Further Studies on Long-term Preservation of Rat Liver: Celsior versus UW Solution

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Abstract. The effects of Celsior solution were compared with those of the University of Wisconsin solution (UW) after 18 or 48 hours of cold storage in a perfused rat liver model. Lactate dehydrogenase (LDH), hyaluronic acid (HA) uptake, thiobarbituric acid-reactive substances (TBARS), tissue reduced (GSH) and oxidized glutathione (GSSG) and ATP were evaluated. Histochemical in situ evaluation of LDH and reactive oxygen species (ROS) were also performed. No significant difference in LDH release, HA uptake, TBARS, ATP levels and GSH/GSSG ratio were observed between UW and Celsior solution when the livers were preserved for 18 hours. By contrast, when preservation was performed for 48 hours, LDH release, TBARS and ROS formation were higher and the ATP levels, GSH/GSSG ratio and HA uptake were lower in the liver preserved by Celsior as compared with UW. Celsior solution was as effective as UW in liver preservation up to 18 hours but the superiority of UW over Celsior solution was obtained when liver was preserved for 48 hours.

Orthotopic liver transplantation (OLT) has become a well established therapy for end-stage liver disease of various etiologies. The University of Wisconsin (UW) solution is the most widely used preservation solution for liver storage. Celsior solution, usually used for heart transplantation, has also been proposed as a cold-storage solution for liver, but its use is still under investigation. Valero et al. have demonstrated that Celsior is similar in its effectiveness to UW for preservation of liver obtained from non-heart-beating donors in experimental pig liver transplantation after 6 hours of cold storage (1). Van As et al. have shown that there was less hepatocellular injury in livers preserved in UW and less reperfusion injury in livers preserved in Celsior, in pig liver transplantation (2). In their experimental setup, livers were stored in UW or Celsior for only 3 hours and the endothelial cell injury was similar in both solutions. Celsior appeared to protect both hepatocytes and sinusoidal endothelial cells from ischemia-reperfusion injury in a canine liver transplantation model, but the organ in this study was stored at 4°C for only 4 hours (3). Preliminary reports suggest that, similar to UW solution, Celsior is appropriate for clinical use in liver transplantation, but in these studies the cold ischemic time considered was no more than 7 hours (4). UW and Celsior are equally effective in preventing rat liver death for periods of up to 16 hours of cold storage but after 24 hours rat livers were best preserved in UW (5). Howden et al. have demonstrated that Celsior is not effective in long-term liver preservation in orthotopic rat liver transplantation after 24 hours of cold preservation (6).

Preservation solutions allowing the time span of preservation to be prolonged and the survival of all types of liver cells to be improved are still being sought. While in the literature the prolongation of UW use for 48 hours has been reported (7-8), to our knowledge, no studies have been performed to compare Celsior with UW after 48 hours of liver preservation at 4°C. Recent data also reported analysis of liver damage after up to 48 hours of cold storage as a valid approach for studying reperfusion injury in liver transplants (9).

In this study, the efficiency of Celsior on hepatocellular and endothelial injury was compared to the most common clinically used liver preservation solution, UW, after 18 or 48 hours of cold storage followed by reperfusion in a model of isolated rat liver (10).

Materials and Methods

N-(2-hydroxyethyl)-piperazine-N’-(2-ethanesulfonic acid) (HEPES) and all chemicals were purchased from Sigma (Milano, Italy).
Male Wistar rats (250-300 g) were allowed free access to water and food in all the experiments. The use and care of animals in this experimental study was approved by the Italian Ministry of Health and by the University Commission for Animal Care. Rats were anaesthetised with sodium pentobarbital (40 mg/kg i.p.) and livers were perfused in a nonrecirculating system. Briefly, the bile duct was cannulated with polyethylene PE 50 tubing and 250 IU of heparin was injected intravenously. An intravenous catheter (16-gauge) was inserted into the portal vein and perfusion begun immediately at a rate of 4 ml/min/g of liver. An identical catheter was introduced into the thoracic inferior vena cava. Livers were perfused with Krebs–Henseleit (KH) medium containing 118 mM NaCl, 4.7 mM KCl, 1.2 mM MgSO₄, 1.2 mM KH₂PO₄, 2.5 mM CaCl₂, 25 mM NaHCO₃ and 20 mM HEPES (pH 7.4) maintained at 37°C under a continuous flushing of 95% O₂ and 5% CO₂ mixture, resulting in an oxygen concentration of 500 to 600 mmHg. In the experimental groups, the livers were flushed out in situ with ice-cold UW or Celsior for 2 minutes, maintained in the ice-cold solution and placed in plastic containers surrounded by ice. The composition of the used preservation solutions is shown in Table I. After 18 or 48 hours of storage, the livers were reperfused at 37°C for 60 min with KH medium. The portal venous pressure was continuously measured throughout the perfusion by means of a water column connected to the portal vein inflow catheter. Precalibration was performed each time just before connecting the liver to the circuit. The initial flow rate was 1 ml/min/g, which was gradually increased to 4 ml/min/g over 5 min (10).

**Assays.** Hepatocyte viability was assessed by release of lactate dehydrogenase (LDH) into the effluent perfusate obtained in the first 5 minutes (wash-out) and during reperfusion (11). Sinusoidal endothelial cells (SECs) take up and metabolize more than 90% of circulating hyaluronic acid (HA) (12-13). Hyaluronic acid (HA) (150 μg/l) was infused in the reservoir to achieve a steady state level. The percentage of HA taken up by SECs during 60 minutes of reperfusion was used as a parameter for SEC function. HA uptake was evaluated by a sandwich-binding enzyme assay (Hyaluronic acid, Chugai Corp., Tokyo, Japan).

The hepatic concentration of total glutathione was measured by the enzymatic recycling method as described by Tieze (14). Oxidized glutathione (GSSG) was determined after derivatization of reduced glutathione (GSH) with 2-vinylpyridine (15). Tissue ATP was measured by the luciferin-luciferase method with the ATP Bioluminescence Assay Kit CLS II (Roche Molecular Biochemicals, Milano, Italy).

Protein was assayed by the method of Lowry et al., with bovine serum albumin as standard (16).

Lipid peroxidation was monitored in the perfusate by measuring the formation of thiobarbituric acid-reactive substances (TBARS) (17).

For histochemical assays, liver samples were snap frozen in liquid nitrogen at the end of the reperfusion period. Unfixed serial frozen sections were used. Glycogen content was assessed with the periodic acid-Schiff (PAS) reaction. LDH activity was demonstrated with the tetrazolium salt method (18) and reactive oxygen species (ROS) with the diaminobenzidine-Mn²⁺-Co²⁺ method (19).

**Statistical analysis.** Data are presented as the mean±SE from at least five independent experiments. Statistical analysis for multiple comparisons was performed by one-way ANOVA test with Bonferroni’s corrections.

### Table I. Components of the UW and Celsior preservation solutions.

<table>
<thead>
<tr>
<th>Component</th>
<th>UW</th>
<th>Celsior</th>
</tr>
</thead>
<tbody>
<tr>
<td>Na⁺ (mmol/l)</td>
<td>27</td>
<td>100</td>
</tr>
<tr>
<td>K⁺ (mmol/l)</td>
<td>125</td>
<td>15</td>
</tr>
<tr>
<td>Mg²⁺ (mmol/l)</td>
<td>5</td>
<td>13</td>
</tr>
<tr>
<td>Ca²⁺ (mmol/l)</td>
<td>2</td>
<td>0.25</td>
</tr>
<tr>
<td>Hes (g/l)</td>
<td>105</td>
<td>80</td>
</tr>
<tr>
<td>Promamitol (g/l)</td>
<td>30</td>
<td>60</td>
</tr>
<tr>
<td>Lactobionic acid (mmol/l)</td>
<td>100</td>
<td>50</td>
</tr>
<tr>
<td>Histidine (mmol/l)</td>
<td>30</td>
<td>30</td>
</tr>
<tr>
<td>Glutathione (mmol/l)</td>
<td>3</td>
<td>3</td>
</tr>
<tr>
<td>Allopurinol (mmol/l)</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>Adenosine (mmol/l)</td>
<td>20</td>
<td>5</td>
</tr>
<tr>
<td>pH</td>
<td>7.4</td>
<td>7.3</td>
</tr>
</tbody>
</table>

### Results

**Biochemical assays.** LDH release was similar during the reperfusion period using both the UW and the Celsior solution after 18 hours of cold storage (Figure 1A). Only a significant reduction of LDH levels in the first 5 min of reperfusion (washout) was observed in the livers preserved in UW when compared with livers preserved in Celsior (Figure 1A). By contrast, during the reperfusion period, which followed 48 hours of cold storage, significantly higher LDH was released in the effluent perfusate when livers were preserved in Celsior than in those stored in UW (Figure 1B). After 18-h cold storage, bile flow at the steady state was best preserved using UW as compared with Celsior (0.4±0.7 versus 0.29±0.3 μl/min/g, respectively) (5). No bile production was observed after 48 hours of liver preservation (20). No significant difference in perfusion pressure during both UW and Celsior preservation was observed (data not shown).

In order to evaluate the endothelial cell function we used the HA uptake test (13). Figure 2 shows that during reperfusion, there was no significant difference in HA levels between the two groups of livers preserved for 18 hours at 4°C. A marked decrease in HA uptake was observed after 48 hours of cold storage: the drop was significantly greater in the livers preserved with Celsior than in those stored with UW (Figure 2).

The analysis of GSH/GSSG ratio showed no significant differences between the livers stored either in Celsior or in UW after 18 hours of preservation (Table II). Similarly, the evaluation of lipid peroxidation in the perfusate exhibited similar levels in both solutions. In contrast after 48 hours of cold storage, the livers exhibited a significant reduction in GSH/GSSG ratio (Table II). In the liver preserved with UW,
GSH/GSSG ratios were higher than in the livers stored with Celsior (Table II). Lipid peroxidation in the perfusate was lower using UW than Celsior solution (Table II).

Tissue ATP levels were also investigated in isolated perfused livers at the end of reperfusion. As shown in Table II no significant differences in ATP were found between the two solutions after 18 hours at 4°C. Hepatic levels of ATP markedly decreased in both solutions after 48 hours of cold storage, especially for livers stored with Celsior (Table II).

**Table II. Hepatic reduced/oxidized glutathione ratio (GSH/GSSG), tissue ATP levels and TBARS formation in the perfusate after 60 min reperfusion in rat livers preserved for 18 or 48 hours in UW or Celsior.**

<table>
<thead>
<tr>
<th>Groups</th>
<th>GSH/GSSG (nmol/mg protein)</th>
<th>ATP (nmol/ml)</th>
<th>TBARS (nmol/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control liver*</td>
<td>17.5±1.7</td>
<td>11.4±2.1</td>
<td>0.3±0.09</td>
</tr>
<tr>
<td>18 hours</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>UW</td>
<td>8.9±3.4*</td>
<td>1.9±0.4*</td>
<td>1.07±0.4*</td>
</tr>
<tr>
<td>Celsior</td>
<td>10.5±2.8*</td>
<td>1.8±0.2*</td>
<td>1.11±0.5*</td>
</tr>
<tr>
<td>48 hours</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>UW</td>
<td>6.2±0.4*</td>
<td>1.3±0.2*</td>
<td>1.71±0.5*</td>
</tr>
<tr>
<td>Celsior</td>
<td>4.5±0.6*,*</td>
<td>0.9±0.2*,*</td>
<td>2.82±0.2*,*</td>
</tr>
</tbody>
</table>

*Control liver before cold storage. There were 5 rats in each group. *p<0.05 versus control; †p<0.05 versus UW.

**Figure 1.** Effect of cold storage on LDH release using UW and Celsior. Livers were preserved in UW or Celsior for 18 (panel A) and 48 hours (panel B); 60 min of reperfusion was performed after cold storage. LDH activity was evaluated in the effluent perfusate obtained in the first 5 minutes (wash-out) and during reperfusion. The results are means of 4-6 different experiments ± S.E.M. Statistical significance: *p<0.05.

**Figure 2.** Effect of cold storage on liver HA uptake using UW and Celsior. Livers were preserved in UW or Celsior for 18 (panel A) and 48 hours (panel B); 60 min of reperfusion was performed after cold storage. Hyaluronic acid (HA) (150 μg/l) was infused in the reservoir during the liver perfusion. The results are means of 4-6 different experiments ± S.E.M. Statistical significance: *p<0.05.
Histochemical assays. In livers cold-preserved for 18 hours and reperfused for 1 hour, no significant differences of LDH activity or ROS production patterns were observed between the two types of preservation solutions (data not shown). Changes were seen only when cold preservation was carried out for 48 hours. In this case, glycogen content was much lower than in control, non-cold-preserved liver, especially for Celsior-preserved livers (data not shown).

Slides stained to demonstrate LDH activity of liver reperfused after 48 hours cold preservation with UW solution showed a well-preserved parenchyma with typical LDH activity zonation, decreasing from periportal to pericentral hepatocytes (Figure 3a). In livers cold-preserved with Celsior solution, periportal and pericentral hepatocytes displayed high activity but hepatocytes in the mid-zone appeared to be injured and had a much lower LDH activity (Figure 3b); the LDH found in the perfusion medium might correspond to LDH activity leaked from mid-zonal hepatocytes.

As far as ROS production is concerned, in livers cold-preserved with UW, the reaction was present but heterogeneous only in peri-portal hepatocytes (Figure 3c). By contrast, in livers cold-preserved with Celsior solution, the ROS reaction was extremely intense in periportal hepatocytes and sinusoidal cells (Figure 3d); mid-zonal hepatocytes were ROS negative for UW-preserved livers but showed low-to-moderate reaction when preservation was made with Celsior (not shown). Pericentral hepatocytes were negative for both solutions. Staining in hepatocytes was shown to be mainly due to superoxide anions and singlet oxygen produced by mitochondria (20); in sinusoidal cells staining may be produced during phagocytosis. Mitochondrial activity and phagocytosis thus appear more intense for Celsior-preserved livers than UW-preserved ones. The higher concentration of TBARS in the perfusate in Celsior-preserved livers than those preserved with UW might be correlated both to the higher ROS production and to the lower reductive capacity (GSH/GSSG) in these livers.

Discussion
Preservation injury and its consequences during reperfusion are responsible for primary graft dysfunction. Use of the UW solution increased the time span for organ preservation for
transplantation, thereby enhancing the margin of safety and allowing a more effective use of organs. The new Celsior solution has been proposed for liver transplantation but little data relating to the long-term cold preservation of the liver have been reported. We compared the effects of Celsior with the UW on graft function after 18 or 48 hours of cold storage and 60 min reperfusion. We previously reported the use of this experimental model (21). Moreover, other authors have used this experimental procedure to test damage to rat liver stored for transplantation (9, 10, 22).

UW and Celsior are almost equally effective preservation solutions in liver transplantation when the storage time span is around 12-16 hours (23-24), but the long-term efficacy of Celsior for liver preservation is still controversial. In this study, we performed experiments in which the livers were preserved in UW and Celsior for 18 hours and did not find any significant difference as shown by the analysis of LDH activity, oxidative stress and ATP content. But the effectiveness of these two solutions differed considerably when the organ was preserved for 48 hours. Previous studies have reported that isolated hepatocytes expressed metabotropic glutamate receptors as indicated by RT-PCR analysis and Western blot analysis (25). We have recently reported that the activation of these receptors significantly reduces cell viability in hepatocytes exposed to hypoxia, whereas the antagonist is protective against hypoxic damage in hepatocytes (26). Glutamate is an ingredient of Celsior (20 mmol/l), added as a precursor for ATP synthesis, and may be detrimental for liver preservation.

Liver sinusoidal endothelial cells were particularly vulnerable to cold ischemia-reperfusion and their impairment plays a crucial role in primary dysfunction following liver transplantation (27). Hyaluronic acid uptake by isolated perfused rat liver, utilized as an index of hepatic sinusoidal endothelial cell function, makes it possible to quantitatively assess the function of these cells in a whole-organ model environment (28). HA uptake reflects an impaired endothelial cell function caused by prolonged cold preservation/reperfusion. We measured HA uptake and the use of UW improved liver sinusoidal endothelial cells viability to a much greater extent than Celsior.

ROS have been implicated in the pathogenesis of hepatic reperfusion injury and the intracellular levels of GSH are certainly involved in the pathophysiology of ischemia-reperfusion (29). Previous data, using a pig liver transplantation model, demonstrated that the intrahepatic GSH and GSSG levels and GSH/GSSG ratio were not significantly different using UW or Celsior after 12 hours of storage at 4°C (30). Our findings show that the GSH/GSSG ratio and TBARS formation were similar after 18 hours of cold storage, while the GSH/GSSG ratio was higher and TBARS formation was lower in the livers preserved for 48 hours in UW than in those stored with Celsior; these data suggest a greater effectiveness of UW in maintaining the ROS scavenger ability of the liver after very long periods of preservation.

ATP resynthesis during reperfusion after liver preservation has been shown to be well correlated with the function of transplanted grafts (31). ATP was slightly superior in the UW group after 18 hours of cold storage but it was not statistically different. Higher ATP levels and the glycogen amount evaluated using the UW solution are correlated with higher cell viability as compared with the liver preserved in Celsior after 48 hours at 4°C. Posttransplant liver function is very sensitive to cold storage conditions and, to our knowledge, Celsior solution has not been tested for long-term (48 to 72 hour) preservation as reported by Southard (32).

The results presented here demonstrate that the degree of hepatic injury was similar in 18-hour preserved livers while UW provided better preservation of the liver than Celsior after 48 hours of cold storage. The isolated perfused liver has proven to be a valid model for evaluating stresses induced specifically during the cold storage phase (9, 13-14). In conclusion, this study confirms that Celsior solution was as effective as UW in liver preservation up to 18 hours at 4°C. Furthermore, these data demonstrate that UW is the better solution when the livers are preserved for 48 hours, in this experimental model. Celsior is probably useful for periods of cold storage of up to 16-18 hours but it is not an effective solution for very long-term cold preservation of the liver.

Acknowledgements

We dedicate this paper to Roberta Bertone, who just in time managed to see the beginning of this research before a tragic flight accident in November 2005. The authors thank Mr. Gaetano Viani for his skilful technical assistance and Professor Anthony Baldry for revising the English. The authors thank the Chugai Corp., Tokyo, Japan, for supporting our research with the Hyaluronic acid kits. This work was supported by MIUR-COFIN 2006 and by F.A.R.- University of Pavia.

References


Received May 13, 2008
Revised August 8, 2008
Accepted August 22, 2008