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In vitro evaluation of donor liver preservation fluids on human hepatocyte function

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Abstract Successful liver transplantation depends on adequate preservation of cellular function. We therefore tested the effects of two currently used liver preservation fluids, Euro-Collins (EC) solution and University of Wisconsin (UW) solution, on the viability and some functional activities of hepatocytes isolated from human livers. Cells in primary culture were maintained under hypoxic (95 % N_2/5 % CO_2) and hypothermic (4 °C) conditions for 24 h, either in EC or UW solution. This treatment did not result in significant hepatocyte damage, as judged by phase contrast microscopy, intracellular LDH release, and the MTT mitochondrial test. However, neutral red uptake indicated that lysosomal functions were slightly affected (35 % decrease) when compared to control conditions. At the end of the hypoxia/hypothermia period, hepatocyte monolayers were incubated at 37 °C under normoxic conditions for 24 h, in order to simulate the reperfusion of a transplanted liver. Three drugs – midazolam, diazepam, zidovudine – were used as diagnostic substrates to check the metabolic abilities of human hepatocytes replaced in normal conditions. Both phase I (hydroxylation, demethylation) and phase II (glucuronidation) metabolic reactions were affected by the hypoxia/hypothermia shock. Indeed, a 30 %–50 % decrease in these activities was observed as compared to values obtained in control hepatocytes. No difference could, however, be found at the cellular level regarding the solution used for cold storage. These results suggest that the superiority of UW over EC solution, already reported in clinical practice after transplantation of preserved human livers, was not due to a better preservation of the hepatocytes.

Key words Liver preservation, UW solution, EC solution · Preservation, liver, UW solution, EC solution · Hepatocytes, liver preservation · UW solution, EC solution, liver preservation · EC solution, UW solution, liver preservation

Introduction

The ultimate objective of organ preservation is unrestricted and immediate graft function after transplantation. To date, the preferred method of preserving livers in clinical practice has been simple cold storage [1]. Cold storage of the graft in modified Collins' solution or in its more recent derivative, Euro-Collins (EC) solution, has provided acceptable graft function with preservation time up to 8 h. With the development of the University of Wisconsin (UW) solution, 12- to 18 h preservation periods have become possible that have resulted in a dramatic improvement in the conditions and clinical results of liver transplantation [2]. Longer preserva-

tion times are, however, necessary to enable assessment of the viability of the harvested organ, to apply immunomodulation of the recipient, and to perform crossmatching in repeat or highly sensitized transplant candidates. In that context, improvements in the current preservation methods, both in duration of safe storage and in quality of graft function in the early postreimplantation phase, are still necessary [8].

Several models are available for studying the effects of preservation on the quality of liver preservation, including the isolated perfused liver and orthotopic liver transplantation in animals. These models, however, are not particularly well suited for the study of multiple variables, such as differences in ionic, osmotic, and oncotic compositions of preservation fluids, or for detailed analysis of multiple metabolic pathways in the liver [23].

Potential uses for isolated hepatocytes as model systems are now well recognized in various areas, including pharmacotoxicological research [7, 15] and treatment of acute liver failure [9, 28, 34]. Since major differences exist in various liver functions between laboratory animals and humans, human hepatocytes constitute the most relevant model for investigating hepatic functions in vitro [5, 13]. We have studied the efficacy of two commonly used liver preservation fluids in maintaining human hepatocyte viability by measuring the cytotoxic effects induced by long term hypothermia and hypoxia. The resulting functional impairment of the cells was also assessed by comparing the metabolic profiles of diagnostic substrates in hepatocytes cultured under hypoxia/hypothermia and conventional conditions.

Material and methods

Obtaining human hepatocytes and use

Isolation

Human hepatocytes were obtained using a two step collagenase perfusion technique [14] from two whole livers taken under strict ethical conditions from multiorgan donors and from three large liver biopsies from elective hepatectomy for cancer. Whole livers were extensively flushed with 4 °C UW solution through the portal vein and the abdominal aorta. The organ was then transported on ice to the laboratory; this time lag never exceeded 30 min. Liver biopsies were anatomically divided according to the biliovascular systematization at the healthy margin of fresh operative specimen. Biopsies were then immersed in 4 °C UW solution and immediately transported on ice to the laboratory. Livers were placed in a perfusion apparatus and perfused simultaneously via the the portal and arterial vessels with a calcium-free Hepes buffer at 20 °C [Na HCO_3 0.187 %, NaCl 0.83 %, KCl 0.05 %, glucose 0.1 %, 4-(2-Hydroxyethyl)-piperazine ethane sulfonic acid 0.24 %, pH 7.6]. Then, livers were perfused with a calcium- and magnesium-free Hank's buffer at 37 °C, and finally with a 37 °C collagenase solution (0.05 % in Hepes buffer containing 0.2 % $CaCl_2$) under recirculation and continuous oxygenation. After 15 min, the surrounding Glisson's capsule was disrupted, and hepatocytes were resuspended in a washing medium and filtered through a 60-μm nylon mesh. Three washes in L15 Leibovitz (L15) medium supplemented with 10 % newborn calf serum, followed by centrifugation at 50 *g*, allowed for the removal of tissue debris and non-parenchymal cells. The hepatocytes in the final pellet were immediatly used for primary culture.

The mean viability of isolated human hepatocytes, as estimated by the erythrosin B exclusion test, was 68 %. The average quantity was 24.6 billion cells.

Primary culture

Hepatocytes were resuspended in medium I (Williams' E, pH 7.4) containing 10 % fetal calf serum and supplemented with insulin 0.1 UI/ml, penicillin 50 UI/ml, netilmicin 50 μg/ml, and streptomicin 50 μg/ml. Cells were seeded in 35-mm diameter dishes (0.8×10^6 cells/1.5 ml of medium I), previously coated with type I rat tail collagen (10 μg/cm^2), and incubated at 37 °C under a humidified 5 % CO_2 atmosphere. Medium I was removed after 10 h of adhesion and replaced by 4 ml of the same medium without fetal calf serum but containing 10^{-6} M dexamethasone (medium II).

Preservation and reperfusion

After an overall incubation period of 24 h, the culture medium was removed and replaced volume per volume with either EC or UW solution. Dishes were then placed at 4 °C for 24 h in airtight 10-l bags (Glove-bag, Instruments for Research and Industry) filled with 95 % N_2, 5 % CO_2 atmosphere. Measurements of the PO_2 in culture supernatants (Blood gas system 278, CIBA-Corning Diagnostics) showed that hypoxia was obtained within 30 min, reaching a PO_2 plateau of 60 mmHg. At the end of the preservation period, samples were harvested for cytotoxicity assays. Reperfusion was further obtained by replacing cells in medium II and in normoxic conditions (95 % air, 5 % CO_2) at 37 °C for 24 h. A PO_2plateau of 120 mmHg in the medium was reached in 30 min. Control cultures were carried out in medium II and incubated at 37 °C under a humidified 95 % air, 5 % CO_2 atmosphere for the duration of various experiments. All cultures were routinely examined under phase-contrast microscopy before cytotoxicity assays and removal of medium.

Cytotoxicity assays after hypoxia/reoxygenation

LDH determination

Lactate dehydrogenase (LDH) activity was measured in culture supernatants and cell extracts as previously described [39]. LDH activity was assayed spectrophotometrically at 334 nm (Uvikon 360, Kontron) by monitoring the appearance of NAD^+ from NADH at 30 °C, using pyruvate as substrate. Results were expressed as the extracellular LDH/total LDH ratio (%).

MTT reduction

Dimethyl – 4,5 – thiazolyl – 2,3 – diphenyl – 2,5–2H – tetrazolium bromide (MTT) is a soluble, pale yellow salt that is reduced by mitochondrial succinate dehydrogenase to form an insoluble dark blue formazan product [10]. The cells were washed once before addition of MTT (0.05 %). After 2 h of incubation at 37 °C, the form-

azan blue formed in the cells was assessed by measuring of the optical density at 550 nm (MR 7000, Dynatech). Results were expressed as percentage of the control values.

Neutral red test

Cells were preloaded with a neutral red solution for 3 h at 37 °C. Then, this solution was discarded and replaced with a formol-calcium solution in distilled water. After 1 min, the cells were washed with phosphate-buffered saline at 4 °C before addition of an alcohol (50 %)-acetic acid (1 %) solution [3]. Optical density was measured at 550 nm. Results were expressed in percentage by reference to the values obtained from the control cultures.

Biotransformation of drugs after reperfusion

After removal of the medium, one of three radiolabeled drugs was added: midazolam (MDZ), diazepam (DZP), or azidodeoxythymidine (AZT). After a 24-h exposure period of the radiolabel with the cell monolayer under conventional culture conditions, the unchanged drug and its metabolites were analyzed in the extracellular medium. Similar experiments were performed in control cultures.

MDZ and DZP biotransformation

Experiments were initiated as previously described [11], by the addition of ^{14}C MDZ or ^{14}C DZP (0.45 μCi/ml) to achieve a final concentration of 50 μM and 10 μM, respectively. After the 24-h exposure period, the extracellular medium was analyzed with high-performance liquid chromatography (HPLC) using a chromatograph equipped with an automatic injector (715 Ultra WISP, Waters) and a terminal computer (Nec, Baseline 810). All analyses were performed on a reversed-phase C 18 column (25 × 0.4 cm; pore size 10 μm; a Bondapak, Millipore). Elution was carried out at 1.2 ml/min along a linear gradient of 50/50 (v/v) methyl alcohol/ultrapure water (Milli Q plus, Waters) for 30 min. Column temperature was maintained at 25 °C; absorbance was recorded at 254 nm. Eluent from the column was directed to a continuous flow liquid scintillation detector (Flow One Radiomatic Instruments). Chromatographic profiles of unchanged MDZ or DZP and their metabolites were recorded. Results were expressed as the percentage of metabolized drug.

AZT biotransformation

Experiments were initiated by the addition of an isotopic dilution of ^{3}H AZT to achieve a final concentration of 100 μM. All analyses were performed at 25 °C by HPLC on a reversed-phase C 18 column (25 × 0.46 cm; pore size 5 μm; Interchrom). Elution was carried out at 1.2 ml/min along a nonlinear gradient of phosphate buffer (50 mM, pH 6.5)/acetonitrile for 35 min. Absorbance was recorded at 280 nm. Radiolabeled compounds were detected by continuous flow liquid scintillation. Results were expressed as the percentage of metabolized drug.

Statistical analysis

Experiments were performed on five different human hepatocyte batches. Data from unpreserved and hypothermically preserved hepatocytes from the same organ were used. For all tests, at least, two independent experiments were performed. For each medium, the values of each experiment were the mean of three to six cultures. Data were expressed as the mean and the standard deviation. Different groups of values were compared with a two-way ANOVA. The significant threshold was fixed at 0.05.

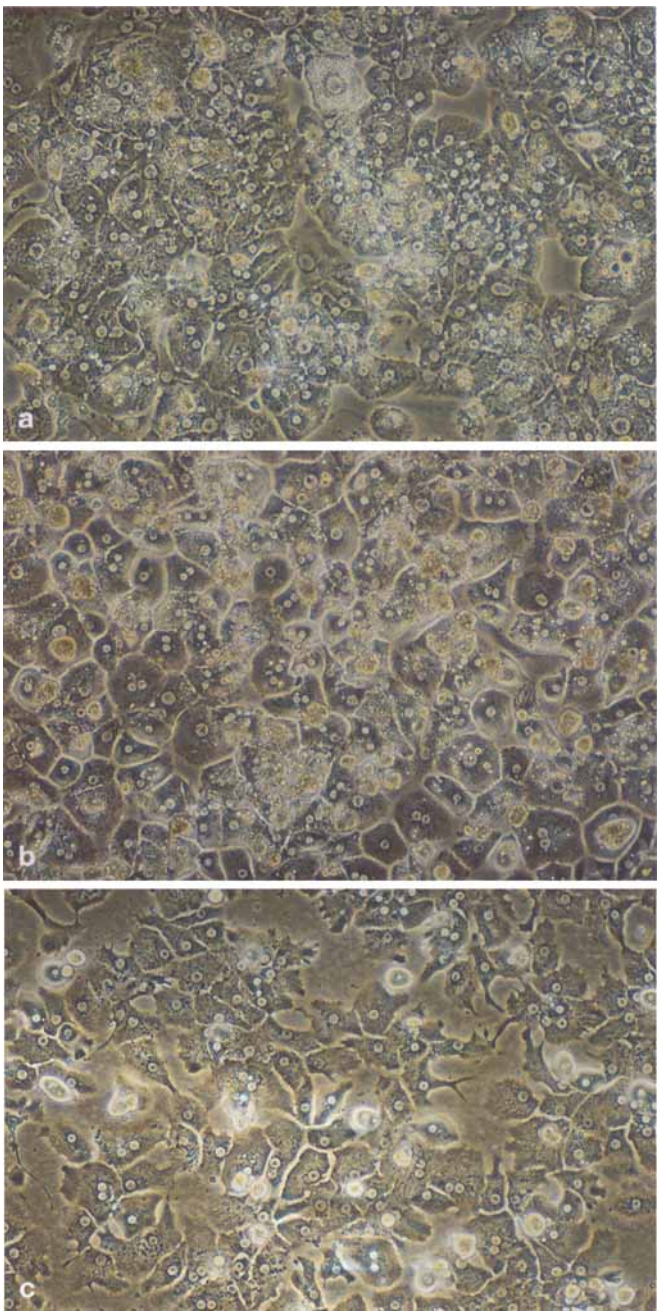

Fig. 1A–C Morphology. Light microscopic appearance of: **A** unpreserved and **B,C** hypothermically preserved primary culture of human hepatocytes

Table 1 Influence of hypothermic preservation on cell viability. The viability of nonpreserved (*NPH*) and 24-h hypothermic (4 °C) preserved (*HPH*) hepatocytes was assessed by LDH release (*LDH*), reduction of a tetrazolium salt (*MTT*), and red neutral uptake (*NR*). Values represent mean ± SD of five different experiments in triplicate. MTT reduction and NR uptake by HPH are expressed as a percentage of the values obtained in NPH

Parameter	NPH	HPH	
		EC	UW
LDH (%)	11.7 ± 5.1	7.9 ± 4*, **	5.1 ± 1 *, **
MTT (%)	*100*	101 ± 6.9 **	100 ± 32 **
NR (%)	*100*	66.5 ± 6.7*, **	64.5 ± 7.1*, **

* $P < 0.001$ between preserved and nonpreserved hepatocytes
** Nonsignificant difference between the two preservation fluids

Results

Morphology

Phase-contrast microscopic examination showed that, as with control hepatocytes, primary culture of human hepatocytes hypothermically preserved for 24 h in either EC or UW solution maintained large cords of contiguous granular epithelial cells after 24 h of culture. EC solution, however, appeared to induce hepatocyte swelling, whereas cellular shrinkage was noted in cultures placed in UW solution (Fig. 1).

Cell viability

As shown in Table 1, which reports cell viability parameters such as LDH release, MTT, and NR tests, primary cultures of hepatocytes did not seem to be affected by the hypoxia/hypothermia treament. Furthermore, LDH release, as measured by the extracellular LDH/total LDH ratio, decreased significantly in preserved cells when compared to unpreserved cells, indicating a positive effect of these preservation conditions on hepatocyte viability. However, neutral red uptake was 33.5 % and 35.5 % lower in EC and UW cultures, respectively, than in control cultures. Finally, none of these three cytotoxicity tests showed a significant difference between EC and UW cells.

Functional activities

The time course of disappearance of unchanged radiolabeled drugs and the subsequent appearance of their respective metabolites in the extracellular compartment were studied for 24 h both in preserved and unpreserved human hepatocytes (Fig. 2).

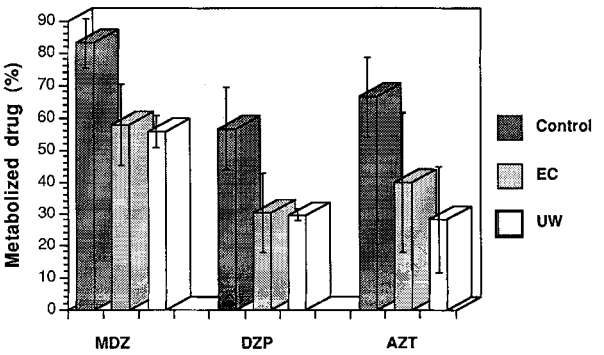

Fig. 2 Biotransformation of drugs. Metabolism of midazolam (*MDZ*), diazepam (*DZP*), and azidodeoxythymidine (*AZT*) by human hepatocytes in primary culture after cold preservation in Euro-Collins (*EC*) or University of Wisconsin (UW) solution. The values of drug metabolism are compared with those obtained in control cultures of nonpreserved hepatocytes; they are expressed as the metabolized compounds/total drug ratio (%). Data are expressed as means ± SD of five independent assays

MDZ metabolism

Figure 3 illustrates the HPLC extracellular radiochromatograms of MDZ and its metabolites after biotransformation by unpreserved hepatocytes, EC hepatocytes, and UW hepatocytes. In all cases, HPLC profiles showed both phase I (mono- and dihydroxylated derivatives) and phase II (polar derivatives) metabolite formation. The major metabolites were polar derivatives: GLU-MDZ resulting from the conjugation of hydroxylated derivatives to glucuronic acid, and an unknown compound probably due to a sulfo conjugation reaction. Hydroxylated derivatives (Di-OH-, 4-OH-, and mainly 1-OH-MDZ) represented only a minor part of all the biotransformation products. As far as hypothermically preserved hepatocytes were concerned, MDZ biotransformation was significantly reduced when compared to that observed in unpreserved ones ($P = 0.03$). No difference was, however, obtained between EC and UW cells.

DZP metabolism

HPLC profiles of extracellular media showed phase I and phase II metabolites of DZP (Fig. 4). Phase I metabolites included N-demethylated and hydroxylated derivatives referred to as N-desmethyl DZP, temazepam, and oxazepam. Phase II metabolites resulted from the conjugation of 4'-hydroxylated derivatives of diazepam to glucuronic acid. DZP biotransformation by hypothermically preserved hepatocytes was significantly less extensive than that of unpreserved cells ($P = 0.007$), but no difference could be found between EC and UW cells.

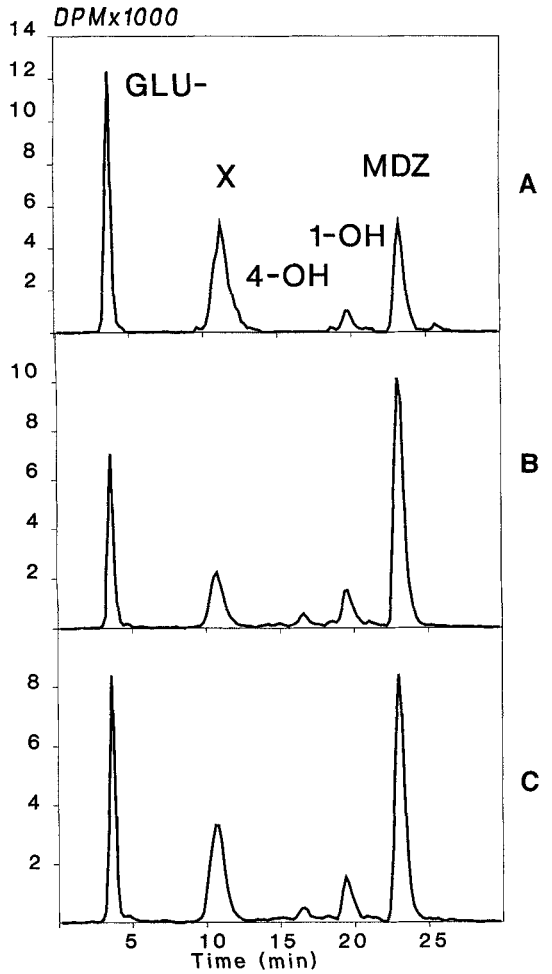

Fig. 3A–C HPLC chromatogram of MDZ and its metabolites: **A** unpreserved and **B,C** hypothermically preserved human hepatocytes in primary culture incubated for 24 h with ^{14}C MDZ 50 μM. Metabolites were identified according to their retention times relative to those of standards (*1-OH* and *4-OH* hydroxylated derivatives, *GLU-* glucuronide, *MDZ* unchanged midazolam, *X* unknown metabolite)

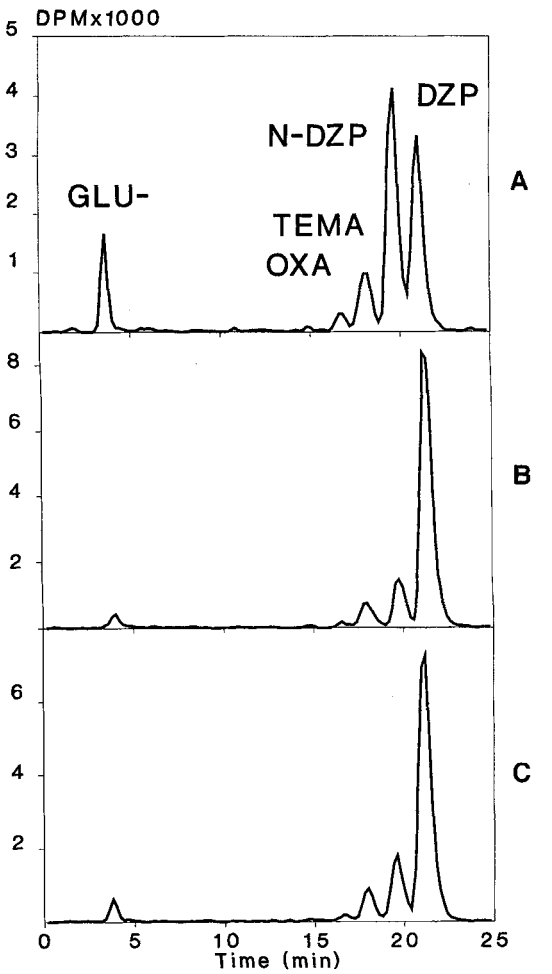

Fig. 4A–C HPLC chromatogram of DZP and its metabolites: **A** unpreserved and **B,C** hypothermically preserved human hepatocytes in primary culture incubated for 24 h with ^{14}C DZP 10 μM. Metabolites were identified according to their retention times relative to those of standards (*DZP* unchanged diazepam, *GLU-* glucuronide, *N-DZP* N-desmethyl diazepam, *TEMA* temazepam, *OXA* oxazepam)

AZT metabolism

As shown in Fig. 5, the major metabolite excreted in the extracellular medium was a polar derivative referred to as GLU-AZT, resulting from the conjugation of AZT to glucuronic acid. Phase I metabolite (3'-amino-3'-deoxythymidine, or AMT) represented only a minor part. AZT biotransformation by hypothermically preserved hepatocytes showed a 50 % decrease compared to that of unpreserved cells ($P = 0.02$), but no difference could be found between EC and UW cells.

These data obtained in vitro correlate well to those already published for these drugs in vivo in patients [21, 32, 36].

Discussion

Jamieson et al. [18] first demonstrated the efficacy of the UW solution in long-term preservation of the liver in dogs. Its first clinical application for liver transplantation was reported by Kalayoglu et al. [19]. The greater efficacy of this solution over other preservation mediums has been documented in humans as well as in animals [2, 31]. The advantages of UW over EC solution is due mainly to protection of the vascular endothelium [4, 25], which is selectively damaged using the ischemia-hypothermia conservation procedure [24].

Most data concerning preservation mediums and the mechanisms of ischemia-perfusion have been obtained

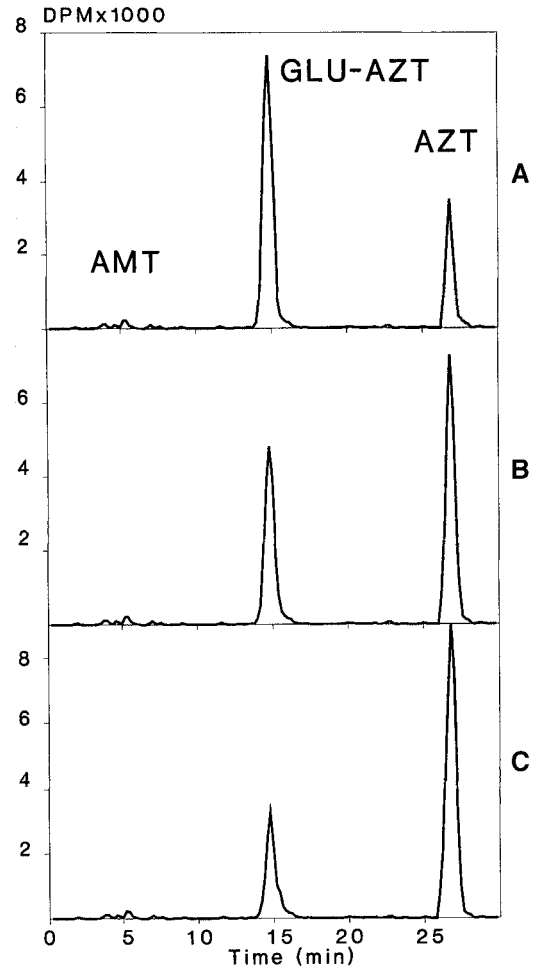

Fig.5A–C HPLC chromatogram of AZT and its metabolites: **A** unpreserved and **B,C** hypothermically preserved human hepatocytes in primary culture incubated for 24 h with an isotopic dilution of methyl ^{3}H AZT 100 μM. Metabolites were identified according to their retention times relative to those of standards, *AMT* 3'-amino-3'-deoxythymidine, *AZT* unchanged azidodeoxythymidine, *GLU-AZT* glucuronide)

from experimental orthotopic liver transplantation performed in animals. While graft viability after transplantation is the ultimate proof of the efficacy of a preservation solution, this approach presents several drawbacks. The model requires extensive facilities and does not allow for a rapid discrimination of specific factors that could be important in improving the quality and duration of organ preservation [23]. Moreover, in addition to the ethical problem raised by animal experimentation, extrapolation of data to humans remains hazardous because of the wide interspecies variability in hepatic functions [5]. For these reasons and given the heterogeneity of cell populations in the whole liver [13], the use of human hepatocytes may provide relevant information.

A major problem with employing human hepatocytes is obtaining them on a regular basis; because of the increasing development and success of organ transplantation, adequate amounts of fresh normal liver portions are becoming rare. This has led us to vigorous attempts to find suitable methods for hepatocyte isolation from small samples such as liver biopsies harvested at the healthy margin of fresh operative specimen of elective hepatectomy for cancer. As previously reported [12], combined portal and arterial perfusion have led to increased viability and yield of hepatocytes.

Several studies involving preservation solutions have been carried out using the hepatocyte model [17, 20, 22, 23, 26, 29, 30, 35, 37]. Most have been limited to the evaluation of a few liver functions immediately after the preservation period. To our knowledge, no extensive study has been conducted on human hepatocytes. Up until now, two hepatocyte models have been proposed. The first is primary culture under normoxia and hypothermia (simulating conventional cold storage) followed by standard medium and temperature conditions (simulating reperfusion) [17, 26, 29, 30, 35]. The second model is suspension with constant stirring and bubbling (simulating preservation by continuous microperfusion) followed by warming and reoxygenation (simulating reperfusion) [20, 22, 23, 37]. We chose to use the primary culture model because hepatocytes survive only a few hours in suspension [13, 14] and, thus, are unsuitable for the long-term studies of xenobiotic metabolism. Hepatocytes in primary culture form regular monolayers of polygonal cells and exhibit ultrastructural features as well as most of the biochemical and metabolic functions typical of hepatocytes in the intact liver for several days [13, 14].

The hypoxia-reoxygenation model that we used was designed to mimic ischemia-reperfusion of the organ in clinical practice as closely as possible, although anaerobiosis was not achieved during cold storage. Two preservation solutions were compared: UW because it is the reference solution used in clinical practice [2], and EC, because it shows a much lower efficacy in vivo [25, 33]. The effects of hypoxia-reoxygenation were evaluated at two times: first, immediately after reoxygenation to determine the cell damage induced by hypothermia and hypoxia and, second, 24 h after hepatocyte culture under normal conditions to assess the ability of hepatocytes to restore their functions following oxidative stress.

The parameters used for these purposes were toxicological tests and analysis of the metabolism of diagnostic substrates. Cytotoxicity was assessed by means of classical subcellular activity markers including mitochondrial reduction of MTT [10], lysosomal uptake of neutral red [3], and LDH release [6]. Various teams have proposed other methods for evaluating cell function, such as determination of protein synthesis, measurement of 7-et-

hoxyresorufin O-deethylase (EROD), pentoxyresorufin O-dealkylase (PROD) activities, or P450 cytochromes markers, and assessment of glutathione or malonyldialdehyde concentrations [17, 20, 22, 23, 26, 29, 30, 35, 37]. Evaluation of the metabolism of xenobiotics seemed particularly well suited to the clinical setting of liver transplantation in which patients receive extensive medication especially during the postoperative period. The drugs tested were chosen because their hepatic biotransformations involve both phase I and phase II reactions of different intracellular pathways and, therefore, a wide range of enzymatic activities are covered.

Using a similar in vitro model, Poullain et al. [26] compared the cell viability after cold storage in UW for 24 h with that obtained in hepatocytes cultured under standard conditions. Immediately after preservation, cell viability determined by trypan blue exclusion was only slightly lower than in fresh cells, but after seeding the capacity of preserved cells to metabolize drugs (paracetamol, phenacetin, and procainamide) was significantly lower. Our results confirm these observations. The overall cytotoxic effect resulting from hypoxic-hypothermic preservation was not different from that observed in controls, but the metabolism of the three xenobiotics was significantly lower.

Little data comparing the efficacy of UW and EC solutions in the protection of liver parenchymal cells is available. The study by Michel et al. [25] involved orthotopic transplantation in the rat after preservation of the liver in UW or EC solution, associated with isolation and culture of hepatocytes. The results indicated that the greater efficacy of UW over EC solution was related to a better protection of the vascular endothelium of the organ, whereas the viability of hepatocytes that were isolated and then placed in culture was the same regardless of solution used for liver preservation. However, assessment of viability was based only on measurement of LDH and production of cetones. Our results showed no difference between these two solutions for hepatocytes cultured after cold storage. Cells placed in UW solution showed less swelling than cells placed in EC solution. However, this advantage, which is in agreement with previously published histological data from whole livers [16, 33], was not confirmed by the cytotoxicity tests used, and metabolism of xenobiotics was qualitatively and quantitatively the same. These results support earlier evidence that UW solution cannot be used alone as a preservation medium for primary hepatocyte culture [17, 26], and strongly suggest that the clinically obvious superiority of UW over EC solution is not due to a better preservation of the hepatocyte itself.

Primary human hepatocyte cultures appear to be a suitable model for studying the effects of hypothermic preservation on viability and metabolic activity and, thus, would be useful in improving preservation solutions. However, several factors limit extrapolation of data obtained with this model to preservation of the whole liver. The first is related to the heterogeneity of the cell populations in the liver and to the three dimensional organization of the organ. In this regard, the importance of endothelial cells and of the vascular network in preservation of the organ has been well documented [4, 24, 25]. The fact that the recipient's blood is the reperfusion solution during orthotopic transplantation is probably a major factor in the survival of the graft. Indeed, it seems that through the formation of aggregates, blood components modify the flow and homogeneity of reperfusion [27]. Activated macrophages can locally induce the formation and release of oxygen-derived free radicals and other toxic mediators, thus contributing to secondary injury to hepatocytes [38]. Another obstacle to the understanding of the mechanisms underlying recovery of liver function after cold storage is the complex composition of plasma [23].

Because of these difficulties, this in vitro model must be used in association with perfused whole liver models and/or orthotopic transplantation experiments in animals or clinical study data in humans. However, its full potential should also be considered in the perspective of alternative treatment strategies of acute liver insufficiency involving bioartificial hybrid systems [28] or hepatocyte transplantation [9].

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