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A preservation solution with polyethylene glycol and calcium: a possible multiorgan liquid

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Abstract The addition of polyethylene glycol (PEG) to hepatocyte storage medium is known to decrease lipid peroxidation and swelling and to protect the cell cytoskeleton from cold. We therefore decided to investigate the effect of substituting PEG for hydroxyethyl starch (HES) in an extracellular-like UW solution, with and without Ca++, on rat liver preservation. Isolated perfused rat livers were used to assess graft injury after 24h of cold storage. Four groups of preserved livers ($n=6$ for each group) were compared to controls (non preserved livers, $n=11$). For this purpose, Belzer solution (K+-UW, group 1) was stepwise modified. Group 2 (Na+-UW) was treated with the same liquid, however with inverted concentrations of Na+ and K+. Group 3 was preserved in the first experimental solution (EPS-1) with Ca++ (0.5mM) added to the Na+-UW solution. In the EPS-2 (group 4), PEG-35 (0.03mM) was substituted for HES. The last group, EPS-3 (group 5) was treated with the same compounds as EPS-2, but without Ca++. After 24h of cold storage and 120min normothermic reperfusion, there was no statistical difference in transaminases (ALT and AST) release between the control and the Na+-UW groups. Furthermore, rat livers preserved in Na+-UW solution released less ($P<0.05$) ALT and AST and excreted more ($P<0.05$) indocyanine green (ICG) than livers preserved in K+-UW solution. The addition of 0.5mM Ca++ to Na+-UW solution (EPS-1) dramatically increased ($P<0.05$) parenchymal (ALT, AST) and non parenchymal (creatine kinase-BB) cellular injury. The substitution of PEG (0.03mM) for HES (EPS-2) reduced ($P<0.05$) membrane injuries due to Ca++ while bile flow was statistically increased ($P<0.05$). Finally, the omission of Ca++ from EPS-2, that is EPS-3, has no statistically significant effect on the studied parameters. PEG effectively protected the rat liver grafts from the onset of hypothermic ischemia-reperfusion and Ca++ damages and thus may be a valuable additive to preservation solutions.

Keywords UW Cold-storage solution · Liver preservation · Isolated perfused rat liver · Colloids · PEG · HES

Abbreviations *HES* Hydroxyethyl starch · *ICG* Indocyanine green · *IPRL* Isolated perfused rat liver · *PEG* Polyethylene glycol

Introduction

From harvest to implantation, liver grafts are continuously exposed to cold- and warm-ischemia-reperfusion injury. As a result, microvascular hemodynamics are impaired [4], Kupffer cells are activated [32], and hepatocytes are necrosed [31]. On the cellular level, energy charge depletion [9, 11], mitochondrial dysfunction [29], calcium influx [17], and release of oxygen free radicals [22] are involved in graft non-function. These factors have only moderate individual effects but they act in synergy after reperfusion [24].

The introduction of University of Wisconsin (UW) cold storage solution to clinical practice has significantly improved long-term graft function. It has extended preservation times for intra-abdominal organs, simplified logistics, and increased the number of organs available for sharing and transplantation [28]. Two major refinements of this cold-storage liquid have further decreased graft damage: the inclusion of osmotically active compounds to prevent hypothermically-induced cell swelling, and free radical scavengers, which reduce protein and membrane impairment during ischemia and subsequent reperfusion [3].

UW solution contains many compounds, and the role of each has been extensively studied. However, some of their benefits remain controversial. It has been shown that the removal of adenosine, allopurinol, raffinose, phosphate buffer, and insulin did not affect the efficiency of the UW solution [6, 15, 16, 24, 26, 34].

On one hand, the role of hydroxyethyl starch (HES) in cold preservation is still questionable. According to Biguzas [6] and Howden [15], it does not seem to be essential for short-term preservation of the liver and the kidney. Some results, however, suggest better long-term storage if it is included [1], and its addition to UW solution appeared to protect the hepatic architecture prior to reperfusion [24]. With the isolated perfused rat liver model, Ar'Rajab [2] found that HES could effectively be replaced by Dextran 40. On the other hand, we demonstrated recently that extracellular-like UW solution (high-Na+, low-K+ UW solution) preserves rat liver [5] and kidney [12, 23] more efficiently than the intracellular-like UW solution (high-K+, low-Na+).

During cold storage, energy depletion leads to an impairment of Na+-K+ ATPase function with the dissipation of ion gradients across cell membranes and a rapid increase in intracellular Ca++ [8, 18]. At a sustained concentration, Ca++ activates phospholipases and proteases, leading to cell destruction. To prevent these deleterious effects, Ca++ was removed from several abdominal organ storage solutions. Ca++ is, however, essential for heart preservation, and, for the purpose of compiling a multi-organ-preservation solution, it is of interest to know whether the liver can endure Ca++.

For this study we used isolated perfused rat liver (IPRL) to evaluate the efficiency of substituting polyethylene glycol (PEG-35) for HES in Na+-UW solution. We also compared the effect on parenchymal and non-parenchymal cells of adding Ca++ to the storage solution in the presence of HES or PEG.

Materials and methods

Animals

Male inbred Sprague Dawley rats (Iffa-Credo, France) weighing 180–200g were the organ donors. They had free access to water and a standard pellet diet. All animals were given human care and handled in compliance with French regulations, as well as with the "Principles of Laboratory Animal Care" (NIH publication N° 86–23, revised 1985).

Liver harvesting and storage conditions

Rats were anesthetized with an intraperitoneal injection of sodium pentobarbital (6g%, 0.1ml/100g of body weight). The surgical technique was as previously described [5]. Briefly, after cannulation of the common bile duct, livers were flushed with chilled preservation solution (4°C) by means of a catheter inserted into the aorta. After cooling, a second catheter was inserted into the portal vein, and the whole liver was excised and trimmed of surrounding tissues. *Ex situ*, the portal vein catheter was used to complete liver rinsing. Fifty milliliters of preservation solution were infused through the aorta and the portal vein. Then the livers were preserved with further 75ml of the same solution for 24h at 4°C. Control livers (without preservation) were perfused with the oxygenated perfusion medium (described below in organ perfusion) at 37°C immediately after they were harvested.

Experimental groups and preservation solutions

The study comprised 41 rats divided between a control group without preservation ($n=11$) and five preserved groups of $n=6$ for each group. The composition of the tested preservation solutions is shown in Table 1. The K+-UW solution is the original Belzer liquid without dexamethasone, insulin or antibiotics (group 1). It was modified in a stepwise fashion. The Na+-UW solution [23] contains all the ingredients of the K+-UW solution but with inverted concentrations of K+ and Na+ (group 2). The experimental preservation solution 1 (EPS-1, group 3) is a Na+-UW solution containing Ca++ (0.5mM). EPS-2 (group 4) is the result of PEG-35 (0.03mM) substitution for HES (0.25mM, MW 200,000) in EPS-1. EPS-3 (group 5) is a Ca++-free EPS-2.

Organ perfusion

All livers were perfused at 37°C via the portal vein in a closed and controlled pressure circuit. As described previously [5], the perfusion liquid consisted of a cell culture medium (William's medium E, BioWhitaker) with a Krebs-Henseleit-like electrolyte composition with 5% of albumin as oncotic supply. The medium (150ml) was continuously gassed with a 95% O_2-5% CO_2 mixture. Time point 0 was when the portal catheter was satisfactorily established in the circuit. During the first 15min of perfusion, the flow was progressively increased in order to stabilize the portal pressure at 12–14mm Hg, reaching a level of around 30ml/min. Thirty minutes after the

Table 1. The composition of the tested preservation solutions

Component	K^+-UW	Na^+-UW	EPS-1	EPS-2	EPS-3
HES (mM)	0.25	0.25	0.25	–	–
PEG-35 (mM)	–	–	–	0.03	0.03
Lactobionic acid (mM)	100	100	100	100	100
Raffinose (mM)	30	30	30	30	30
$MgSO_4$ (mM)	5	5	5	5	5
KH_2PO_4 (mM)	25	25	25	25	25
Glutathione (mM)	3	3	3	3	3
Adenosine (mM)	5	5	5	5	5
Allopurinol (mM)	1	1	1	1	1
Na^+ (mM)	30	125	125	125	125
K^+ (mM)	125	30	30	30	30
Ca^{++} (mM)	–	–	0.5	0.5	–
Osmolality (mosmol/kg)	320	320	320	320	320
pH	7.2–7.4	7.2–7.4	7.2–7.4	7.2–7.4	7.2–7.4

onset of the perfusion (t_{30}), 0.5mg of indocyanine green (ICG, Becton Dickinson Microbiology Systems) was added to the perfusate.

Liver function study

Bile was collected at 30-min intervals, and the volume was estimated by weighing. The concentrations of ICG in diluted perfusate (t_{30}) and bile samples (t_{120}) were measured at 800nm with an UV-visible spectrophotometer [32]. Bile ICG excretion was expressed as a percentage of perfusate content (t_{120} bile/t_{30} perfusate *100).

Caval outflow perfusate samples were taken for AST and ALT kinetic measurements at 340nm on a Hitachi 747 analyzer. Serial samples of caval outflow perfusate were taken for measurement of total creatine kinase (CK) and CK-BB isoenzyme activities [30]. Total CK activity was determined with a commercial kinetic UV reagent (BioMèrieux, Charbonnières-les-Bains, France). 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).

Statistical analysis

Results are expressed as mean ± SE. Data between groups were compared using the ANOVA test, followed by the Fisher's protected least-significant difference test (PLSD). Statistical significance was defined as $P < 0.05$.

Results

After 24h of cold storage and 120min of normothermic reperfusion, all the biological and functional parameters (Figs. 1, 2, 3, 4 and 5) measured in K+-UW stored livers were statistically impaired ($P < 0.05$) compared to controls. No statistical difference was found for transaminase release between controls and Na+-UW stored livers. Furthermore, the Na+-UW preserved livers released less AST and ALT ($P < 0.05$, 9.7 ± 2.5 vs 31.0 ± 11.3 iu/l, 120min, Fig. 2), and excreted more ICG ($P < 0.05$, 23.3 ± 3.8 vs $10.5 \pm 1.7\%$, Fig. 5) into bile than those preserved in the K+-UW solution. No statistical difference between the two stored groups for bile flow and perfusate CK-BB activity was observed.

The addition of Ca++ to the Na+-UW solution (EPS-1) dramatically increased perfusate transaminase ($P < 0.05$, Na+-UW vs EPS-1) and CK-BB ($P < 0.05$, Na+-UW vs EPS-1) release, but it had no effect on ICG excretion and bile flow. The substitution of PEG for HES (EPS-2) suppressed the deleterious effects of Ca++. Perfusate AST and ALT contents were significantly decreased in EPS-2 vs EPS-1 ($P < 0.05$, 17.7 ± 2.9 vs 57.7 ± 11.6 iu/l, 120min, Fig. 2) and close to the levels of the control and the Na+-UW groups. Bile flow in the EPS-2 group was increased in comparison to EPS-1 ($P < 0.05$, 0.56 ± 0.06 vs 0.33 ± 0.02µl/min per g, 120min, Fig. 4). More surprisingly, the latter reached control bile flow and was higher than those of K+-UW and Na+-UW groups ($P < 0.05$, Fig. 4). In addition, the level of CK-BB isoenzyme activity reached after 30min of reperfusion was lower in the EPS-2 than in the EPS-1 group, although not significantly. Finally, the lack of Ca++ (EPS-3) has no statistical effect on the different parameters (EPS-2 vs EPS-3) except for creatine kinase-BB isoenzyme release which was lower, at 30min, in EPS-3 than in EPS-2 ($P < 0.05$, 13 ± 3 vs 34 ± 8 iu/l).

Discussion

In the field of organ transplantation, graft damage subsequent to ischemia-reperfusion is one of the chief hindrances to successful outcome. With the development of new effective preservation solutions, liver transplantation became the established therapy for various end-stage liver diseases. UW cold storage solution, optimised for the preservation of abdominal organs, is now widely used in human multiorgan harvest, and is superior to any previous liquids [28].

Our preceding studies have already demonstrated that the simple inversion of K+ and Na+ concentrations in UW solution improves rat liver graft function after 24h of cold preservation [5]. The same improvements were observed with the isolated perfused rat kidney [23] and renal transplant [12] models. In the present study, we confirm

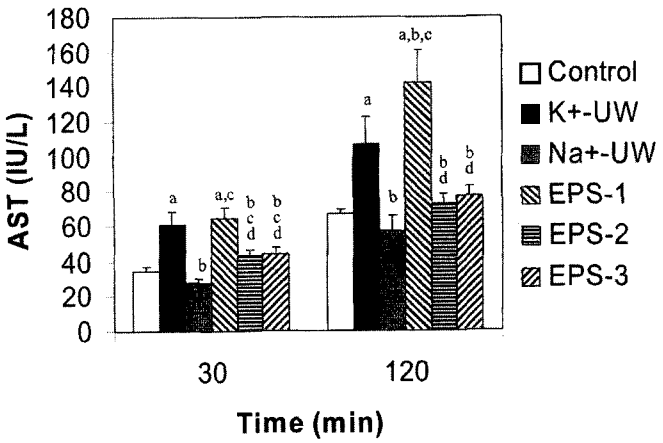

Fig. 1. Perfusate aspartate aminotransferase during 120 min of normothermic reperfusion. Data are expressed as mean ± SEM (controls: $n=11$; preserved groups: $n=6$). *a* $P<0.05$ vs. controls; *b* $P<0.05$ vs. K^+-UW; *c* $P<0.05$ vs. Na^+-UW; *d* $P<0.05$ vs. EPS-1

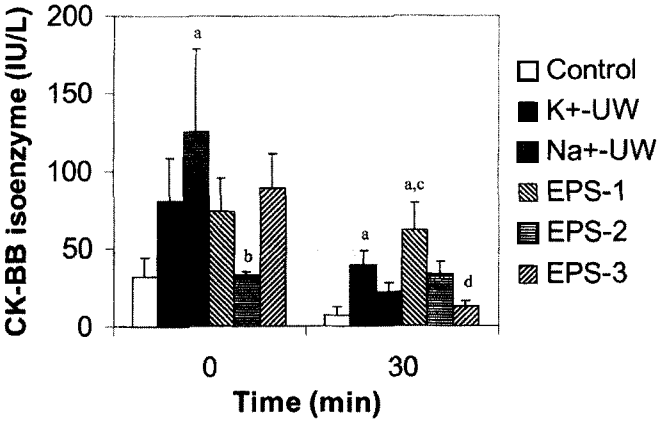

Fig. 3. Perfusate creatine kinase-BB isoenzyme activity at the beginning of the normothermic reperfusion (t_0) and after 30 min of normothermic reperfusion (t_{30}). Data are expressed as mean ± SEM (controls: $n=11$; preserved groups: $n=6$). *a* $P<0.05$ vs. controls; *b* $P<0.05$ vs. K^+-UW; *c* $P<0.05$ vs. Na^+-UW; *d* $P<0.05$ vs. EPS-1

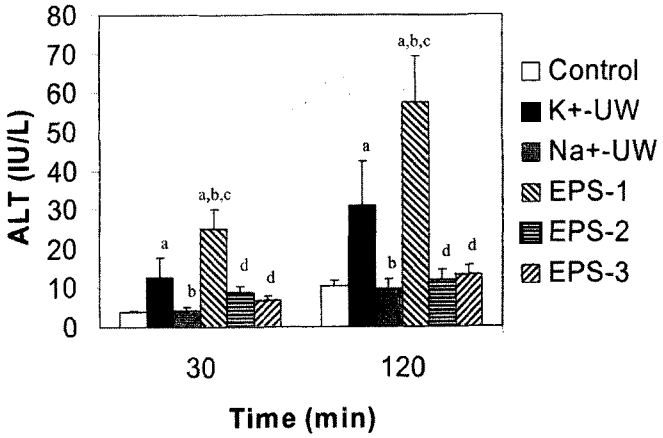

Fig. 2. Perfusate alanine aminotransferase during 120 min of normothermic reperfusion. Data are expressed as mean ± SEM (controls: $n=11$; preserved groups: $n=6$). *a* $P<0.05$ vs. controls; *b* $P<0.05$ vs. K^+-UW; *c* $P<0.05$ vs. Na^+-UW; *d* $P<0.05$ vs. EPS-1

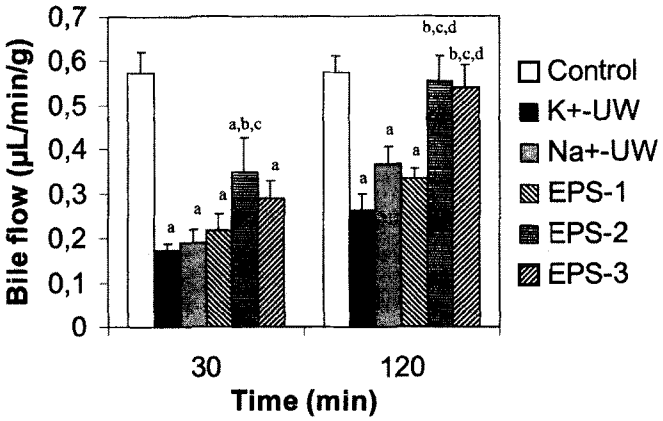

Fig. 4. Bile flow during 120 min of normothermic reperfusion. Data are expressed as mean ± SEM (controls: $n=11$; preserved groups: $n=6$). *a* $P<0.05$ vs. controls; *b* $P<0.05$ vs. K^+-UW; *c* $P<0.05$ vs. Na^+-UW; *d* $P<0.05$ vs. EPS-1

that the Na+-UW solution preserves the rat liver from reperfusion injury better than the K+-UW liquid.

One aim of this study was to evaluate the influence of adding Ca++. Although Ca++ is important for cell viability [21], its exact role under hypothermic preservation conditions is unknown. Ischemia and hypothermia profoundly alter Ca++ distribution [17]. Impairment of Ca++ homeostasis may be associated with the activation of Ca++-dependent enzymes, such as proteases and lipases, and the loss of mitochondrial function. To avoid the negative effects of intracellular accumulation of Ca++, this ion has been excluded from several preservation solutions.

In the present study, we observed that on one hand, the addition of Ca++ to the Na+-UW solution induced a dramatic increase in transaminase release, indicating a loss of parenchymal integrity. On the other hand, assuming that CK-BB isoenzyme is an appropriate marker of endothelial cell damage [30], Ca++ profoundly alters the non parenchymal integrity of cells after normothermic reperfusion. Indeed, between Na+-UW and EPS-1 there is no statistical difference in CK-BB levels at t_0 (these samples correspond to CK-BB released during cold preservation), unlike CK-BB levels at t_{30}, which represent reperfusion injury. Livers preserved in the PEG-rich EPS-2 and EPS-3 released less CK-BB than those stored in the HES-rich EPS-1. Therefore, the substitution of PEG for HES in the extracellular-like UW solution protected rat liver from damages due to Ca++.

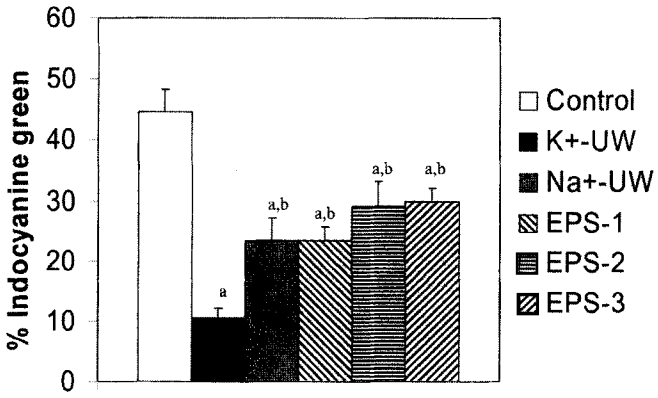

Fig. 5. Bile indocyanine green excretion during 90 min of normothermic reperfusion (from t_{30} to t_{120}). Data are expressed as mean ± SEM (controls: $n=11$; preserved groups: $n=6$). *a* $P<0.05$ vs. controls; *b* $P<0.05$ vs K^+-UW

Calcium addition to the HES-based Na+-UW solution is injurious. This might imply that the liver exchanges sodium for calcium or protons [18]. Moreover, Kupffer cells have been implicated in liver injury and graft failure after liver transplantation, and their activation is reperfusion-dependent [32]. It has been shown that they contain voltage-dependent Ca++ channels which are activated during ischemia [14]. In support of this observation, Takei et al. [27] found that the L-type Ca++ channel blocker (nisoldipine) improved graft survival after cold storage and transplantation of rat livers. In our study, the Ca++-induced loss of parenchymal and non parenchymal membrane integrity after preservation in EPS-1 may be related to activation of Kupffer cells, as extracellular Ca++ is essential for their activation [14].

We tested PEG substitution for HES in the Na+-UW solution. HES was added to the UW solution to prevent expansion of the interstitial space during the flush-out period [3]. We carried out this substitution for the following reasons: it is easily available and cheaper than HES; beside its oncotic properties, PEG interacts with the cell membranes; it is a non-toxic, water-soluble synthetic polymer with a variety of effects on biological systems. Its use in organ preservation is unconventional, although some studies have shown that it protects many organs. Several authors have reported that the addition of PEG to UW liquid improved the outcomes of pancreas [35], heart [33] and kidney [13] grafts. However, DenButter [10] found that the replacement of HES by PEG-8 was less effective for 48-h liver preservation than UW solution, while PEG-20 caused a greater release of liver enzymes into the perfusion medium than either HTK or UW solution. In the present study, we used PEG-35. The expected benefit of a higher PEG molecular weight is an increased oncotic pressure.

The substitution of PEG for HES in the Ca++-rich Na+-UW solution (EPS-2) improved graft integrity and function, and provided protection from Ca++-induced injuries. It appears that the mechanism by which PEG acts does not entirely depend on its osmotic properties since, at the concentration used (0.03mM), it does not significantly contribute to the preservation solution's osmolality. Preliminary experiments with a higher PEG concentration (0.25mM) suggested an increase of cytolysis (ALT and AST) and an impairment of ICG clearance (data not shown).

It has been shown that hypothermic preservation of cultured hepatocytes with PEG prevented cell swelling and blebbing [20] and reduced the deleterious effects of cold on the cytoskeleton [25]. Moreover, PEG has been found to be an effective antioxidant which suppressed lipid peroxidation after rewarming cold-stored hepatocytes [19]. The mechanism by which it improves cellular viability under preservation conditions has not been thoroughly elucidated. PEG has been shown to bind to phospholipids and to accumulate in the cell membranes [7]. Thus, by coating liver cells, and particularly the non parenchymal cells which are in direct contact with preservation solution, PEG may constitute a barrier restricting the passage of ions (e.g. Ca++) in spite of ischemia and hypothermia. To corroborate this hypothesis, we observed that Ca++ was hepatotoxic when the Na+-UW solution was HES-rich (EPS-1) whereas it was not deleterious when the Na+-UW solution was PEG-rich (EPS-2). Furthermore, the Ca++ omission from PEG-based Na+-UW solution (EPS-3) did not change the EPS-2 results. This confirms that PEG, unlike HES, protects liver grafts from Ca++ and cold storage injuries.

Although the importance of the colloid appears to be central in this work, we did not include a group without HES and PEG. Adam et al. show, that with UW solution, the maximal preservation time for rat livers, allowing 100% survival after transplantation, was 18h, and the omission of HES entails a diminution of this survival rate when the preservation duration reached or exceeded 12h [1]. As we preserved livers for 24h, a supplementary group without colloid was not essential.

In conclusion, PEG can be efficiently substituted for HES in the extracellular UW solution, resulting in an improvement of all the functional parameters of the preserved livers. While Ca++ is not appropriate for use with HES, PEG abolished its toxic effect, and a PEG-Ca++ rich solution (EPS-2) produces better results than standard K+-HES or even Na+-HES solutions, as calcium is essential for heart preservation. If similar results are obtained with the kidney, a first step to a multi-organ solution could be taken.

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