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High-Na^+ low-K^+ UW cold storage solution reduces reperfusion injuries of the rat liver graft

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Abstract The isolated perfused rat liver model was used to assess graft viability after 24 h of cold preservation. Two solutions were compared for liver preservation: Belzer's original UW solution (high-K^+ UW) and a solution containing the same components but with inverted concentrations of sodium and potassium (high-Na^+ UW). During the 120 min of normothermic reperfusion, livers preserved in the high-Na^+ UW solution released lower levels of creatine kinase-BB isoenzyme, transaminases (ALT and AST), and potassium than those preserved in the high-K^+ UW solution. Bile flow and biliary excretion of indocyanine green increased when livers were preserved in the high-Na^+ UW solution. We found no statistical differences for oxygen consumption and tissue ATP concentration. The results of this study support the concept that a high-Na^+ UW solution is a more effective means of preserving rat livers, at least after 24 h of cold-storage and 120 min of reperfusion in the isolated perfused model, than the original high-K^+ UW solution. Liver preservation in the high-Na^+ UW solution reduces damage to sinusoidal endothelial and hepatocellular cells. The use of an extracellular-like Belzer cold storage solution eliminates potassium-related problems in cold preservation and subsequent normothermic reperfusion while keeping all the qualities of the original UW solution.

Key words UW solution, rat liver, preservation · Preservation, rat liver, modified UW solution · Rat liver, preservation, modified UW solution

Introduction

Transplantation has become the most appropriate treatment for end-stage liver failure, but it requires exposure of the graft to ischemia for variable periods of time. Each graft undergoes damage during hypothermic storage and subsequent normothermic reperfusion [7, 21]. Several studies have investigated the mechanisms of tissue injury, termed "cold ischemia-reperfusion injury", that are responsible for primary nonfunction [6, 7]. They showed that the sinusoidal endothelial cells are damaged during hypothermic preservation, leading to microcirculatory disturbances and leukocyte-endothelial cell adherence after normothermic reperfusion. As an ultimate result of microvascular reperfusion failure, hypoxia, necrosis of hepatocytes, and nonfunction of the liver graft occur.

Graft quality depends partly on the cold storage solution used [22]. The development and use of Belzer's University of Wisconsin (UW) solution have revolution-

ized hepatic transplantation. The superiority of UW solution over all previously used solutions is now widely acknowledged, based on both experimental [14, 15] and clinical [16, 30] evidence. Preservation in UW solution increases the time of storage. The decrease in endothelial cell death after reperfusion of livers stored in UW solution parallels the improvement in graft survival after transplantation.

Much work has been done to improve the effectiveness of UW solution by simplifying its composition or by adding other compounds. Some of these studies have tested the inversion of the Na^+/K^+ ratio of UW solution, making the solution, initially an intracellular milieu, an extracellular preparation. They have shown that the high-Na^+ UW solution is at least as effective [13, 33], if not more effective, than the high-K^+ UW version [23, 25]. However, none of these studies have evaluated liver microcirculation after preservation in the high-Na^+ UW solution.

In this study we evaluated microvascular disturbances, assessed by indocyanine green clearance and the release of creatine kinase-BB (CK-BB) isoenzyme in the perfusate, after reperfusion of rat livers preserved in the original, intracellular UW solution (Na^+ 30 mM/K^+ 125 mM) versus the same solution but with an inverted Na^+/K^+ ratio. In addition, we compared the metabolic and functional capacities of the preserved livers.

Materials and methods

Animals

Male inbred Wistar rats (Iffa-Credo, St Germain-sur-l'Arbresle, France) weighing 300 g were used as organ donors. Before the experiments, they had free access to water and a standard pellet diet. Animals were handled in compliance with French regulations and the "Principles of laboratory and animal care" (NIH publication No. 86–36, revised 1985).

Cold storage solutions

Two cold storage solutions, prepared in our pharmaceutical department, were compared. The first was Belzer's original UW solution (high-K^+ UW solution, K^+ 125 mM/Na^+ 30 mM) and the second was a solution containing the same compounds as the original UW solution but with high-Na^+ and low-K^+ concentrations (high-Na^+ UW solution, Na^+ 125 mM/K^+ 30 mM) [24].

Liver harvesting and experimental groups

Rats were anesthetized by an intraperitoneal injection of sodium pentobarbital (6 g%, 0.1 ml/100 g body weight). Two hundred and fifty international units (IU) of heparin per hundred grams of body weight were injected into the femoral vein. A laparotomy was performed first by an abdominal midline incision and then by a bilateral transverse extension, and a catheter (Venocath 18G) was inserted into the common bile duct for bile collection. Then, the aorta, vena cava, and portal vein were dissected. Organs were washed out, with one of the cold storage solutions, by means of a catheter (Venocath 16G) inserted into the aorta, at a constant flow rate of 3 ml/min. To allow exsanguination, the abdominal vena cava was cut off and catheterized, the thoracic vena cava was ligated. During aortic perfusion alone, the portal flow (returning from the splanchnic area) was sufficient to flush the liver graft. In this way, kidneys, intestine, pancreas, and liver were washed out [4, 32]. A third catheter (Venocath 14G) was inserted into the portal vein, and the whole liver was then excised and trimmed of adhering tissue. On the back table, 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.

For each solution, two groups of five rats were used:

1. A control group in which livers were rinsed with high-K^+ UW solution or with high-Na^+ UW solution, then perfused for 120 min in a closed circuit immediately after harvesting, and
2. A preservation group in which livers were rinsed and stored in a small container with 75 ml of either solution for 24 h at 4 °C; the organs were then perfused for 120 min in a closed circuit.

Before reperfusion, all livers were washed out with the perfusate to eliminate the cold storage solution.

Liver perfusion

The perfusate consisted of a cell culture medium (William's medium E, BioWhitaker) with a plasma-like electrolyte composition [2]. We added albumin (5 g%) as oncotic supply. The medium was continuously gassed with a 95% O_2-5% CO_2 mixture and the pH adjusted to 7.30–7.40 with sodium bicarbonate.

A continuous and closed perfusion circuit built in our laboratory was used. Briefly, the liver perfusion system consisted of a thermostatically controlled, medical grade, acrylic plastic container that was divided into two parts: a tank containing the perfusate (150 ml) and a platform supporting the liver. The perfusate circulated by means of a peristaltic pump, passed through a macrofilter and a bubble trap and into the liver. The perfusion pressure was continuously monitored by an in-line manometer within the portal vein cannula. All of the livers were perfused for 120 min at 37 °C.

Liver function study

During an equilibration period of about 30 min after the onset of perfusion, the portal flow was progressively increased. Portal pressure was continuously monitored and was kept in the physiological range. Serial samples of perfusate were taken for measurement of total CK and CK-BB isoenzyme activity [8, 31]. Total CK activity was determined with a kit (BioMérieux, Charbonnière-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 fluorometric densitometer (SEBIA, France). All CK measurements were done every day.

Bile was collected at 30-min intervals and the volume was estimated by weighing. Thirty minutes after starting perfusion, 1 mg of indocyanine green (ICG) was added to the perfusate and the percentage bile excretion of the administered dose was measured. The concentration of ICG was determined by measuring absorbance at 800 nm of diluted samples using a UV-visible spectrophotometer [5].

Table 1 Portal flow, portal pressure, and CK-BB isoenzyme after 24 h of cold preservation and during the 30-min equilibration period. Data represent the mean ± SE ($n = 5$)

Time	High-K^+ UW			High-Na^+ UW		
	24-h cold preservation	15 min	30 min	24-h cold preservation	15 min	30 min
Portal flow (ml/min per gram)		1.86 ± 0.07	2.18 ± 0.07		1.94 ± 0.07	2.28 ± 0.07
Portal pressure (mm Hg)		12.2 ± 0.4	12.2 ± 0.5		12.6 ± 0.5	12.8 ± 0.7
CK-BB (IU/g)	0.90 ± 0.18	0.69 ± 0.16*	0.90 ± 0.12*	0.54 ± 0.18	0.39 ± 0.09	0.53 ± 0.16

* $P < 0.05$ vs high-Na^+ UW

Following a 30-min equilibration period, caval outflow perfusate samples were taken for electrolyte (Na^+ and K^+), aspartate aminotransferase (AST), and alanine aminotransferases (ALT) measurements on a Hitachi 747 analyzer. In addition, perfusate samples of portal inflow and caval outflow were taken to measure oxygen tension differences using an ABL blood-gas analyzer (Acid Base Laboratory, Radiometer, Copenhagen, Denmark) as an index of oxygen uptake.

At the end of the 120-min perfusion period, samples of tissue were taken to determine tissue ATP concentration by a bioluminescence technique. After rapid removal of the sample and homogenization in cold 5% trichloroacetic acid, the acid insoluble material was removed by cold centrifugation (3000 *g*, 20 min) and the acid supernatant neutralized by 1N NaOH [29]. The homogenates were frozen (–80 °C) until assay (about 1 month). ATP was measured by the bioluminescent firefly luciferase assay [29]. ATP was measured in control livers perfused for 120 min, in 24 h cold-stored livers, and in 24 h cold-stored and 120-min reperfused livers.

Statistical analysis

Results were expressed as the mean ± SE. for five livers in each group. Data between groups were compared with an ANOVA test, followed by PLSD Fischer's test. Statistical significance was defined as a P level below 0.05.

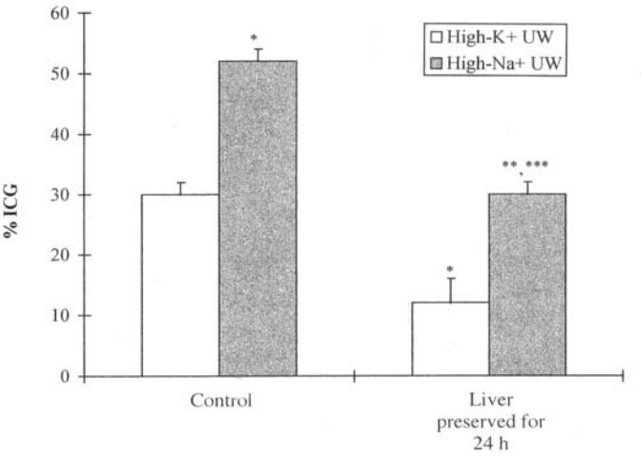

Fig. 1 ICG clearance following 90 min (from 30 to 120 min) of normothermic perfusion. Values are expressed as a percentage of ICG quantity in bile according to ICG quantity in perfusate. Data represent the mean ± SE ($n = 5$) * $P < 0.05$ vs control high-K^+ UW; ** $P < 0.05$ vs control high-Na^+ UW; *** $P < 0.05$ vs preserved high-K^+ UW

Results

Table 1 shows portal flow and pressure and CK-BB isoenzyme activity during the 30-min equilibration period. The CK-BB isoenzyme has been shown to be a marker of sinusoidal lining cell injury [31]. CK-BB isoenzyme activity was also assessed in the preservation solution purged from the preserved liver. We found no statistical differences for portal flow or portal pressure between the two preserved groups. Livers preserved in the high-K^+ UW solution released statistically more CK-BB than those in the high-Na^+ UW solution. This statistical difference was reached at 15 and 30 min of perfusion. The total amount of ICG (expressed as a percentage of the added ICG) excreted into the bile collected between 30 and 120 min is shown in Fig. 1. The added ICG was rapidly taken up by all livers, but the amount excreted into bile was significantly different between the two storage solutions. Both control high-Na^+ UW and preserved high-Na^+ UW groups excreted more ICG into bile than, respectively, the control high-K^+ UW and the preserved high-K^+ UW groups ($P < 0.05$). Control high-Na^+ UW livers excreted 52% of the added ICG. After 24-h preservation in the same solution, ICG clearance declined significantly to 30% ($P < 0.05$). Control high-K^+ UW livers excreted 30%, and only 12% after 24-h preservation ($P < 0.05$). Compared to the respective control groups, ICG clearance declined by 42% in the preserved high-Na^+ UW group and by 60% in the preserved high-K^+ UW group.

The results of bile flow during 120 min of perfusion are summarized in Fig. 2. Bile flow was higher in the control and preserved high-Na^+ UW groups ($P < 0.05$). In both control groups, bile flow started immediately after reperfusion and was significantly higher in the control high-Na^+ UW at 60 and 120 min ($P < 0.05$). In the preserved high-K^+ UW group, bile flow started very slowly, then increased gradually during perfusion. In contrast, in the preserved high-Na^+ UW group, bile

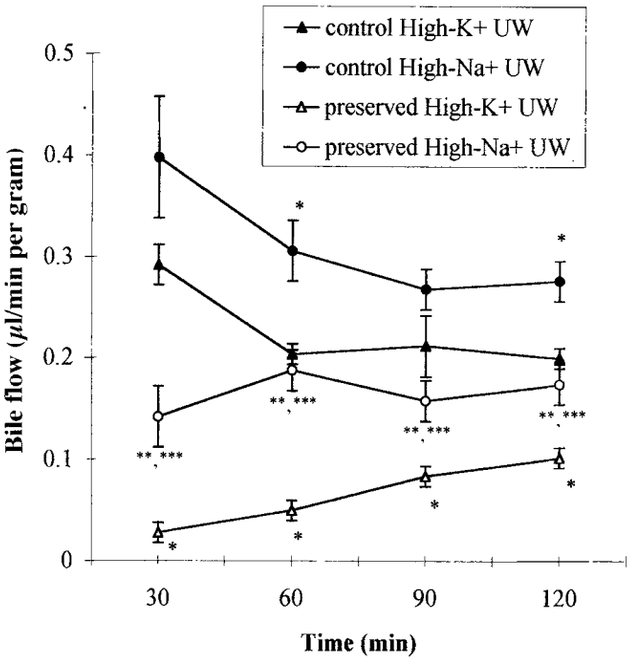

Fig. 2 Bile flow during 120 min of normothermic perfusion. Values are expressed in µl/min per gram liver. Data represent the mean ± SE ($n = 5$) * $P < 0.05$ vs control high-K^+ UW; ** $P < 0.05$ vs control high-Na^+ UW; *** $P < 0.05$ vs preserved high-K^+ UW

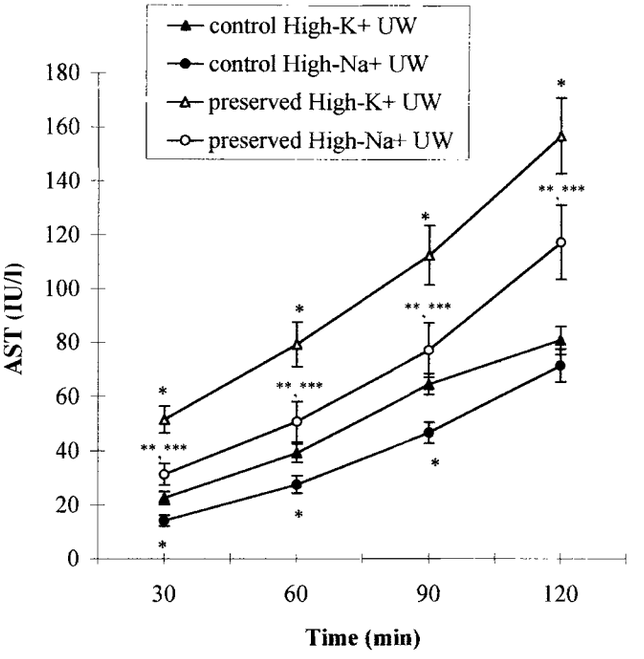

Fig. 3 AST perfusate content during 120 min of normothermic perfusion. Values are expressed in IU/l. Data represent the ± SE ($n = 5$) * $P < 0.05$ vs control high-K^+ UW; ** $P < 0.05$ vs control high-Na^+ UW; *** $P < 0.05$ vs preserved high-K^+ UW

flow was more stable and significantly higher at the different times of reperfusion ($P < 0.05$). In both preserved groups, bile flow was significantly lower than in the respective control groups ($P < 0.05$).

Transaminase activity (AST and ALT) in the perfusate is shown in Figs. 3 and 4. When livers were preserved in the high-Na^+ UW liquid, AST and ALT activity was significantly lower than in the preserved high-K^+ UW group at the different times of reperfusion ($P < 0.05$). In the control high-K^+ UW group, the amount of AST in the perfusate was significantly higher at 30, 60, and 90 min than in the control high-Na^+ UW group ($P < 0.05$), but there was no difference between the two control groups for ALT activity.

The perfusate potassium content during the 120 min of normothermic perfusion is shown in Fig. 5. Before reperfusion, all livers were rinsed with the same perfusate medium at room temperature to eliminate the potassium excess in cold-stored organs. Both the control high-Na^+ UW and the preserved high-Na^+ UW groups excreted less potassium than the respective control high-K^+ UW and preserved high-K^+ UW groups ($P < 0.05$).

Tissue ATP concentrations in control livers, in livers preserved for 24 h in the two different solutions, and in livers preserved for 24 h and reperfused are shown in Fig. 6. We found no statistical difference between the two control groups. After 24-h cold preservation, ATP

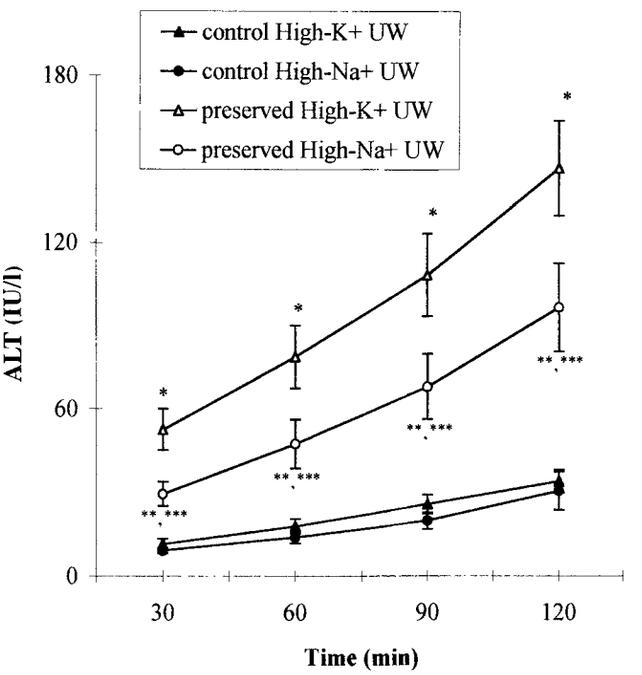

Fig. 4 ALT perfusate content during 120 min of normothermic perfusion. Values are expressed in IU/l. Data represent the mean ± SE ($n = 5$) * $P < 0.05$ vs control high-K^+ UW; ** $P < 0.05$ vs control high-Na^+ UW; *** $P < 0.05$ vs preserved high-K^+ UW

Table 2 Oxygen uptake during 120 min of normothermic perfusion. Data represent the mean ± SE ($n = 5$)

	High-K^+ UW		High-Na^+ UW	
	Control	24-h preservation	Control	24-h preservation
Oxygen uptake	334 ± 9	334 ± 9	344 ± 7	347 ± 6

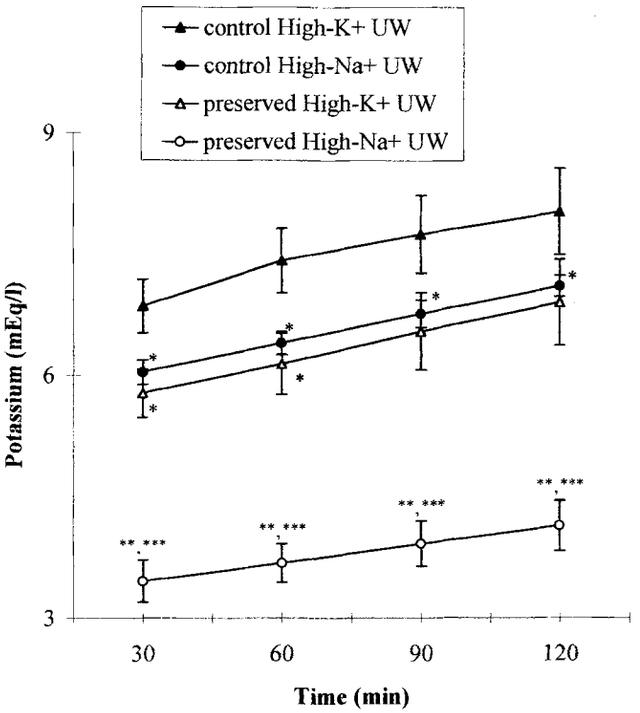

Fig. 5 Potassium perfusate content during 120 min of normothermic perfusion. Values are expressed in mEq/l. Data represent the mean ± SE ($n = 5$) * $P < 0.05$ vs control high-K^+ UW; ** $P < 0.05$ vs control high-Na^+ UW; *** $P < 0.05$ vs preserved high-K^+ UW

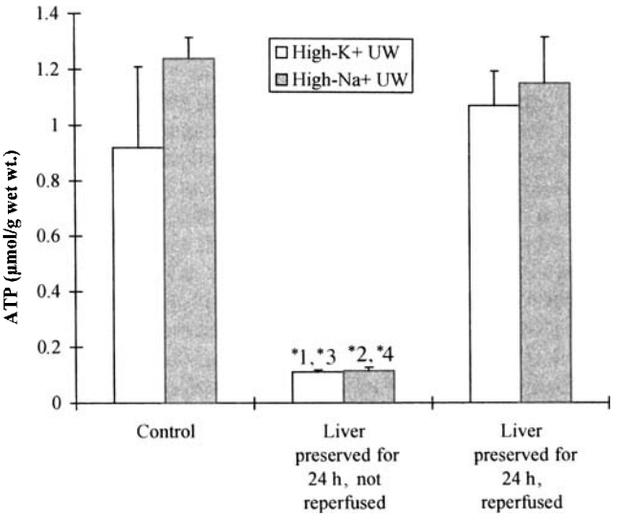

Fig. 6 ATP tissue concentrations. Data represent the mean ± SE. ($n = 5$) ^{*1} $P < 0.05$ vs control high-K^+ UW; ^{*2} $P < 0.05$ vs control high-Na^+ UW; ^{*3} $P < 0.05$ vs preserved and reperfused high-K^+ UW; ^{*4} $P < 0.05$ vs preserved and reperfused high-Na^+ UW

tissue concentrations decreased to ≈ 10% of the respective control groups' basal values. Normothermic reperfusion after cold storage induced a restoration of the ATP level compared to the respective control groups, although no statistical difference was found between the two preserved groups.

Results concerning oxygen uptake are shown in Table 2. The difference in oxygen tension between portal inflow and vena cava outflow was used as an index of oxygen uptake. We found no statistical difference between control and preserved groups for either solution used.

Discussion

Hypothermia remains the basis of all preservation techniques. It does not stop metabolism but slows reaction rates and can, by itself, prolong anoxic tissue viability [1]. Maintaining normal intracellular conditions is a process that requires energy, and, because of the impairment of Na^+/K^+-dependent ATPase by hypothermia and energy depletion, the cell loses its normal volume-regulating capacity and will be damaged. In addition, under cold storage conditions and lack of oxygen, the cell will switch to anaerobic metabolism with the resultant development of metabolic acidosis [20]. Therefore, by combining hypothermia and an appropriate flush-out solution to avoid all these alterations, the storage time can be increased. In this field, the UW solution developed by Belzer has brought about a major improvement in human liver transplantation [30].

The high potassium concentration of the UW solution makes it an intracellular-like preparation. The arguments for using potassium-rich preservation solutions are based on early studies of Keeler et al. [18] and Collins et al. [9]. Indeed, because of the inhibition of Na^+/K^+-dependent ATPase by hypothermia, such solutions prevent the loss of intracellular potassium and the gain of sodium. The Na^+/K^+ gradients are therefore maintained, thus avoiding ionic shifts and damage to the cell.

More recently, with the development of the UW cold storage solution, the need for such a high concentration of potassium was questioned. Indeed, after 48-h preser-

vation and reperfusion of rabbit livers, the inversion of the Na^+/K^+ ratio (100 mM Na^+/40 mM K^+) did not have any effect [13]. No effects were observed with the 155 mM/5 mM Na^+/K^+ ratio on the survival of rat grafts after 20 h of preservation [33]. In contrast, on a similar model and with a Na^+/K^+ ratio of 120 mM/40 mM and 24-h preservation, the rat grafts' survival rate was increased [25]. With a Na^+/K^+ ratio of 140 mM /9 mM, dog livers were better preserved than in the high-K^+ UW solution [23]. These studies demonstrate that what distinguishes the UW solution for organ preservation is the appropriate type and concentration of impermeants to suppress hypothermically induced cell swelling and consequent damage. Gluthatione adds some protection by reducing the formation of cytotoxic end products of oxygen metabolism. Biguzas et al. [3], using the rat kidney isograft model, showed that a concentration of 30–50 mM potassium would be as effective as the usual 120–140 mM of potassium cold storage solution. Ramella et al. [24], using the isolated perfused rat kidney model, showed that functional parameters were improved when kidneys were preserved in the UW solution containing 125 mM of Na^+ and 30 mM of K^+. However, none of these studies has shown any effect on hepatic microcirculation after liver preservation in an extracellular solution.

We used the isolated perfused rat liver model to assess graft viability after 24 h of cold preservation. This model has the advantage of being relatively simple and rapid, and the methods of evaluation are reproducible. It maintains almost normal hepatic architecture, microcirculation, and bile production [10]. However, the functional parameters are below physiological values and undergo progressive changes after 2–3 h of perfusion [10].

In the liver, the CK-BB isoenzyme is an enzyme found mainly in Kupffer cells and endothelial cells [31]. The release of this enzyme into the preserved livers' perfusate after reperfusion indicate a lysis of these nonparenchymal cells. Livers preserved in the high-Na^+ UW cold storage solution released less of the CK-BB isoenzyme than livers preserved in the high-K^+ UW solution. On the other hand, we found no statistical differences in portal flow or portal pressure between the two preserved groups. Therefore, CK-BB release would appear to be directly related to preservation-reperfusion damage and not to mechanical injury during reperfusion.

Indocyanine green (ICG) is a water-soluble dye excreted in almost totally unchanged form in the bile [15]. It has no toxic effects and is cleared specifically by the liver [5]. A positive correlation has been demonstrated between bile formation and dye excretion [26], and between microvascular damage and ICG clearance [27]. In addition, the liver ICG-clearing capacity correlates positively with early graft function/injury [19]. Thus, it is a good marker of graft function. In this study, we found higher ICG excretion in bile when livers were preserved in the high-Na^+ UW solution.

Some investigators have proposed bile flow as a sensitive indicator for preservation quality [15, 26], and a close correlation between bile production and ATP content in the liver has been demonstrated [17]. Our study showed better bile production when livers were preserved in the high-Na^+ UW solution.

The release of enzymes, and particularly of AST, was found to be a good viability index, able to predict the viability of preserved, transplanted livers, and might be used to screen the efficacy of new preservation strategies [12]. In our study, we found lower levels of transaminase release when livers were preserved in the high-Na^+ UW solution. In addition, the ratio of transaminase amounts in preserved livers versus transaminase amounts in control livers was highest during the first 30 min of perfusion; it then reached a plateau for ALT and decreased for AST, reflecting primarily their release from cells damaged during storage and initial reperfusion. This result is in agreement with that of CK-BB.

Potassium release could be used as a marker of membrane damage and cell lysis. We used a plasma-like perfusate, containing 5 mEq/l of potassium, to perfuse livers. Only for livers stored in high-Na^+ UW solution was the amount of potassium in the caval vein lower than that of the perfusate. It could be assumed that this decrease was due to uptake of potassium during the normothermic reperfusion. This uptake probably represents the reaccumulation of potassium lost by the hepatocytes during cold storage. Potassium is accumulated actively in the cell by the Na^+/K^+ ATP-dependent pump; thus, a drop in perfusate potassium suggests that the Na^+/K^+ pump is functioning correctly.

There are several disadvantages to using potassium-rich cold storage solutions. They cause blood vessel constriction and, thus, heterogeneous graft rinsing. If the graft is not efficiently flushed before transplantation, the systemic K^+ rises and causes cardiac arrest in the recipient. Reperfusion injury, characterized by loss of sinusoidal endothelial cells viability and activation of Kupffer cells, occurs after reperfusion of stored livers [6]. Our study is the first one to show a reduction in reperfusion injury and microvascular disturbances for rat livers preserved in a high-Na^+ UW solution. A high potassium concentration depolarizes cell membranes [20]. Furthermore, rat Kupffer cells are known to contain voltage-dependent calcium channels [11]. The increase in cytosolic free Ca^{++} concentrations activate Kupffer cells, inducing damage at reperfusion [7, 28]. With the extracellular solution used in our study containing 30 mM of potassium, the membrane potential would still remain negative [3, 20], and we can hypothesize that Kupffer cells should be less activated and reperfusion injury reduced.

During the 120 min of normothermic perfusion of the control high-Na^+ UW group, we found that the ICG clearance, the level of transaminases, and the bile flow were better than those of the control high-K^+ UW group. These parameters were still better after 24-h preservation. This suggests that the deleterious effect of the potassium begins as soon as the organ is flushed and continues, undiminished, after preservation.

We measured oxygen uptake by hepatocytes and ATP tissue concentration. No differences were found between the groups studied. ATP represents chemical energy indispensable to cellular functioning (biosynthesis of components, transmembranous ionic and molecular transport). ATP is synthesized from glucose degradation by means of two successive biochemical steps: cytosolic glycolysis followed by intramitochondrial oxidative phosphorylation. As cells become anoxic, oxidative phosphorylation ceases and the remaining ATP is consumed very rapidly. During harvesting and cooling, blood flow and, hence, oxygen supply are stopped. Consequently, ATP decreases and the metabolism can function only in anaerobic glycolysis. After reperfusion and rewarming, oxygen is no longer the limiting factor and ATP is regenerated. Since our groups consumed equal amonts of oxygen, one might expect that they would regenerate ATP equally. Mitchell et al. found varying degrees of ATP regeneration in rat livers when stored in different preservation solutions and reperfused at 4 °C with an air-equilibrated perfusate [22]. However, the highest ATP production never exceeded 50% of the amount in control fresh liver. They proposed that the liver's capacity to regenerate ATP is limited by the reduction in mitochondrial function caused by prolonged cold hypoxia. In this study, we found no statistical differences between control groups and preserved groups with regard to ATP regeneration. Furthermore, ATP regeneration represented only about half the amount of fresh liver ATP (ATP content 2.34 ± 0.12 μmol/g wet weight, $n = 5$) [unpublished results]), a result similar to that reported by Mitchell. Thus, we can assume that one limitation to the isolated perfused rat liver model is that full ATP restoration is not possible.

In conclusion, the functional results of this study support the concept that high-Na^+ UW solutions are more effective for preserving the isolated rat liver, at least after 24 h of cold storage, than the original high-K^+ UW solution. We can assume, based on the higher ICG clearance and lower CK-BB release seen during normothermic reperfusion, that rat liver preservation in the extracellular-like cold storage solution reduces microvascular injury. Curiously, we cannot correlate overall functional improvement (hepatocellular and vascular) with ATP status. Thus, the use of sodium-rich solutions provides all of the advantages of the UW cold storage solution without the disadvantages of potassium.

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