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ORIGINAL ARTICLE

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Celsior solution compared with University of Wisconsin solution (UW) and histidine–tryptophan–ketoglutarate solution (HTK) in the protection of human hepatocytes against ischemia–reperfusion injury

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Abstract Celsior, a new preservation solution in thoracic organ transplantation was evaluated for efficacy in cold preservation of human hepatocytes and compared with University of Wisconsin solution (UW) and histidine–tryptophan–ketoglutarate solution (HTK, Custodiol). Human hepatocyte cultures were preserved at 4 °C in Celsior, UW and HTK for 2, 6, 12, 24 and 48 h with 6 h of reperfusion. Levels of lactate dehydrogenase (LDH; cell necrosis), 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT; mitochondrial function), and adenosine 5′-triphosphate (ATP; loss of intracellular energy) were measured. Cell necrosis, mitochondrial dysfunction, and loss of ATP were significantly ($P < 0.001$, $P < 0.001$, $P < 0.002$, respectively) lower in Celsior than in HTK. The amount of cell necrosis and mitochondrial dysfunction in Celsior solution (CS) and UW was equal (P = n.s.) up to 24 h and significantly lower in UW after 48 h ($P < 0.001$). Additionally, the intracellular level of ATP was significantly higher after ischemia ($P < 0.001$) and reperfusion from long-term ischemia (24, 48 h) ($P < 0.002$). We can conclude that Celsior was superior to HTK and equal to UW in the protection of human hepatocytes against cold preservation injury from ischemia and reperfusion. Furthermore, Celsior was effective in long-term preservation of human hepatocytes.

Keywords Human hepatocytes · Liver transplantation · Ischemia–reperfusion

Introduction

Ischemia, intermittent cold preservation, and reperfusion are crucial factors for graft outcome in organ transplantation. Despite these obstacles, transplantation of the heart, lung, liver, pancreas and kidney has become a clinical routine in the past century. Nevertheless, the need for extended ischemia times, the recruitment of marginal donors, and economic aspects have remained major demands in clinical organ transplantation [7, 12, 28]. In this context, preservation solutions are one of the master keys to improve these clinical demands.

During the past decade, the introduction of University of Wisconsin solution (UW) in liver transplantation improved graft and patient survival [24]. Components such as impermeants, an effective buffering system, and the provision of substrates to regenerate high-energy phosphates may have contributed to this result [33]. Histidine–tryptophan–ketoglutarate solution (HTK, Custodiol) was primarily developed for heart preservation and subsequently introduced to the preservation of human liver grafts [8]. Both solutions were routinely used in human liver transplantation, and their efficacy in preventing preservation injury was confirmed in experimental and clinical studies [8, 34].

Celsior solution (CS), a new preservation solution [18], has currently been introduced to heart transplantation and is subsequently also used in lung transplantation [35, 39]. Preliminary data from CS in the preservation of thoracic organs showed improved graft

function after ischemia and reperfusion, compared with results achieved with UW and HTK [19, 27]. Subsequent results with CS in the cold preservation of abdominal organs of animals also showed a protective effect on ischemia and reperfusion injury [3]. Similar to HTK, CS is now being evaluated for its quality of cold preservation in human abdominal organs. There are major differences between CS and HTK in terms of impermeants, buffering systems, and substrates to generate high-energy phosphates, albeit both solutions belong to the extracellular type of preservation fluids.

Only two studies of human liver transplantation that compare CS with UW in short ischemia times have been published so far [15, 20]. The data presented were not sufficient for the efficacy of CS in the preservation of human liver grafts to be assessed, and data evaluating CS, UW, and HTK in a cell-culture model of human hepatocytes are still lacking.

The aim of our study was to assess the efficacy of CS in short-term and long-term cold preservation of human hepatocytes compared with the efficacy of UW and HTK. For this purpose, we calculated the extent of cell necrosis by measuring the release of intracellular lactate dehydrogenase (LDH). Furthermore, we evaluated the functional integrity of hepatocytes after cold preservation by measuring the capability of the mitochondria to reduce 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) and by measuring the content of intracellular adenosine 5′-triphosphate (ATP) to characterize the energy level. In order to assess the dynamic nature of the preservation injury, we determined each parameter after the ischemia phase and a consecutive 6-h period of reperfusion.

Material and methods

Preservation solutions

We obtained University of Wisconsin solution from DuPont (Bad Homburg, Germany), HTK from Dr. Köhler Chemie (Alsbach, Germany), and Celsior from SangStat (Lyon, France). The specific composition of each solution is shown in Table 1.

Isolation and culture of human hepatocytes

Human liver tissue was obtained from patients' ($n = 11$) livers that were partially resected for primary liver carcinoma and colorectal metastasis. Solely oncological principles determined the extension of liver resection, and informed consent was granted by all patients. Directly after resection, a tumour-free piece of liver tissue (10–25 g) was taken from the edge of the resected liver tissue so that a one-cut surface only, was achieved. The sample was placed in cold medium (DMEM Ham's F12 1:1, Biochrom, Berlin, Germany) and stored at 4 °C until it was processed between 30 min and 4 h after resection.

The cells were isolated as described by the method of Seglen and Schröder [31, 32]. Briefly, a small piece of liver tissue was perfused with 0.05% collagenase type IV (Sigma, Munich, Germany).

Table 1 Composition of Celsior, HTK and UW

Component	Celsior	HTK	UW
HAES, g/l			50
Histidine, mmol/l	30	198	
Mannitol, mmol/l	60	30	
Lactobionate, mmol/l	80		105
Raffinose, mmol/l			30
Glutamic acid, mmol/l	20		
Phosphate, mmol/l			25
Glutathione, mmol/l	3		3
Allopurinol, mmol/l			1
Adenosine, mmol/l			5
Sodium, mmol/l	100	15	27
Potassium, mmol/l	15	10	125
Chloride, mmol/l		50	
Calcium, mmol/l	0.25	0.015	
Magnesium, mmol/l	13	4	
Magnesium sulphate, mmol/l			5
Tryptophan, mmol/l		2	
Ketoglutarate		1	
Insulin, U/l			100
Dexamethasone, mg/l			8
Bactrim (penicil.), mg/l			0.5

Differential centrifugation was used to separate hepatocytes from non-parenchymal cells. Cell viability was determined with a trypan-blue exclusion test in a Neubauer chamber. Hepatocytes were cultured in HepatoZYME-SFM (Gibco, Berlin, Germany) supplemented with 5% FCS Gold (PAA, Cölbe, Germany), 2 mmol/l L-glutamine (Gibco), and 50 µg/ml gentamicin (Gibco). For the experiments, 1×10^5 cells/cm^2 were sown on collagen type I-covered 96-well microtitre plates (Primaria, Falcon, Heidelberg, Germany). The cells were used for experiments after overnight cultivation.

Experimental procedures

Cultured human hepatocytes were washed three times with Hank's balanced salt solution (HBSS) without calcium and magnesium at room temperature (PAA), and incubated in UW, HTK and CS for 2, 6, 12, 24 and 48 h. Culture plates were then kept in sealed plastic bags and stored for hypothermic ischemia at 4 °C so that the clinical conditions of cold organ storage could be simulated. After ischemia, we took one part of the hepatocyte culture to measure the preservation injury of each solution by LDH release, MTT reduction, and intracellular ATP level. The second portion of the cells was washed three times with HBSS without calcium and magnesium (PAA) at room temperature. Thereafter, complete cell-culture medium (37 °C) was added, and reperfusion was simulated by further cultivation with 5% CO_2 at 37 °C for 6 h. Hepatocytes that were cultivated under normal culture conditions were used as warm controls for each interval of ischemia and reperfusion.

Assays

LDH

LDH was assessed with a standard assay to calculate cell viability. At different points of time for ischemia and reperfusion, LDH activity was measured in the supernatant and after lysis of the cells with the detergent Triton X-100 (1% in HBSS, 30 min at 37 °C). Released LDH was given as a percentage of total LDH activity.

MTT

Mitochondrial function was further assessed with MTT. Only mitochondria of intact cells reduce MTT to blue-coloured formazan by means of the mitochondrial succinate dehydrogenase system. To the cells was added 5 mg/ml MTT (Sigma), together with fresh culture medium at an amount equal to 10%, and the mixture was incubated for 4 h under culture conditions. The resulting formazan crystals dissolved upon addition of 10% Triton X-100 plus 0.1N HCl in anhydrous isopropanol (Sigma). Absorbance of converted dye was measured at a wavelength of 570 nm with background subtraction at 690 nm. Values ranged between 0.40 and 0.01 of absorbance. In all experiments, reduction of MTT was given as a percentage of the reduction of untreated warm controls.

ATP

Human hepatocytes were sown as described above on 96-well culture plates (White, TopCount, Packard Bioscience, Dreieich, Germany). ATP was measured by a luminescence ATP-detection assay (ATPLite-M, Packard Bioscience) with the microplate reader LumiCount (Packard) according to the manufacturer's instructions. The distribution of each experiment was represented in a standard curve. Values in fivefold-diluted samples ranged between 10,570 and 235 relative luminescence units (RLU). Results are expressed as a percentage of the levels of untreated warm controls.

Statistics

Only isolations of hepatocyte cultures that had achieved the number of cells that were adequate for us to perform the complete study protocol were analyzed ($n = 7$). All assays were carried out eightfold for each hepatocyte isolation. Data are expressed as means ± SE. Student's t-test was performed for statistical analysis. $P < 0.05$ was considered significant.

Results

LDH

In general, as demonstrated in Figs. 1 and 2, we observed an increase in LDH release from human hepatocytes in CS, UW, and HTK during the intervals of ischemia and consecutive reperfusion. The lowest LDH release was found in CS within each interval of ischemia (Fig. 1). Similar results were found in UW, whereas after 12 and 24 h the difference was of statistical significance ($P = 0.001$). In contrast, HTK revealed in each interval of ischemia, except after 2 h, a statistically significant ($P < 0.001$) higher release of LDH than CS did.

With regard to the reperfusion phase in Fig. 2, there was a quite similar course of LDH release in CS and UW. Nevertheless, after reperfusion of hepatocytes from long-term ischemia of 24 and 48 h, the release of LDH in CS was statistically of higher significance ($P = 0.01$) than in UW. When compared with HTK, there was again, overall, a significantly ($P < 0.001$) lower LDH release during reperfusion in CS-preserved hepatocytes.

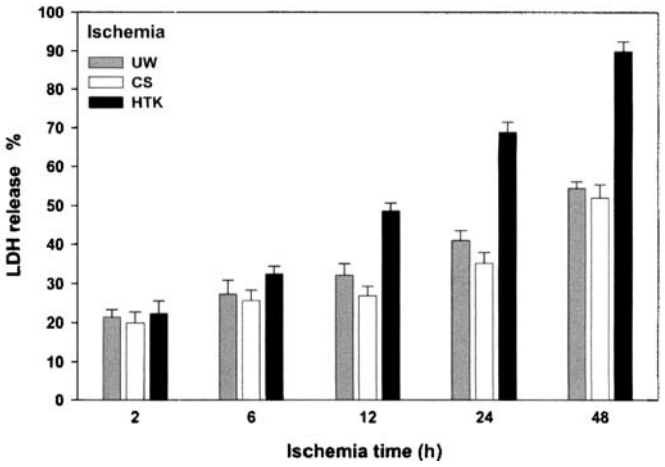

Fig. 1 Release of LDH from human hepatocytes after cold ischemia

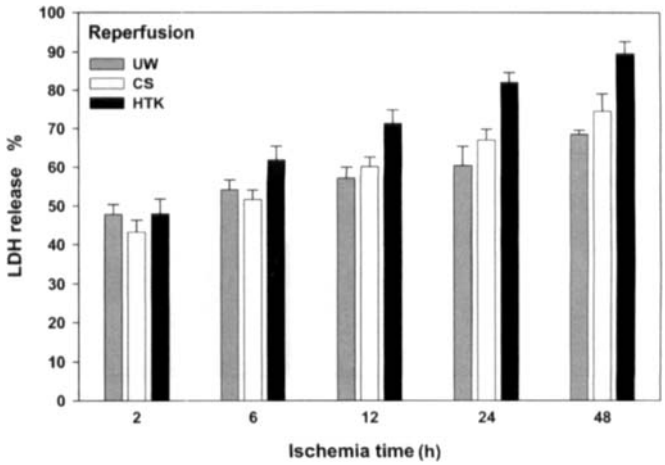

Fig. 2 Release of LDH from human hepatocytes after 6 h of reperfusion from cold ischemia

MTT

MTT reduction in hepatocytes after ischemia and reperfusion was compared with that of untreated warm controls. As shown in Fig. 3, CS and UW demonstrate the same course of MTT reduction after ischemia (P = n.s.). Both preservation solutions revealed, after 6, 12, and 24 h of ischemia, a steady state of MTT reduction. After 6 and 48 h of ischemia, there was a distinct decrease in MTT reduction in CS and UW ($P = 0.001$). In contrast, after each interval of ischemia, HTK showed a remarkable decrease in MTT reduction ($P < 0.03$; Fig. 3. Compared with CS, there was a significant ($P < 0.001$) decrease of MTT reduction in HTK-preserved hepatocytes, except after 2 h of ischemia ($P = 0.03$).

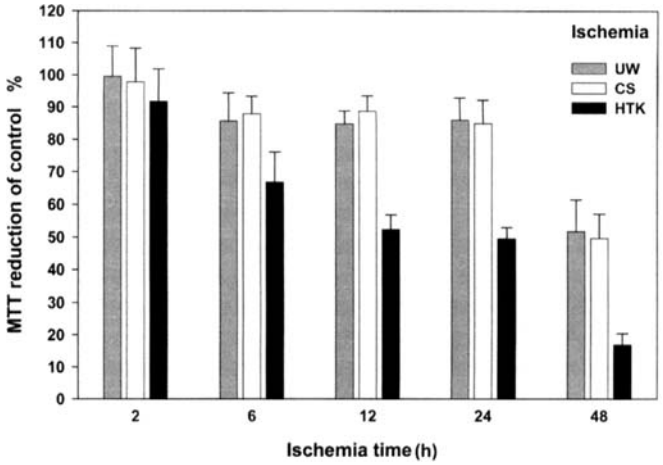

Fig. 3 Reduction of MTT in human hepatocytes after cold ischemia

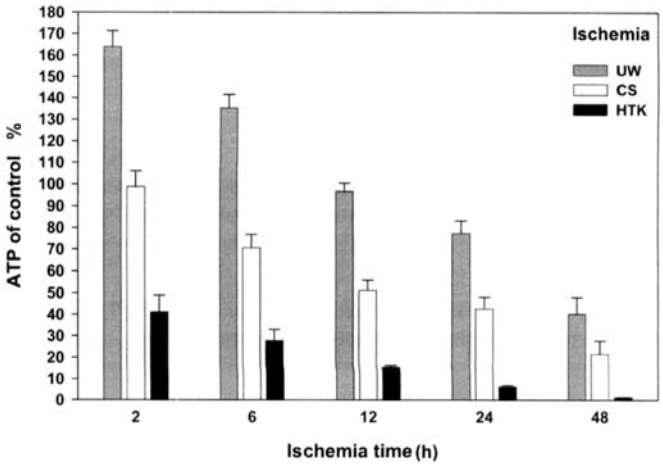

Fig. 5 Level of intracellular ATP in human hepatocytes after cold ischemia

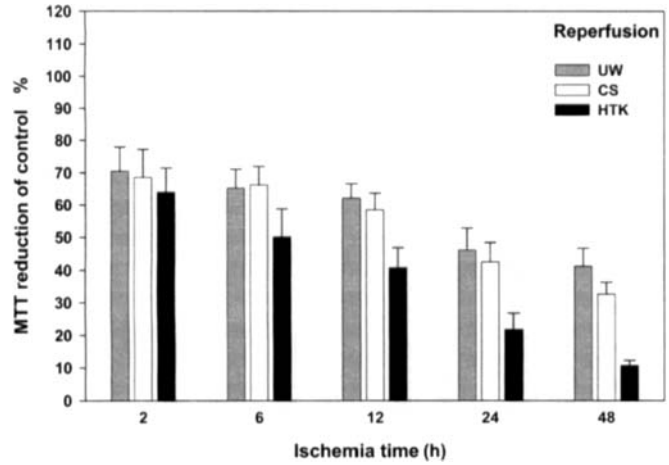

Fig. 4 Reduction of MTT in human hepatocytes after 6 h of reperfusion from cold ischemia

During the reperfusion phase, CS and UW again demonstrated a quite similar course of MTT reduction, except after reperfusion from 48 h of ischemia (Fig. 4). At this interval, the MTT reduction was lower in CS ($P < 0.001$). Furthermore, the reduction of MTT in HTK-preserved hepatocytes decreased again remarkably along the course. Compared with CS after reperfusion from 6 to 48 h of ischemia, HTK showed a significant ($P < 0.001$) decrease in MTT reduction (Fig. 4). The highest loss of MTT reduction was found in all three preservation solutions after reperfusion from 24 h of ischemia.

ATP

The intracellular ATP level after ischemia and reperfusion was compared with that of untreated warm controls. As shown in Fig. 5, the course of hepatocytes' ATP level after each interval of ischemia was different in CS, UW and HTK. The highest level of ATP was found after each interval of ischemia in UW-preserved hepatocytes. Until 6 h of ischemia, hepatocytes exceeded the ATP content of the control, achieving their level only after 12 h of ischemia. In contrast, CS had already reached the ATP level of the control after 2 h of ischemia. Furthermore, CS-preserved hepatocytes revealed, in the intervals of ischemia, a continuous decrease in ATP. The intracellular level of ATP was statistically of higher significance ($P < 0.001$) in UW during all intervals of ischemia than in CS. In contrast, HTK revealed the lowest level of ATP after each interval of ischemia, as shown in Fig. 5. At 48 h of ischemia in HTK, the ATP level of hepatocytes tended towards 0%. Nevertheless, the comparison of the ATP course between CS and HTK revealed a statistically significant ($P < 0.001$) decrease in human hepatocytes for each interval of ischemia.

The analysis of the ATP content in hepatocytes after the phase of reperfusion showed quite different results (Fig. 6). Compared with the levels of ischemia, CS and UW displayed a significant ($P < 0.001$) decrease of intracellular ATP during the reperfusion phase. HTK, in contrast, demonstrated an identical course of ATP level during ischemia and reperfusion. In detail, the course of ATP content in hepatocytes was almost identical in CS and UW after reperfusion from 2, 6 and 12 h of ischemia. With regard to the reperfusion from a long term of ischemia of 24 h, there was a statistically significant ($P = 0.02$) decrease of ATP content in UW, as shown in Fig. 6. However, at reperfusion after 48 h of ischemia, the ATP level was distinctly ($P < 0.001$) higher in UW than in CS. As for HTK, there was a statistically significant decrease ($P < 0.001$) of intracellular ATP after

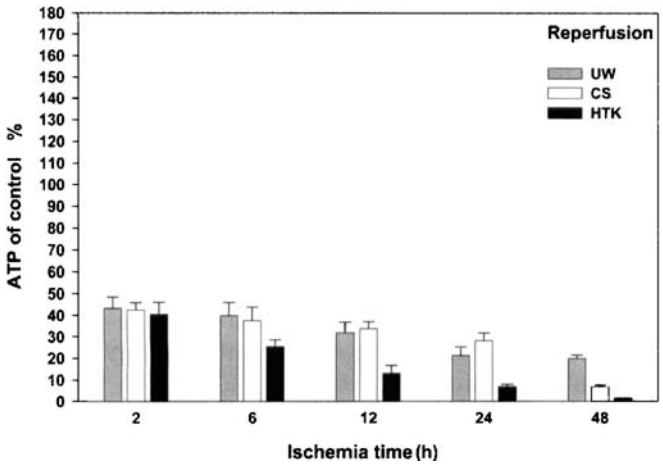

Fig. 6 Level of intracellular ATP in human hepatocytes after 6 h of reperfusion from cold ischemia

reperfusion following 6, 12, 24 and 48 h of ischemia, when compared with intracellular ATP-levels after reperfusion with CS and UW. Except after reperfusion from 2 h of ischemia, the level of intracellular ATP was the same in all preservation solutions.

Discussion

The clinical introduction of CS in the transplantation of thoracic organs poses the question of whether this solution could also be used in the preservation of abdominal organs. Although CS was examined for the preservation of the liver, the kidney and the pancreas of various animal species [4, 38], there is a lack of data on preservation of human organs, such as a cell-culture model. To evaluate the efficacy of CS in the preservation of the human liver, we chose a hepatocyte culture as a suitable model. In particular, the isolation of parenchymal and non-parenchymal liver cells provides one with the opportunity of selective examination. We therefore focused on the preservation injury of the cell membrane, the mitochondrial function, and the intracellular level of ATP, to evaluate the efficacy of CS in liver transplantation.

The maintenance of membrane integrity during ischemia and the reperfusion phase is one hallmark of good protection from organ-preservation solutions [16]. Alterations to membrane integrity as a result of preservation injury were of multifactorial cause and well described as cell necrosis by the measurement of intracellular LDH release [6]. In this context, our results revealed that CS achieved nearly the same efficacy as UW in maintaining the integrity of hepatocyte membranes during various intervals of ischemia and reperfusion. HTK, on the other hand, revealed a significantly higher release of LDH from hepatocytes during the intervals of ischemia and reperfusion. These results confirm that CS, its pattern of impermeants (mannitol, lactobionate) being different from that of UW (hydroxyethyl starch, lactobionate, raffinose), shows the same efficacy in preventing cell necrosis caused by membrane damage. The elevated level of cell necrosis in HTK-preserved hepatocytes might have been caused by the low concentration of the impermeant mannitol, which was supported by the remarkable increase of cell necrosis after long-term ischemia. Even so, our results were in accordance with data from a clinical study in human liver transplantation and in an isolated rat-liver model. Neither study found a difference in the release of intracellular enzymes (LDH, alanine aminotransferase, aspartate aminotransferase) after long-term preservation in CS and UW [5, 15]. Additionally, our results for HTK were in accordance with data from human liver transplantations, which confirmed a significantly higher release of intracellular enzymes than for UW [25].

In order to examine the protection of functional aspects in CS-preserved hepatocytes, we assessed the mitochondrial capacity to reduce MTT, as verified by others [14]. Our results reveal that CS preserves mitochondrial function against preservation injury as well as UW does. CS showed a higher reduction of MTT ($P < 0.001$) during each interval of ischemia and reperfusion than HTK. This was in accordance with our findings of LDH release. Nevertheless, the difference in LDH release and MTT reduction after 48 h of ischemia in UW compared with CS was significant, but at present without relevance to clinical liver transplantation.

Reactive oxygen species were found to play a pivotal role in the injury of cold-preserved hepatocytes [13, 26]. Moreover, a master key in the initiation of mitochondrial damage is the opening of the mitochondrial permeability transition (MPT) pore [10], which was particularly caused by these reactive oxygen species. Preservation solutions, therefore, contain radical scavengers to prevent the MPT pore from opening. Our results in MTT reduction reflect the ability of the preservation solution to catch these oxygen free-radicals by special components. In CS, the high quality of mitochondrial preservation was caused by the presence of multiple-radical scavengers such as glutathione, mannitol, and histidine. UW, in contrast, achieves the same result of MTT reduction with the use of glutathione and allopurinol. Data from human liver transplantation confirm our results of the high quality of mitochondrial preservation in UW that was also valid for CS [21]. Despite the presence of radical scavengers (histidine, mannitol) in HTK, the lower ability for MTT reduction, particularly in long-term ischemia of human hepatocytes, might be explained by a lack of glutathione. According to our isolated cell culture model, glutathione was also found to be highly effective in preventing reperfusion injury from hepatocyte-derived oxygen

free-radicals in a rat-hepatocyte model [37]. This effect was also proven in other organ models [1].

Mitochondria are the principal site of ATP generation and are, therefore, a valid parameter in the assessment of graft viability and function with regard to preservation injury [17, 29]. Our results showed a statistically significantly higher content of intracellular ATP in CS after cold ischemia and reperfusion than in HTK. These findings were in accordance with our data on MTT reduction, which confirmed the improved mitochondrial function in CS after ischemic injury. In contrast to HTK, CS contains glutamic acid, which is known to enhance the tissue levels of ATP [23]. With regard to our results, the impaired mitochondrial function and the lack of any ATP precursor were contributing factors to the inferior outcome of HTK-preserved human hepatocytes.

The comparison of intracellular ATP levels in CS and UW reveals a different pattern. Despite the same efficacy of both solutions to protect mitochondrial function, as shown by MTT reduction, there is a significantly higher content of ATP in UW-preserved hepatocytes after ischemia. Subsequent to reperfusion, the levels in CS and UW were equal. We suppose that the presence of adenosine in UW was responsible for the significantly different ATP level after ischemia from that in CS. Our findings were in accordance with data from UW in pancreas transplantation, which proved the high efficacy of adenosine to increase cellular ATP levels during ischemia [22]. Moreover, we concluded from our data that the significantly higher level of ATP in UW-preserved hepatocytes after ischemia caused no extra beneficial effect with regard to cell necrosis and mitochondrial function during reperfusion. This was verified by our data from LDH release and MTT reduction. In contrast to data from a study with CS in a rat liver-transplantation model, our results from intracellular ATP failed to confirm a significantly higher level after the ischemia phase [36]. However, the results of ATP level after a consecutive reperfusion phase were in accordance with this rat-liver model and another recently performed study from pig-liver transplantation [2]. We assume that the use of different experimental models (human/animal) in each study may explain this finding, as is supposed by others [30].

With regard to long-term preservation, our data clearly demonstrate the efficacy of CS to sustain differential cell function after prolonged cold ischemia. This finding contrasted with data from a rat liver-transplantation model that proved CS to be unsuitable for longer preservation times [11]. Neither the quantity of LDH release nor the reduction of MTT, or the level of intracellular ATP in human hepatocytes after preservation of CS, could explain the deleterious results in this rat-liver model. With regard to the clinical application of CS, a recently published multicentre trial with a mean ischemia time of 16 h confirmed the same efficacy in human kidney transplantation as with UW [9]. CS holds two major advantages for clinical use, particularly over UW. The encouraging results of CS in our study and other experimental and clinical preservation studies of various organs suggest that multi-organ procurement could be performed solely with CS. Furthermore, the noticeably lower price of CS than of UW is an additional advantage in times of progressively falling financial resources in the public health system.

In conclusion, our study proves the superiority of CS over HTK and its parity to UW in the protection of human hepatocytes against cell necrosis, mitochondrial dysfunction, and loss of intracellular energy from ischemia and reperfusion. The impermeants and the ATP precursor in CS were confirmed to be contributing factors to the high level of preservation quality in human hepatocytes. Furthermore, our data also confirmed the efficacy of CS to preserve hepatocytes in long-term ischemia. Despite the methodological limitations of our cell-culture model, we found CS to be suitable for cold preservation of human liver grafts. Further experimental studies with CS for preservation of human non-parenchymal liver cells, e.g. for endothelial cells (in preparation) and clinical studies in human liver transplantation, are to be encouraged.

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