± 12.58
Valine	−27.90 ± 3.46	−12.39 ± 5.93	−24.61 ± 2.33	−1.84 ± 5.51*
Leucine	−46.52 ± 6.35	−29.32 ± 3.48	−40.00 ± 2.54	−5.57 ± 11.64**

[a] Negative values = uptake.
*$p<0.01$; **$p<0.05$ versus Co control livers.

and their median storage duration was 11 h while ours was 24 h. Interestingly, the amino acid metabolism of stored livers was also modified during their reperfusion. In our isolated perfused liver model, the 55 min between 35 and 90 min of reperfusion constitute the period of metabolic study. During that period, livers normally uptake the amino acids provided by the supplemented perfusate (De Bandt et al., 1990) as observed with non-stored control livers. After storage, the uptake of alanine, the major gluconeogenic amino acid, was unchanged showing that the hepatic metabolic function was preserved. However, va-

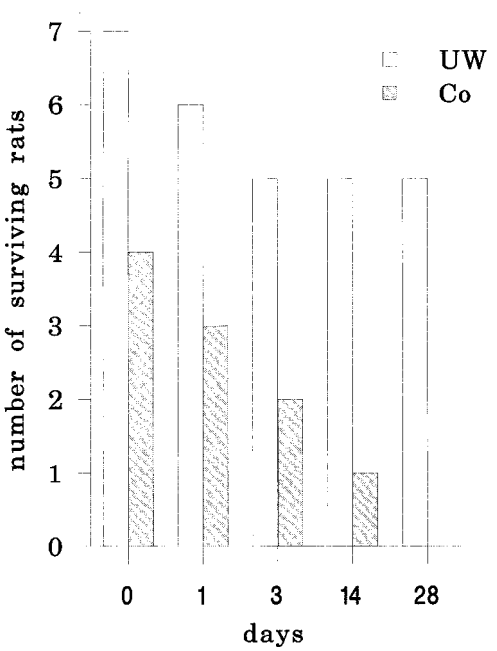

Fig. 3. Survival time after orthotopic liver transplantation in rats. Eighteen h of cold storage of the grafts represented survival conditions with UW preservation solution. Survival rate was decreased after 18 h of preservation in Co solution.

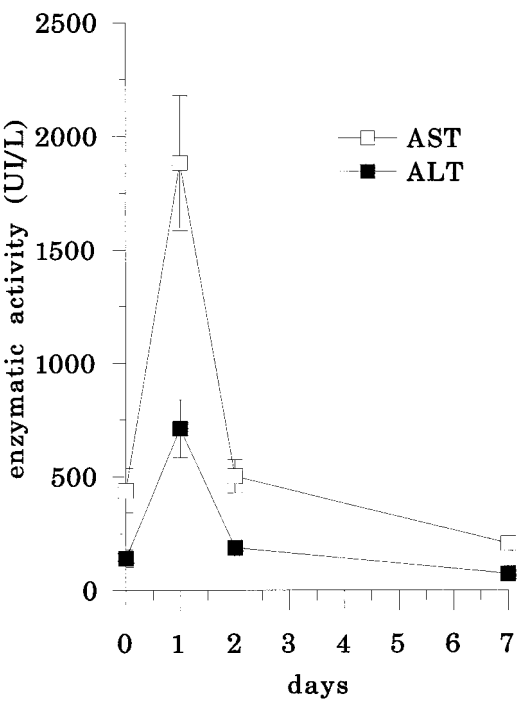

Fig. 4. AST and ALT activities in transplanted rats. After 18 h of cold storage of livers in UW solution, plasmatic AST and ALT activities were measured in surviving recipients 1 h after transplantation and then on first, second and seventh days. The profile of transaminase activities showed a characteristic peak on the first day.

line and leucine fluxes were again particularly affected, showing that proteolysis continued after storage. But the phenomenon was less in the UW group than in the Co group, suggesting that the protective effect of UW was not limited to the cold storage time but extended to reperfusion. Besides their differences regarding amino acid metabolism, stored livers showed rather identical functional behavior in UW and Co groups: compared to non-stored control livers, portal flow rate was transiently decreased at the very beginning of the perfusion and bile production was concomitantly decreased. Both measurements were not discriminative for UW and Co comparison.

In parallel, orthotopic transplantation also showed superiority of UW over Co solution. In preliminary experiments, following 24 h of storage in UW, the survival rate was poor (33% at 3 days and 0% at 7 days; data not shown), so that the preservation time was reduced to 18 h, at which time the 28-day survival rate was more than 70%. These conditions of preservation were therefore considered as survival conditions. Using Co solution, the survival rate following transplantation decreased dramatically to 25% at 7 days.

The main question raised by this study is to know why UW solution is more protective against proteolysis than Co solution. Since the difference between the two solutions consists in three components, the hydroxyethylstarch, adenosine and allopurinol (Boillot et al., 1991), either one of these three products or their combination should be responsible for UW antiproteolytic effect. Originally, these molecules have been included in the composition of UW solution on the basis of their specific activities (Wahlberg et al., 1986), without concern with proteolysis. However, a potential antiproteolytic effect could be suspected for these components. Indeed, hypothermia, the basic principle for storage of grafts leads inevitably to energetic loss which is the starting point for preservation injury (Clavien et al., 1992). Hence, ATP depletion results in (i) xanthine accumulation and xanthine-oxidase activation (McCord, 1985), both responsible for production of oxygenated free radicals whose targets are lipids as well as enzymatic and structural proteins (Clavien et al., 1992); (ii) anaerobic glycolysis generating cellular acidosis associated with the activation of phospholipase and proteases (Gores et al., 1989); (iii) expansion of the interstitial space due to water leakage from the vascular space following Na^+/K^+-ATPase inhibition (Ar'Rajab et al., 1991). As a precursor for adenine nucleotide regeneration, adenosine could indirectly reduce proteolysis by preserving the ATP pool. Allopurinol could also contribute to this protective effect regarding to its specific property to inhibit xanthine-oxidase. However, these explanations remain hypothetical: since ATP synthesis from adenosine is oxygen dependent (Southard et al., 1984), one cannot assume a positive effect of adenosine during the anoxic cold storage but only during oxygenated reperfusion. In addition, allopurinol concentration in UW has been demonstrated to be lower than the concentration necessary to inhibit xanthine-oxidase activity (Metzger et al., 1988; Jaeschke, 1991). Therefore, the more probable support of the antiproteolytic effect of UW remains the hydroxyethylstarch. This component is a stable colloid providing oncotic support and limiting interstitial edema by reducing fluid exchange between vascular and interstitial spaces (Ar'Rajab et al., 1991). In that way, it maintains the hydration state of the liver. Häussinger et al. (1991) have positively identified cellular hydration as a major site of proteolysis control in liver. According to the authors, cell swelling inhibits proteolysis, whereas cell shrinkage stimulates hepatic protein breakdown. Hydroxyethylstarch could therefore inhibit proteolysis through the preservation of cell volume. A recent study of our group (Neveux et al., 1997) evaluating the influence of the deletion of hydroxyethylstarch in UW solution supports the hypothesis: while the intracellular volume measured in cold-stored livers was well maintained in UW, it was significantly reduced in UW without hydroxyethylstarch and was associated with a dramatic increase in proteolysis.

Considering that proteolysis could be a key-process in hepatic failure, the improvement of liver graft viability lies in its inhibition. UW already provides a good protection, but proteolysis although limited still exists. Since the preservation of cell volume seems to be necessary for the

antiproteolytic effect of UW, it would be worth accurately controlling this parameter in order to optimize liver storage.

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