Machine perfusion at 20 °C reduces preservation damage to livers from non-heart beating donors

Andrea Ferrigno a, Vittoria Rizzo b, Eleonora Boncompagni c, Alberto Bianchi a, Enrico Gringeri d, Daniele Neri d, Plinio Richelmi a, Isabel Freitas c, Umberto Cillo d, Mariapia Vairetti a,*

a Department of Internal Medicine and Therapeutics, University of Pavia, Italy
b Department of Biochemistry, IRCCS Policlinico San Matteo, University of Pavia, Italy
c Department of Animal Biology and IGM-CNR, Histochemistry and Cytometry Section, University of Pavia, Italy
d Department of General Surgery and Organ Transplantation, University of Padua, Italy

A R T I C L E I N F O
Article history:
Received 22 January 2010
Accepted 1 February 2011
Available online 17 February 2011

Keywords:
LIVER
NHBD
Cold storage
Machine perfusion
Mitochondria
Bile

A B S T R A C T
We previously reported that machine perfusion (MP) performed at 20 °C enhanced the preservation of steatotic rat livers. Here, we tested whether rat livers retrieved 30 min after cardiac arrest (NHBDs) were better protected by MP at 20 °C than with cold storage. We compared the recovery of livers from NHBDs with organs obtained from heart beating donors (HBDs) preserved by cold storage. MP technique: livers were perfused for 6 h with UW-G modified at 20 °C. Cold storage: livers were perfused in situ and preserved with UW solution at 4 °C. LDH and AST release, mitochondrial glutamate dehydrogenase (GDH), and GSH/GSSG were evaluated. Parameters assessed included: bile production and biliary enzymes; tissue ATP, reduced and oxidized glutathione (GSH/GSSG); protein–SH group concentration. Liver recovery using MP at 20 °C was comparable to recovery with HBDs. MP at 20 °C improves cell survival and gives a better-quality of preservation for livers obtained from NHBDs and may provide a new method for the successful utilization of marginal livers.

© 2011 Elsevier Inc. All rights reserved.

Introduction
Liver transplantation is the treatment of choice for end-stage liver disease and acute liver failure [31]. Currently, donor organs for orthotopic liver transplantation (OLT) are taken almost exclusively from heart beating donors [33]. Uncontrolled non-heart-beating donor (NHBD) livers are not generally utilized for liver transplantation due to the fact that these organs are associated with an increased incidence of primary non function (PNF) both experimentally [26] and clinically [1].

Recently, faced with the increasing shortage of donor organs for transplantation, there is renewed interest in non-heart-beating donor (NHBD) transplantation [27]. NHBDs could be a conspicuous source of organs for transplantation: in various centers 4–10% of transplants are carried out using NHBD livers, but the true potential of NHBD in liver transplantation is probably greatly underestimated [31] including NHBDs the pool of available organs could potentially increase by as much as 20% [36].

Several centers have initiated programs for the procurement of livers from NHBD [27,9] and research groups are evaluating new strategies to better preserve NHBD livers [33,38,5,12] or predict organ survival after transplantation [16,28]. In particular, livers obtained from NHBD and preserved by cold storage have exhibited postoperative biliary complications [15,32] the well-known Achilles'heel of liver transplantation. Therefore new proposals to safeguard against this short-coming are welcome. Currently, cold storage is the gold standard for organ preservation but, because of the increased use of marginal donor organs, interest in experimental and clinical studies on machine perfusion (MP) preservation has been revived [3] in particular in hypothermic MP protected marginal livers such as those from non-heart-beating donors (NHBD) or those containing fat due to alcohol or obesity [6,4]. We have further protected organs, especially steatotic livers, using a subnomenclature temperature during MP: in our previous

Statement of funding: This work was funded by MIUR-COFIN 2006-2008 and by F.A.R. – University of Pavia.
* Corresponding author. Address: Department of Internal Medicine and Therapeutics, University of Pavia, Via Ferrata 9A, 27100 Pavia, Italy. Fax: +39 382 986347.
E-mail address: mariapia.vairetti@unipv.it (M. Vairetti).

0011-2240/$ - see front matter © 2011 Elsevier Inc. All rights reserved.
doi:10.1016/j.cryobiol.2011.02.004
papers, we demonstrated that MP performed at 20 °C reduces fatty liver susceptibility associated to organ preservation, when compared with conventional cold storage. Steatotic liver preserved by subnormothermic MP exhibited a marked reduction in damage evaluated as enzyme and cytokine release, excretory function and energy recovery, usually exacerbated by conventional preservation at 4 °C [41,25].

Based on the above reports, we designed a study to test whether rat liver preservation performed by MP at 20 °C can enhance the functional integrity of livers obtained from NHBDs versus simple cold storage. Furthermore, we compared the recovery of livers from NHBDs with organs obtained from heart beating donors (HBDs) preserved by conventional cold storage.

Materials and methods

Chemicals

N-(2-hydroxyethyl)-piperazine-N′-(2-ethanesulphonic acid) (HEPES) and all chemicals were purchased from Sigma (Milan, Italy).

Animals and surgery

Male Wistar rats (Harlan-Nossan, Italy), weighing 250–300 g, were allowed free access to water and food until the beginning of all experiments. The use and care of animals in this experimental study were approved by the Italian Ministry of Health and by the University Commission for Animal Care. Rats were anesthetized with sodium pentobarbital (40 mg/kg intraperitoneally). After median laparotomy followed by bilateral subcostal incisions, the animals received 500 U of heparin via the inferior vena cava (5000 IU/mL, Marvec Services, Agrate Brianza, MI) in accordance with the experimental method reported in the literature [3]. The use of heparin regards the organs obtained from the controlled NHBD as reported in clinical studies [18]. After 2 min, a phrenotomy was performed to sacrifice the animal. The warm ischemic time started after cessation of blood flow to the liver. During warm ischemia, the bile duct was cannulated with a 50 G polyethylene tubing (Intramed, Becton–Dickinson, Loveton Circle, MD, USA) and the portal vein was cannulated with a 16 G catheter (Johnson and Johnson, Arlington, UK). After 30 min of warm ischemia, the liver was washed with 50 mL Ringer Lactate via the portal vein cannula. The washout solution drained away by cutting the infrahepatic caval vein. The suprahepatic caval vein was then excised, and Johnson, Arlington, UK). After 30 min of warm ischemia, the liver was removed. Then, livers were preserved for 6 h by either cold storage using UW–Johnson and Johnson, Arlington, UK). After 30 min of warm ischemia, the liver was washed with 50 mL Ringer Lactate via the portal vein cannula. The washout solution drained away by cutting the infrahepatic caval vein. The suprahepatic caval vein was then excised, and Johnson, Arlington, UK). After 30 min of warm ischemia, the liver was removed. Then, livers were preserved for 6 h by either cold storage using UW–

Cold storage preservation

After washout, the livers were flushed in situ with ice-cold UW for 2 min and maintained at 4 °C in this solution for 6 h.

Normothermic reperfusion

Normothermic reperfusion with KH (2 h at 37 °C) was performed with the same perfusion system used for MP preservation. An identical set up was applied to MP or cold storage preserved livers.

Assays

Hepatocyte viability was assessed by release, into the effluent perfusate, of aspartate transaminase (AST) and amino transaminase (ALT) determined by an automated Hitachi 747 analyzer (Roche/Hitachi, Indianapolis, IN, USA); lactate dehydrogenase (LDH) release was evaluated as previously described [2]. Total bile production was measured during MP and reperfusion periods; gamma-glutamyl transpeptidase (γGT), aspartate transaminase (AST), alanine transaminase (ALT) and alkaline phosphatase (AP) levels in bile were determined by an automated Hitachi 747 analyzer. At the end of the reperfusion, tissue samples were quickly removed and frozen in liquid nitrogen. Damage to mitochondria was estimated by the glutamate dehydrogenase activity, spectrophotometrically assayed as described by Ellis and Goldberg [13].

The hepatic concentration of total glutathione was measured by an enzymatic method (Cayman Chemical Co., Ann Arbor, MI): oxidized glutathione (GSSG) was determined after derivatization of reduced glutathione (GSSG) with 2-vinylpyridine [17]. Protein–SH groups were evaluated by the method of Di Monte et al. [11]. Protein content was assayed by the method of Lowry et al. [23]. Tissue ATP was measured by the luciferin-luciferase method with the ATP Bioluminescence Assay Kit CLS II (Roche Molecular Biochemicals, Milan, Italy).

Histochemistry

Liver pieces were rapidly removed after normothermic reperfusion, fixed in 2% p-formaldehyde in 0.1 M phosphate buffer at pH 7.4 for 24 h and processed routinely until they were embedded in Paraplast wax. Sections were cut at 7μm and stained with Hematoxylin and Eosin (H&E) for histological examination or with the periodic acid–Schiff reaction (PAS) for glycogen visualization. To appraise the severity of hepatic injury, H&E-stained sections were evaluated by a point-counting method on an ordinal scale as follows: Grade 0, minimal or no evidence of injury; Grade 1, mild injury consisting of cytoplasmic vacuolation and focal nuclear pyknosis; Grade 2, moderate-to-severe injury with extensive nuclear pyknosis, cytoplasmic hypereosinophilia, and loss of intercellular borders; and Grade 3, severe necrosis with disintegration of hepatic cords, hemorrhage, and neutrophil infiltration [34].
Statistical analysis

Data are presented as the mean ± SE. Statistical analysis for multiple comparisons was performed by one-way ANOVA test with Bonferroni’s corrections.

Results

Hepatocellular injury during machine perfusion (MP) preservation of livers from NHBDs

Samples of perfusate, collected every 10 min, showed that MP at 20 °C induced an enzyme release of ALT (18.5 ± 5 mU/mL), LDH 5.2 ± 0.8 mU/min/g as well as GDH (0.49 ± 0.1 mU/min/g). The bile flow observed was 0.04 ± 0.01 μl/g liver.

Portal pressure

During 6 h of MP preservation, a moderate decrease in portal pressure was observed in livers preserved by MP at 20 °C (from 5.6 ± 0.4 to 3.9 ± 0.2 mm Hg). At the end of reperfusion, no difference in portal pressure between livers obtained from HBDs or NHBDs was observed (data not shown).

Liver viability during reperfusion

The evaluation of parenchymal integrity after cold storage preservation showed a notable release of the AST and LDH during reperfusion in livers obtained from NHBDs as compared with heart beating donors (HBDs) (Fig. 1). Indeed, the enzyme release during reperfusion showed a decrease when livers from NHBDs were preserved with MP at 20 °C: the perfusate activities of AST and LDH were significantly decreased as compared with preservation by conventional cold storage (Fig. 1). The injury in livers from NHBDs preserved with MP 20 °C was similar to that observed with control livers preserved by conventional cold storage (Fig. 2B). On the contrary, lower levels of biliary enzymes were observed in livers from NHBDs preserved by MP at 20 °C as compared with cold storage: γ-GT was 48 ± 12, and 188 ± 39 mU/ml (p < 0.05) in livers preserved by MP 20 °C and cold storage, respectively.

Mitochondrial injury

Glutamate dehydrogenase (GDH) activity was evaluated, as index of damage to mitochondrial integrity, in livers obtained from NHBDs and preserved by MP or cold storage. GDH activity into the perfusate was attenuated by MP at 20 °C (Fig. 3A).

Energetic status

To evaluate the ATP depletion after 30’ warm ischemia respect to controls, we measured ATP and ATP/ADP ratio in HBD and NHBD livers, immediately after washout: ATP and ATP/ADP ratio was significantly higher in HBD livers respect to NHBD (Table 1). We also evaluated the energy status of hepatic biopsies obtained at the end of the reperfusion: the ATP levels were significantly higher in livers preserved by MP at 20 °C compared with cold storage preservation (Fig. 3B). In livers from HBDs the ATP content was similar to the content obtained in NHBD preserved by MP at 20 °C.

Reduced and oxidized glutathione (GSH and GSSG) levels and protein-SH groups

Hepatic glutathione (GSH and GSSG), which plays a central role in the defense of cells against free radical species (ROS), was evaluated at the end of reperfusion period: higher GSH/GSSG ratio was...
found in livers preserved by MP at 20 °C as compared with cold storage (Fig. 4A). Non significant differences were observed in livers obtained from HBDs (Fig. 4A). A correlation was also performed with tissue protein-SH groups. As indicated in Fig. 4B a decrease in SH groups was observed both in liver preserved with cold storage than those preserved by MP at 20 °C or obtained from HBDs (*p < 0.05 and **p < 0.05). These are the mean results of 5–7 different experiments ± SEM. 

**Morphology**

The degree of histological hepatocyte damage strongly differed between livers preserved by cold storage and MP (Fig. 5). Cold storage and reperfusion caused marked damage to the parenchyma with sinusoid dilatation, extensive areas of nuclear pyknosis and cytoplasmic vacuolation and various necrotic cells detached from the parenchyma (Fig. 6A). The best preservation was obtained when livers were submitted to MP at 20 °C and reperfusion (Fig. 6B) with well-preserved hepatic architecture but with slight sinusoid dilatation. The morphology observed with MP at 20 °C

---

**Table 1**

<table>
<thead>
<tr>
<th>ATP (nmol/mg prot)</th>
<th>ATP/ADP</th>
</tr>
</thead>
<tbody>
<tr>
<td>Livers from HBD</td>
<td>9.2 ± 1.6</td>
</tr>
<tr>
<td>Livers from NHBD</td>
<td>1.7 ± 0.2</td>
</tr>
</tbody>
</table>

These are the mean results of 5–7 different experiments ± SEM. *p < 0.05 versus HBD.
preservation was comparable with preservation observed in HBDs (data not shown) [40].

Glycogen stores

Glycogen stores in hepatocytes of NHBD donors submitted to cold storage followed by reperfusion were rare (Fig. 6c) whereas they were maintained in the centrolobular areas after MP20 followed by reperfusion (Fig. 6d). Similar findings were found for livers obtained from HBDs (data not shown) [40].

Discussion

Our results demonstrate that MP preservation at 20 °C improves cell survival reducing mitochondrial damage as well as complications with bile duct structures, in livers obtained from NHBDs as compared with conventional cold storage. MP under moderate hypothermia conditions provides a preservation/reperfusion injury level that did not differ from the damage observed in livers from HBDs preserved by cold storage.

The shortage of liver grafts due to the increase in the number of patients requiring liver transplantation suggests the use of NHBDs as an additional source that would increase the donor pool. In the NHBD donors, circulatory arrest has occurred before organ procurement: Casavilla et al. (1995) suggested that the use of controlled or uncontrolled NHBDs lead to acceptable graft function [8].

The preservation solution mainly used for liver MP in both clinical and experimental settings is UW-G. We also performed experiments using UW-G solution during preservation of organs obtained from NHBDs and an increase in hepatic necrosis and perfusion pressure were observed (data not shown). We decided to use a UW-G modified solution on the basis of our previous experience in MP liver preservation [39–41]. In particular, the colloid hydroxyethylstarch (HES) used for hypothermic preservation and known to cause microcirculatory disturbances in NHBD livers, was not added [24]; we used a UW-G containing 1.25 mM CaCl₂ as previously tested [40]: calcium deprivation was shown to induce cholestasis due, at least in part, to a disturbance in osmotic equilibrium [35]; NAC was added in UW-G owing to its antioxidant and microcirculatory relaxant properties [29].

Mitochondria recovery during reperfusion

The literature shows that a marked decrease in the hepatic ATP level occurs in ischemic livers because phosphorylation is rapidly and seriously affected by ischemia [22]: in our model ATP levels dramatically decrease after 30 min of warm ischemia respect to non ischemic livers. Furthermore a significant decrease in hepatic ATP levels associated with a concomitant increase in mitochondrial GDH activity in the perfusate occurred after 2 h of reperfusion in livers from NHBDs preserved with conventional cold storage. But we also demonstrated the ability of MP at 20 °C to reduce mitochondrial damage allowing the synthesis of ATP during reperfusion. These findings confirm our previous work using livers from steatotic rats: the energy status was higher using the MP at 20 °C technique as compared with cold storage [41].

The underlying mechanisms involved in liver preservation by MP have not been elucidated yet. However, probably during MP at 20 °C a reduction in reactive oxygen species production occurs as demonstrated by the increase in GSH levels and protein thiol groups. The increased tolerance of livers from NHBDs to isch-
mia/reperfusion may partially be explained by a reduction in oxidative stress associated to better mitochondria preservation using subnormothermic temperatures. In addition, the higher content of glycogen of NHBDs livers submitted to MP at 20°C, compared with cold storage is in keeping with higher ATP levels. This suggests that enough energy stores are present after a 6-h preservation of livers with MP at 20°C to allow a greater capacity to resume functionality after transplantation than after cold storage. Glycogen may help in maintaining tissue stores of high-energy adenylates during the course of the preservation; it may furthermore contribute, through the pentose phosphate pathway, to maintain the NADPH pool necessary for regeneration of reduced GSH. In fact, better liver preservation has been demonstrated when hepatic glycogen, consumed during early reperfusion, is maintained close to control levels [20,21]. Furthermore, the high glycogen content in MP 20°C preserved livers suggests an adequate oxygen supply during organ preservation phase. In fact, without an appropriate oxygen supply, in anaerobic conditions, liver needs to switch to lactic fermentation, leading to a great glycogen degradation. When MP is conducted at a temperature of 0–4°C, the liver oxygen requirements is below 0.3 μmol/min/g [14]. At 20°C, the liver oxygen requirements rise to 0.8 μmol/min/g [14]. A non-oxygenated solution supplies up to 0.4 μmol/min/g of oxygen using a flow of 3.5 ml/min/g, sufficient for hypothermic MP, but not for subnormothermic temperatures (data not shown). Oxygen supply in our model of MP at 20°C is up to 1.4 μmol/min/g, so fulfilling the liver requirements.

**Biliary tract preservation**

Previous studies have demonstrated a relationship between bile flow rates and cell ATP levels under various conditions and have also shown that hepatic ATP levels are related to the survival rates of the transplanted animals [19,37]. The decrease in bile production observed in the grafts obtained from NHBDs preserved with cold storage reflects the reduction in the energy status of these grafts. On the contrary, probably due to the subnormothermic temperature used, MP at 20°C exerts a positive effect by increasing both bile production and the ATP content.

Biliary complications after orthotopic liver transplantation (OLT) remain the Achille’s heel of this operation especially in organs from NHBDs [7]. Bile analysis after cold ischemia represents a tool when assessing the integrity of biliary epithelial cells after cold ischemia–reperfusion of rat livers [30]. Interestingly, a retrospective review of liver transplant patients demonstrated that liver grafts procured from NHBDs showed a higher re-transplantation rate due to ischemic tract biliary lesion combined with severe intrahepatic cholestasis [18,10]. Of particular interest is the fact that our data demonstrated that MP at 20°C increases bile production and decreases biliary enzyme release thus potentially reducing biliary complications that usually occur after cold storage preservation. In addition, a comparison between livers from NHBDs preserved by MP at 20°C or organs from HBDs preserved by cold storage demonstrated no difference in bile flow and biliary enzymes.

The use of NHBDs for liver transplantation could be made possible by procedures for reducing intervention that reduce the effects of warm ischemia: the use of MP under a moderate hypothermia might provide a new method for the successful utilization of marginal livers such as those obtained from NHBDs. Recently van Gulik [42] reported that MP preservation offers new prospects for the use of predamaged livers. The preservation time could become a window in which the liver can be exposed to pharmacological interventions to reduce preservation injury especially in particularly vulnerable organs such those from NHBDs.

In principle, MP 20°C could imply a microbial growth in perfusion medium. Antibiotics could be included in the medium used for MP20, however they might not be necessary since the mechanical...
perfusion of organs is performed using a conduction system that provides a sealed, sterile and protected environment.

In conclusion, the results of the present study support our previous data on fatty liver preservation: MP at 20 °C protects organs against ischemia/reperfusion injury and represent a new approach for marginal liver preservation. Interestingly, the preservation of these marginal organs by MP at 20 °C produces damage comparable with damage observed in control groups obtained respectively from lean rats or HBDs.

Because no other experimental data on subnormothermic MP has so far been available, future experimental investigations in a liver transplantation model are needed to put these results into perspective.

Acknowledgments

This work was funded by MIUR-COFIN 2006-2008 and by F.A.R.-University of Pavia. We thank Mr Gaetano Viani for his skilful technical assistance and Mrs. Nicoletta Breda for the editing assistance. We also thank Prof. Anthony Baldry for revising the English.

References


