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Hassen Ben Abdennebi
Jean-Paul Steghens
Jacqueline Margonari
Silvina Ramella-Virieux
Alain Barbieux
Olivier Boillot

Evaluation of parenchymal and nonparenchymal cell injury after different conditions of storage and reperfusion

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H. Ben Abdennebi (✉) · J. Margonari
Laboratoire de Recherches Chirurgicales,
INSERM U.281, Pavillon P5,
Hôpital Edouard Herriot,
F-69437 Lyon Cedex 03, France
Fax: + 33 4 72 33 00 44

J.-P. Steghens
Laboratoire de Biochimie,
Hôpital Edouard Herriot,
F-69437 Lyon Cedex 03, France

S. Ramella-Virieux
Laboratoire d'Exploration Fonctionnelle
Rénale,
Hôpital Edouard Herriot,
F-69437 Lyon Cedex 03, France

A. Barbieux
Pharmacie,
Hôpital Edouard Herriot,
F-69437 Lyon Cedex 03, France

O. Boillot
Unité de Transplantation Hépatique,
Hôpital Edouard Herriot,
F-69437 Lyon Cedex 03, France

Abstract We used the isolated perfused rat liver model (IPRL) to assess parenchymal and nonparenchymal cell integrity after different conditions of storage and reperfusion. Two studies were performed. In study 1, the IPRL was applied to evaluate the effects of 30 min of normothermic reperfusion with Elohes solution, enriched William's medium (Wif), or Carolina rinse solution (CRS) following 24 h of cold preservation in high-K^+ or high-Na^+ UW solution. As indicated by creatine kinase-BB (CK-BB) release, reperfusion with CRS provided greater protection of endothelial cells after storage in high-K^+ UW solution than after storage in high-Na^+ UW solution. In study 2, livers were cold-preserved (24 h, 4 °C) in either high-K^+ or high-Na^+ UW solution, then flushed with either CRS or Wif solution at room temperature before reperfusion (120 min, 37 °C) with 5 % albumin-William's medium E. There was no statistical difference between the rinse solutions for bile flow and transaminases release. However, CRS improved bile indocyanine green excretion, which is known to be a marker of parenchymal and nonparenchymal cell integrity. Therefore, we can assume that this rinse solution protects rat liver grafts from reperfusion-induced microvascular damage.

Key words Isolated perfused rat liver, cold preservation · Creatine kinase-BB isoenzyme · Carolina rinse solution

Introduction

Recent evidence indicates that primary injury to liver grafts is associated with disruption of the sinusoidal endothelium during hypothermic preservation and subsequent reperfusion [6]. As a consequence, microvascular hemodynamics are impaired, Kupffer cells are activated, and hepatocytes necrosed [6, 27, 28]. The clinical use of University of Wisconsin (UW) cold storage solution for liver preservation reduces graft failure [36]. This solution contains compounds that prevent the detrimental effects of toxic oxygen free radicals released during the reperfusion-reoxygenation period [1]. However, this potassium-enriched solution must be flushed out from the graft just before revascularization. The terminal rinse is a standard practice in liver preservation

with UW solution to avoid potassium overload. Thus, all UW solution compounds are flushed out at the moment they are needed again. Recently, another solution – Carolina rinse solution (CRS) – has been used to wash out preservation solutions left in the graft [18]. It has been shown that this solution reduces Kupffer cell activation after reperfusion and improves bile flow and microvascular blood flow in both rat liver transplantation and isolated perfused rat liver models [12, 14, 18, 29, 30, 33]. Egawa et al., however, showed that the terminal rinse is harmful in rat liver preservation [16].

The isolated perfused rat liver (IPRL) is commonly used in many areas of research, especially in graft preservation [19]. The model is simple and reproducible and allows preservation-reperfusion injuries to be studied under standardized conditions. However, the perfused liver should be considered a dying organ, as hepatic functions deteriorate progressively and separately. After preservation, when grafts are in a depleted state, the dying process is accelerated. Viability indicators are, therefore, of great importance for studies on the isolated perfused rat liver.

The purpose of the present study was to evaluate the effect of different rinse solutions on parenchymal and nonparenchymal cell injuries after different conditions of storage and reperfusion. This evaluation is based mainly on perfusate creatine kinase-BB isoenzyme (CK-BB) release and bile indocyanine green (ICG) excretion.

Materials and methods

Animals

Male inbred Wistar rats (Iffa-Credo, France) weighing 300 g were used as organ donors. Before the experiments, they had free access to water and a standard pellet diet. All animals were given humane care and handled in compliance with French regulations, as well as with the "Principles of Laboratory and Animal Care" (NIH publication No. 86-36, revised 1985).

Liver harvesting

Rats were anesthetized by an intraperitoneal injection of sodium pentobarbital (6 g%, 0.1 ml/100 g of body weight). Two hundred and fifty international units of heparin per hundred grams of body weight were injected into the femoral vein. A laparotomy was performed, first by an abdominal midline incision, then by a bilateral transverse extension, and a catheter (Venocath 18-G) was inserted into the common bile duct for bile collection. The aorta, vena cava, and portal vein were then dissected. Organs were washed out with either the high-K^+ UW solution (Belzer's original UW solution, K^+ 125 mM/Na^+ 30 mM) or the high-Na^+ solution (Belzer's UW solution with inverted concentrations of Na^+ and K^+) [31] by mean of a catheter (Venocath 16-G) inserted into the aorta at a constant flow rate of 3 ml/min. Both cold preservation solutions contain all of Belzer's UW solution molecules. They are prepared in our pharmaceutical department, enclosed in special PVC bags with a gas barrier (for glutathione preservation), and used as they are provided without added compounds.

To allow exsanguination, the abdominal vena cava was cutt off and catheterized. The thoracic vena cava was ligated. A third catheter (Venocath 14-G) was inserted into the portal vein and the whole liver was then excised and trimmed of adhering tissue. Ex situ, the portal vein catheter was used to complete liver rinsing (3 ml/min). The total volume infused through the aorta and portal vein was about 50 ml. Livers were preserved in either high-K^+ UW or high-Na^+ UW solution (75 ml) at 4 °C for 24 h.

Organ perfusion

All livers were perfused at 37 °C via the portal vein in a closed and controlled pressure circuit [2]. The perfusate (100 ml) was continuously gassed with a 95 % O_2-5 % CO_2 mixture.

Experimental design

Two studies were conducted.

Study 1

Thirty experiments were designed to determine the effectiveness of three different solutions in preventing lethal sinusoidal endothelial cell injury, assessed by perfusate CK-BB isoenzyme, after cold preservation and reperfusion. To this end, rat livers were washed out and preserved for 24 h at 4 °C in high-K^+ UW or high-Na^+ UW solution. Afterwards, livers were perfused for 30 min at 37 °C with Elohes solution (n = 5 for each preservation solution; 6 % hydroxyethyl starch, 0.9 % NaCl) or CRS (n = 5 for each preservation solution; 115 mM NaCl, 5 mM KCl, 1.3 mM $CaCl_2$, 1 mM KH_2PO_4, 1.2 mM $MgSO_4$, 1 mM allopurinol, 1 mM desferrioxamine 3 mM glutathione, 2 μM nicardipine, 200 μM adenosine, 10 mM fructose, 10 mM glucose, 100 U/l insulin, 20 mM MOPS, and 5 mM glycine) or William's medium E (n = 5 for each preservation solution, Bio Whittaker) [39] to which we added 5 % albumin, 10 mM fructose, 5 mM alanine, 2 mM glutamine, 2.5 mM glycine, pH 7.4, 300 mosmol/kg; we called this solution the Wif solution. We included no hydroxyethyl starch in the CRS since the study by Currin et al. has shown that, with the IPRL model, it is not an important compound in the solution [14]. During the 30 min of perfusion, the three solutions were oxygen-saturated. The portal flow rate was increased gradually (from 0.5–1 to 2.5–3 ml/min per gram) and portal pressure was kept within the physiological range (10–12 mm Hg).

Study 2

Twenty-eight experiments were undertaken to assess the effect of the use of CRS and Wif solution as terminal rinse solutions on hepatocellular integrity. Four groups were studied (preservation/rinse). In the groups with high-K^+ UW/Wif (n = 7) and with high-Na^+ UW/Wif (n = 7), rat livers were washed out and preserved in cold (4 °C) high-K^+ UW or high-Na^+ UW solution for 24 h, respectively. Immediately before reperfusion, the preservation solutions were rinsed with 15 ml of Wif solution through the portal vein (hydrostatic pressure 8 cm H_2O, room temperature). Livers in the groups with high-K^+ UW/CRS (n = 7) and high-Na^+ UW/CRS (n = 7) were washed out and preserved in the high-K^+ UW or high-Na^+ solution (24 h at 4 °C), respectively. Then, before reper-

fusion, they were flushed with 15 ml of CRS through the portal vein (hydrostatic pressure 8 cm H_2O, room temperature). Afterwards, all of the livers were perfused in a closed circuit at 37 °C for 120 min. The perfusate was an oxygen-saturated Williams's medium E containing only 5 % albumin as oncotic supply. During the perfusion, we assessed bile indocyanine green (ICG) excretion, bile flow, and transaminase levels.

Liver function study

Serial samples of caval outflow perfusate were taken for measurement of total CK and CK-BB isoenzyme activity [11, 37]. Total CK activity was determined with a commercial reagent (BioMérieux, Charbonnières-les-Bains, France) according to the IFCC method. CK isoenzymes were separated electrophoretically on agarose gels with a Paragon kit (Beckman Instruments, France). After electrophoresis, CK isoenzymes were detected under UV light, and gels were scanned with a fluorimetric densitometer (SEBIA, France). All of the CK measurements were done without deep freezing samples.

ICG (Becton Dickinson Microbiology Systems) is unstable in an aqueous solution, so it is prepared just before perfusion experiments with the solvent provided by the manufacturer. Concentration standards were also prepared with the same solvent. One milligram of ICG was added to the perfusate. The concentration of ICG was determined by measuring absorbance at 800 nm of diluted bile samples with a UV-visible spectrophotometer [5]. Bile was collected at 30-min intervals and the volume was estimated by weighing. Caval outflow perfusate samples were taken for aspartate aminotransferase (AST) and alanine aminotransferase (ALT) measurements on a Hitachi 747 analyzer, according to the IFCC method.

Statistical analysis

Results were expressed as the mean ± SEM. Data between groups were compared with the ANOVA test, followed by PLSD Fischer's test. Statistical significance was defined as a P level below 0.05.

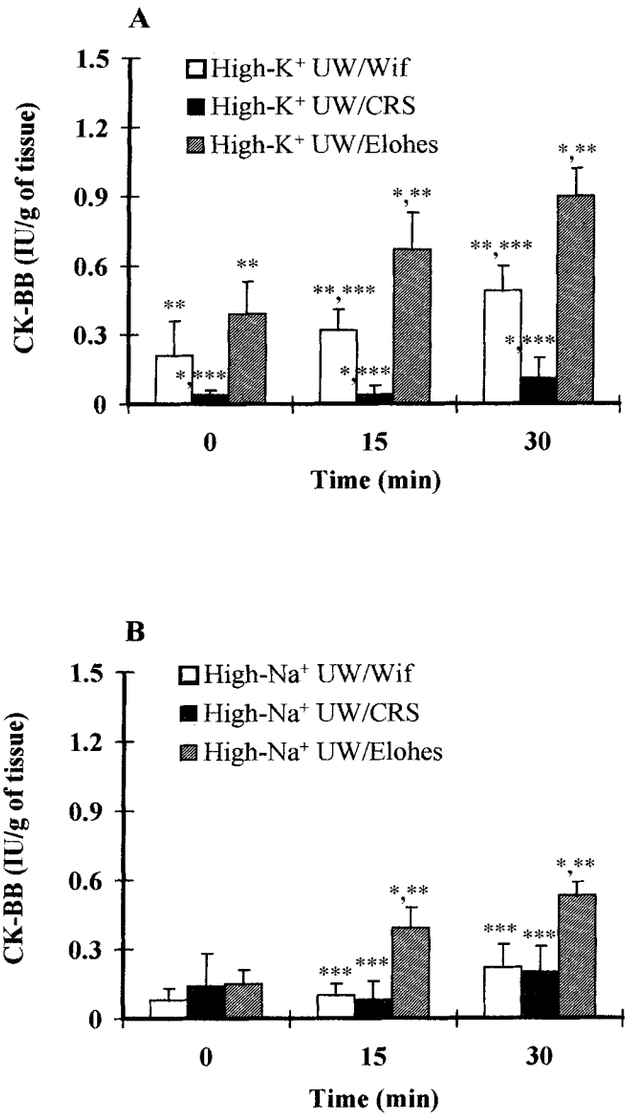

Fig. 1 A, B Perfusate CK-BB isoenzyme release. Rat livers were washed out and preserved in: **A** high-K^+ UW or **B** high-Na^+ UW solution for 24 h at 4 °C, then perfused with oxygen-saturated Wif, CRS, or Elohes solutions for 30 min at 37 °C. Data are expressed as mean ± SEM ($n = 5$) * $P < 0.05$ vs Wif; ** $P < 0.05$ vs CRS; *** $P < 0.05$ vs Elohes

Results

Study 1

After 24 h of cold storage in high-K^+ UW solution and 30 min of reperfusion with oxygenated CRS, rat livers released significantly less CK-BB isoenzyme in the perfusate than those reperfused with the Wif or Elohes solutions (Fig. 1 A, $P < 0.05$ vs Wif and Elohes solutions). Furthermore, livers perfused with the Elohes solution exhibited marked sinusoidal endothelial cell injury, as reflected in higher perfusate CK-BB isoenzyme release (Fig. 1 A, $P < 0.05$ vs CRS and Wif solution). When rat livers were washed out and preserved in the high-Na^+ UW solution, we observed no statistical difference between CRS and Wif solutions during the 30 min of perfusion (Fig. 1 B). Again, livers perfused with the Elohes solution released the highest rate of CK-BB isoenzyme in the perfusate (Fig. 1 B, $P < 0.05$ vs CRS and Wif solution).

Study 2

Figure 2 shows the total amount of ICG excreted into the bile (expressed as a percentage of the added ICG) between 30 and 120 min of perfusion. The groups of

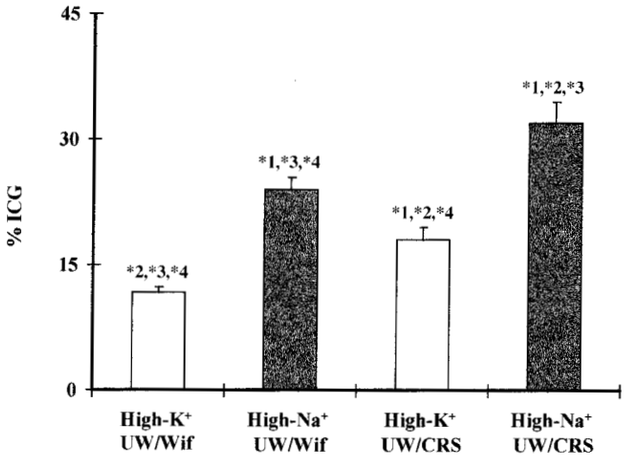

Fig. 2 Bile ICG excretion during 90 min (from 30 to 120 min) of perfusion. Rat livers were washed out and preserved in either high-K^+ UW or high-Na^+ UW solution for 24 h at 4 °C. Immediately before reperfusion, the preservation solutions were flushed out with Wif or CRS solution and perfused at 37 °C for 120 min. ICG is expressed as the percentage bile excretion of the administered dose. Data are expressed as mean ± SEM ($n = 7$) $^{*1}P < 0.05$ vs high-K^+ UW/Wif; $^{*2}P < 0.05$ vs high-Na^+ UW/Wif; $^{*3}P < 0.05$ vs high-K^+ UW/CRS; $^{*4}P < 0.05$ vs high-Na^+ UW/CRS

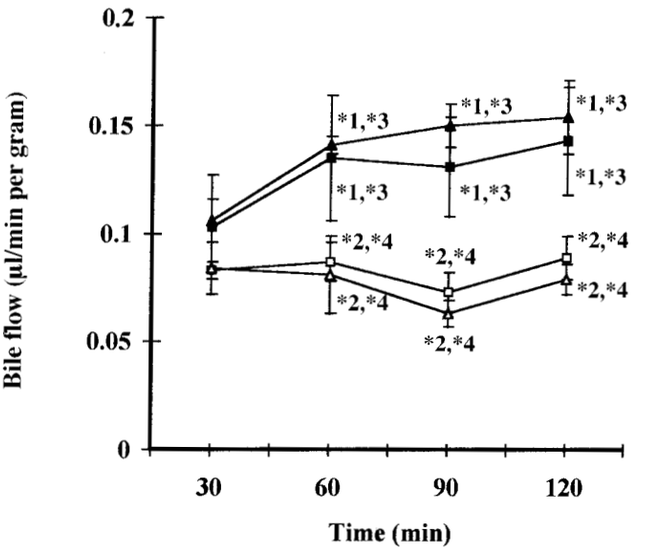

Fig. 3 Bile flow during 120 min of perfusion. Rat livers were washed out and preserved in high-K^+ UW or high-Na^+ UW solution for 24 h at 4 °C. Immediately before reperfusion, the preservation solutions were flushed out with Wif or CRS solution and perfused at 37 °C for 120 min. Data are expressed as mean ± SEM ($n = 7$) $^{*1}P < 0.05$ vs high-K^+ UW/Wif; $^{*2}P < 0.05$ vs high-Na^+ UW/Wif; $^{*3}P < 0.05$ vs high-K^+ UW/CRS; $^{*4}P < 0.05$ vs high-Na^+ UW/CRS. –△– High-K^+ UW/CRS; –□– High-K^+ UW/Wif; –▲– High-Na^+ UW/CRS; –■– High-Na^+ UW/Wif

livers preserved in the high-Na^+ UW solution (high-Na^+ UW/Wif and high-Na^+ UW/CRS groups) exhibited higher ICG clearance ($P < 0.05$) than the groups of livers preserved in the high-K^+ UW solution (high-K^+ UW/Wif and high-K^+ UW/CRS groups). On the other hand, the ICG clearance was always highest ($P < 0.05$) when CRS was used to flush the preservation solutions. Almost the same pattern was observed for bile flow (Fig. 3), AST (Fig. 4), and ALT (Fig. 5) release. The groups of livers preserved in the high-Na^+ UW solution (high-Na^+ UW/Wif and high-Na^+ UW/CRS groups) exhibited the highest bile flow ($P < 0.05$) and released the lowest amounts of AST and ALT in the perfusate ($P < 0.05$). However, we found no statistical differences between CRS and Wif solutions for these three parameters.

Discussion

In organ transplantation, primary graft dysfunction and/or nonfunction caused by preservation and subsequent reperfusion injuries are still major problems. The extent of the damage depends on the ischemic injury, itself determined by the type of preservation solution and the duration of cold storage. The damage is also due to other pathophysiological mechanisms activated upon reperfusion and reoxygenation [10]. Reperfusion injury is characterized by activation of the Kupffer cells and loss of viability of the sinusoidal endothelial cells. In experimental practice, numerous interventions have been proposed to counteract reperfusion injuries. For example, the use of CRS to flush out the cold storage solution just before revascularization results in a reduction in rat graft failure [18, 29, 30]. With the IPRL model, virtually no nonparenchymal cell death occurred, as indicated by the absence of trypan blue staining, when the livers were perfused with CRS [12, 14]. In addition, scanning electron microscopy showed a smooth endothelial cell surface after CRS perfusion, corresponding to an intact cell membrane [14].

In the liver, CK-BB isoenzyme was found mainly in Kupffer cells and endothelial cells [37]. The release of this enzyme into the perfusate after reperfusion points to a lysis of these nonparenchymal cells. In human liver transplantation, Chazouillères et al. [8] observed a marked liberation of CK after graft reperfusion, while Karayalçin et al. [23] found no positive correlation between CK-BB release and the different graft function parameters. Differences in the results of the two studies may be due to the timing of the effluent collection. Indeed, in the first report, effluent was collected during the first minutes of graft reperfusion; however, in the second study, effluent was collected from the liver graft rinse before revascularization. It has been shown that endothelial cell death is a reperfusion-induced injury [25]. Therefore, liver CK-BB release is reperfusion-de-

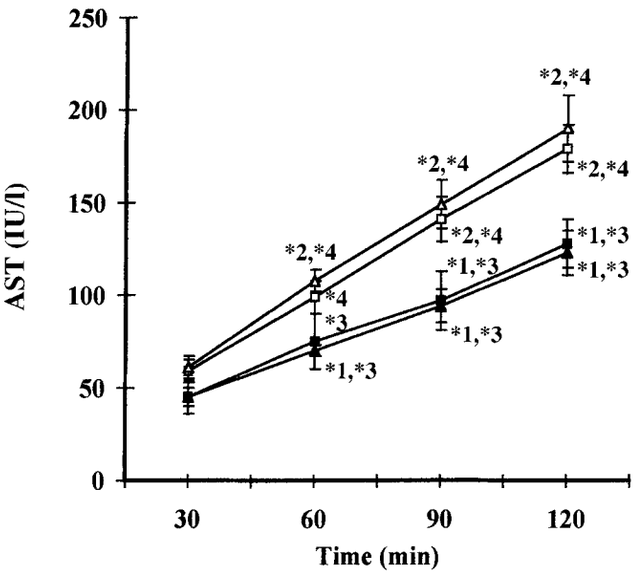

Fig. 4 Perfusate AST rate during 120 min of perfusion. Rat livers were washed out and preserved in high-K^+ UW or high-Na^+ UW solution for 24 h at 4 °C. Immediately before reperfusion, the preservation solutions were flushed out with Wif or CRS solution and perfused at 37 °C for 120 min. Data are expressed as mean ± SEM ($n = 7$) $^{*1}P < 0.05$ vs high-K^+ UW/Wif; $^{*2}P < 0.05$ vs high-Na^+ UW/Wif; $^{*3}P < 0.05$ vs high-K^+ UW/CRS; $^{*4}P < 0.05$ vs high-Na^+ UW/CRS. –△– High-K^+ UW/CRS; –□– High-K^+ UW/Wif; –▲– High-Na^+ UW/CRS; –■– High-Na^+ UW/Wif

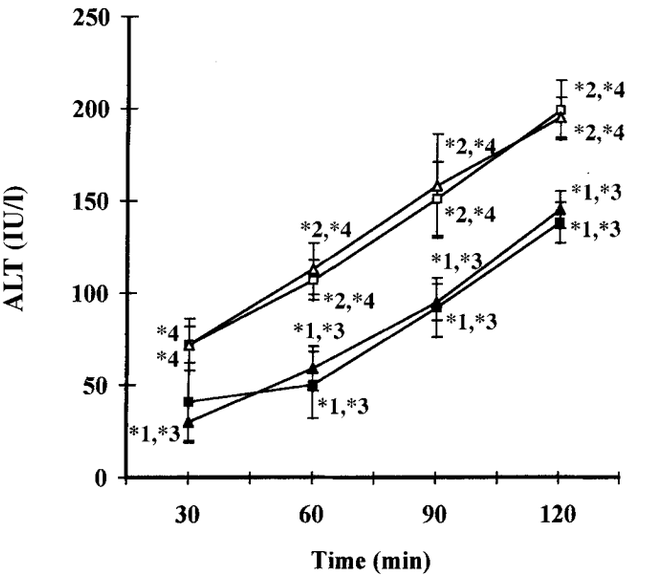

Fig. 5 Perfusate ALT rate during 120 min of perfusion. Rat livers were washed out and preserved in high-K^+ UW or high-Na^+ UW solution for 24 h at 4 °C. Immediately before reperfusion, the preservation solutions were flushed out with Wif or CRS solution and perfused at 37 °C for 120 min. Data are expressed as mean ± SEM ($n = 7$) $^{*1}P < 0.05$ vs high-K^+ UW/Wif; $^{*2}P < 0.05$ vs high-Na^+ UW/Wif; $^{*3}P < 0.05$ vs high-K^+ UW/CRS; $^{*4}P < 0.05$ vs high-Na^+ UW/CRS. –△– High-K^+ UW/CRS; –□– High-K^+ UW/Wif; –▲– High-Na^+ UW/CRS; –■– High-Na^+ UW/Wif

pendent. In our study, less CK-BB was released when the preserved livers were perfused with CRS. Assuming that the isoenzyme is an appropriate marker of endothelial cell injury, the results would indicate that CRS is effective in significantly reducing nonparenchymal cell damage. This is in accordance with other reports on the beneficial effect of the rinse solution.

In this study, we used three different solutions to perfuse the cold-preserved rat livers. During the first 10–12 min of perfusion, a period that corresponds to the stabilization of portal pressure, the portal flow was increased manually and gradually from 0.5–1 ml/min per gram to 2.5–3 ml/min per gram while maintaining a portal pressure of between 10 and 12 mm Hg. We found no statistical differences in portal pressure between the different groups (data not shown). However, we observed that livers perfused with Wif and Elohes solutions released greater amounts of CK-BB isoenzyme than livers perfused with CRS. The highest perfusate rates were exhibited by the Elohes solution. Furthermore, we found a substantial difference between the three solutions in perfusate CK-BB at time 0 (Fig. 1 A). Time 0 corresponds to an initial observation time point when the rat liver was satisfactorily established in the isolated perfusion circuit and coincides with 2–3 min after the onset of normothermic perfusion. These results show that CK-BB release is directly related to preservation-reperfusion damage and not to mechanical injury due to perfusion. This damage is rapid and depends on the composition of the perfusate.

The Elohes solution contains only hydroxyethyl starch (HES). It has been shown that modified CRS without HES is as effective as the original CRS [14]. Wif solution has a plasma-like electrolyte composition containing a mixture of amino acids and vitamins. Several amino acids are known to be effective in preventing proteolysis [38]. Glycine is known to be a cytoprotective molecule [15]. Vitamins participate in multiple metabolic reactions involved in the maintenance of cell membrane integrity and could play a direct role as antioxidants [26]. CRS contains the electrolytes of Ringer's solution, antioxidants (allopurinol, desferrioxamine, and glutathione), substrates for ATP regeneration (fructose, glucose, and adenosine), a calcium channel blocker (nicardipine), insulin, and a mildly acidic pH [14]. Currin et al. have shown that CRS's acidic pH was the single most important protective factor against endothelial cell death [14]. It has been shown that, during liver ischemia, acidosis greatly inhibits cell death caused by anoxia and metabolic inhibition [13]. In addition, it is

known that when rat livers are reperfused with a slightly acidic buffer, endothelial cell death is greatly reduced after cold storage [7]. The superiority of CRS over the Wif solution is probably due to its acidic pH.

Recently, we showed that a high-Na^+ UW solution was more effective in preserving the rat liver [3] and kidneys [17, 31, 32] than a high-K^+ UW solution. In this study, we preserved rat livers in the two UW cold storage solution variants. We found that livers preserved in the high-Na^+ UW solution released less CK-BB isoenzyme into the perfusate than livers preserved in the high-K^+ UW solution during 30 min of perfusion with the Wif, CRS, and Elohes solutions. In addition, we found no statistical difference between the high-Na^+ UW/Wif group and the high-Na^+ UW/CRS group for CK-BB isoenzyme release. These results suggest that CRS would be less effective in maintaining nonparenchymal cell integrity when preservation injury is decreased.

The IPRL model is now a well-established technique, widely used to study liver preservation [22]. In spite of the availability of other techniques (hepatocyte culture, for example), it remains convenient for an evaluation of global hepatic function. In contrast to other in vitro models, the IPRL maintains hepatic architecture, cell polarity, and bile flow. In addition, it allows repeated sampling of the perfusate and easy exposure of the liver to different concentrations of test substances. The perfused liver, however, can only be used for perfusion of short duration. It should be used as a screening technique, especially after liver cold preservation because hepatic functions are in a depleted state.

The major problem with the IPRL model is correlating measurements of hepatic function with hepatic viability. Several tests have been proposed in the literature [21, 22], but ICG clearance or its bile excretion are scarcely used. ICG has several advantages: it has no toxic effects, it binds rapidly to plasma albumin and, consequently, it Spills into the vascular volume [9], its bile recovery in unchanged form is complete after intravenous administration [20], there is no uptake of ICG by extrahepatic tissues [20], there is no urinary excretion [9], and it is easy to measure. A correlation has been shown between microvascular damage and hepatic ICG clearance in a swine liver transplantation model [34]. In addition, it was shown that the clearing capacity of the donor liver correlated positively with early graft function/injury after transplantation [24]. Because of these advantages, ICG was assessed in this study. As we used an IPRL model in a closed circuit and with low portal flow rates, it is technically easier to assess ICG excreted in the bile than that disappearing from the perfusion medium. That is why the term "bile ICG excretion" is used instead of "ICG clearance". We found no statistical difference between cold storage solution flush-out with either CRS or Wif solution for bile flow and transaminase levels. However, a statistically significant difference was reached with bile ICG excretion. The removal of ICG from plasma depends on blood flow [4], and its recovery in bile depends on hepatocyte status [20]. We can infer that ICG recovery in bile reflects functional perfusate flow as well as hepatocyte viability. On the other hand, perfusate AST and ALT release may serve as a marker of hepatocellular damage. We found that CRS improves bile ICG excretion but does not decrease transaminase levels. These results indicate that CRS mainly prevents manifestations of microvascular injury. It has been shown that this rinse solution counteracts reperfusion injuries by improving hepatic microcirculation. This improvement also depends on the quality of the storage solution.

Early graft function was estimated on the basis of bile production after reperfusion. It has been shown that bile flow provides more information about global liver function than do hepatocellular enzyme levels [22]. Post et al. found that bile flow and excretion of bile acids during the first 90 min after rat liver transplantation were improved by the application of CRS [29]. We found no statistical difference between CRS and Wif solution for bile flow during 120 min of normothermic perfusion. In the IPRL model, it has been shown that bile production is under the control of different mechanisms that are dependent upon cellular energy [35]. Hepatic perfusate flow is, therefore, thought not to be a critical determinant of bile secretion. Since the primary reason for using CRS is to mantain sinusoidal endothelium integrity, and since the perfusate flow is maintained in a "physiological" range, it seems normal that we found no statistical effect on bile flow.

In conclusion, our results confirm the superiority of high-Na^+ UW over high-K^+ UW solution for liver cold preservation and provide further support for the effectiveness of CRS. Its beneficial effect is evident in cases of microvascular injury with high-K^+ UW solution; however, this is significantly lessened by the use of the high-Na^+ UW variant. Studying parenchymal injury and function revealed that CRS did not offer protection from hepatocellular injury but rather allowed an improvement in perfusate CK-BB release and bile ICG excretion.

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