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Induction of Necrosis and DNA Fragmentation During Hypothermic Preservation of Hepatocytes in UW, HTK, and Celsior Solutions

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Donor cells can be preserved in University of Wisconsin (UW), histidine-tryptophan-ketoglutarate (HTK), or Celsior solution. However, differences in efficacy and mode of action in preventing hypothermia-induced cell injury have not been unequivocally clarified. Therefore, we investigated and compared necrotic and apoptotic cell death of freshly isolated primary porcine hepatocytes after hypothermic preservation in UW, HTK, and Celsior solutions and subsequent normothermic culturing. Hepatocytes were isolated from porcine livers, divided in fractions, and hypothermically (4°C) stored in phosphate-buffered saline (PBS), UW, HTK, or Celsior solution. Cell necrosis and apoptosis were assessed after 24- and 48-h hypothermic storage and after 24-h normothermic culturing following the hypothermic preservation periods. Necrosis was assessed by trypan blue exclusion, lactate dehydrogenase (LDH) release, and mitochondrial 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT) reduction. Apoptosis was assessed by the induction of histone-associated DNA fragments and cellular caspase-3 activity. Trypan blue exclusion, LDH release, and MTT reduction of hypothermically preserved hepatocytes showed a decrease in cell viability of more than 50% during the first 24 h of hypothermic preservation. Cell viability was further decreased after 48-h preservation. DNA fragmentation was slightly enhanced in hepatocytes after preservation in all solutions, but caspase-3 activity was not significantly increased in these cells. Normothermic culturing of hypothermically preserved cells further decreased cell viability as assessed by LDH release and MTT reduction. Normothermic culturing of hypothermically preserved hepatocytes induced DNA fragmentation, but caspase-3 activity was not enhanced in these cells. Trypan blue exclusion, LDH leakage, and MTT reduction demonstrated the highest cell viability after storage in Celsior, and DNA fragmentation was the lowest in cells that had been stored in PBS and UW solutions. None of the preservation solutions tested in this study was capable of adequately preventing cell death of isolated porcine hepatocytes after 24-h hypothermic preservation and subsequent 24-h normothermic culturing. Culturing of isolated and hypothermically preserved hepatocytes induces DNA fragmentation, but does not lead to caspase-3 activation. With respect to necrosis and DNA fragmentation of hypothermically preserved cells, UW and Celsior were superior to PBS and HTK solutions in this model of isolated porcine hepatocyte preservation.

Key words: Hepatocytes; Transplantation; Preservation; Liver; Necrosis; Apoptosis

INTRODUCTION

The success of organ and cell transplantation depends, among other things, on the method of preservation. Upon excision of the graft and isolation of cells, an ischemic condition is created that results in cell injury and eventually cell death. Today, the most common method to prevent cell injury in grafts is to store the organs on ice after they have been perfused with a cold solution that has been designed for this purpose. This method results in prolonged storage times with minimal complications of graft function after transplantation (35). The use of hypothermic storage conditions and preservation solutions has also been propagated for short-term storage of isolated cells.

The preservation solution most widely used for liver transplantation is the University of Wisconsin (UW) solution (23,35). This so-called cellular type solution consists of high concentrations of impermeable anions and potassium as well as a high viscosity. Alternatively, the solution developed by Bretschneider [histidine-tryptophan-ketoglutarate (HTK)] can be used for hypothermic preservation of liver grafts (5,11,23). The formula of the HTK solution is different from UW solution and is based

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on a high histidine concentration, which results in a potent buffer. The solution contains a potassium concentration in the physiological range, and the viscosity of the solution is low. Recently, a new solution for hypothermic preservation has been introduced that combines the key compounds of both UW and HTK solutions. This solution, called Celsior, has shown to be effective in hypothermic liver preservation in clinical settings (19,23,42).

The mechanisms that lead to cell injury during hypothermic preservation are complex and involve both ischemia-induced cell death and sensitization of cells to reperfusion-induced cell damage after reflow of the graft in the recipient. Much is known about the events that occur during hypothermic ischemia/reperfusion in the liver [for review see (3,18)], and the preservation solutions that are commonly used (i.e., UW, HTK, and Celsior) each have been designed to counteract these events. However, the potential use of these solutions in hypothermic preservation and subsequent transplantation of hepatocytes as well as the distinctive role of each of the components of these preservation solutions in hypothermic ischemia and reperfusion injury have not been unequivocally clarified. Comparative studies at the cellular level involving all three solutions will potentially identify key components of hypothermic preservation solutions and might also elucidate the mode of action of the solutions, but have not been performed yet.

One of the mechanisms involved in cell injury is apoptosis. In recent years, increasing evidence has been obtained for the occurrence of apoptosis during the early phase of liver graft reperfusion in humans after hypothermic preservation (1,7,9,16,22,25,28,32,43). It has been shown that after transplantation an average of 30% of the subcapsular hepatocytes show signs of apoptosis (1). However, the effects of storage conditions on the induction of apoptosis is unknown. So far, the design of UW, HTK, and Celsior solutions has mainly focused on reducing hypothermic preservation damage, thereby investigating biochemical and morphological criteria of cell death in the outcome of transplantation experiments in animals and humans. Little attention has been paid to the role of apoptosis during hypothermic storage and subsequent return to normothermic conditions (1,7,18,38).

Therefore, we designed this study to test the following hypotheses: 1) prolonged hypothermic preservation of isolated hepatocytes induces cell necrosis and sensitizes cells for apoptosis or necrosis during normothermic reperfusion, and 2) the extent of both necrosis and apoptosis during hypothermic preservation and subsequent normothermic reperfusion is influenced by the composition of the preservation solution. To this end, hepatocytes were isolated from pig livers and stored at 4°C. The hypothermically stored cells were subsequently cultured at 37°C, simulating the sequence of storage and oxygenated, normothermic reperfusion. Cell necrosis was studied by the assessment of membrane integrity using trypan blue exclusion and lactate dehydrogenase (LDH) release. Mitochondrial 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT) reduction activity was measured as parameter of cell necrosis and apoptosis. Apoptosis was assessed by measuring histone-associated low molecular weight (LMW) DNA fragments and caspase-3 activity.

MATERIALS AND METHODS

Chemicals and Solutions

Unless otherwise stated, all chemicals were purchased from Merck (Darmstadt, Germany). Atropine and dexamethasone were from Centrafarm (Etten-Leur, The Netherlands). Isoflurane was from Abbott Laboratories (Queensborough, UK). Fetal bovine serum (FBS), gentamycine, glutamine, Williams' medium E, and a mixture of penicillin, streptomycin, and fungizone were from BioWhittaker (BioWhittaker Europe, Verviers, Belgium). Fluconazol (Diflucan® I.V.) was from Pfizer Inc. (New York, USA). Insulin (Actrapid®) was from Novo Nordisk A/S (Bagsvaerd, Denmark). Liberase™ RH was from Roche (Almere, The Netherlands). Nimatek was from Eurovet (Bladel, The Netherlands) and heparin from Leo Pharmaceutical Products (Weesp, The Netherlands). Penicillin-G (disodium salt) was obtained from Yamanouchi (Leiderdorp, The Netherlands). Stresnil was obtained from Janssen Pharmaceutica (Tilburg, The Netherlands), and vancomycin was from Eli Lilly (Nieuwegein, The Netherlands).

Animals

Livers were obtained from pigs of either sex weighing 20–60 kg. Animals were obtained from Fa. Vendrig (Amsterdam, The Netherlands) and housed at the Central Animal Institute Amsterdam at the Academic Medical Centre (Amsterdam, The Netherlands). The Animal Ethic Committee of the University of Amsterdam had approved the use of these animals for this experimental protocol. The animals had been fed a standard chow for pigs, and had free access to tap water. The animals were anesthetized with a mixture of O_2/N_2O (2:3) and isoflurane (0.4–1%) after premedication with 10 mg/kg ketamine (Nimatek™), 2 mg/kg azaperon (Stresnil™), and 0.02 mg/kg atropine. After cannulation of the portal vein, the liver was flushed with cold Ringer's glucose solution (NPBI, Emmer-Compascuum, The Netherlands) containing 10.000 IU/L heparin. Subsequently, the liver was carefully dissected and excised.

Cell Isolation

Hepatocytes were isolated according to the protocol described by Seglen (33). Briefly, excised livers were perfused with 3 L of an oxygenated calcium-free solution composed of 142 mM NaCl, 6.7 mM KCl, 3.4 mM

hydroxyethylpiperazine-N′-2-ethylsulfonic acid (HEPES), 100,000 IU/L penicillin-G, 40 mg/L gentamycine, 100 mg/L vancomycin, 2 mg/L fluconazol, and set at pH 7.4 with NaOH (37°C) at a rate of 100 ml/min. Subsequently, livers were perfused for 45 min at 37°C with a recirculating and oxygenated digestion solution at a rate of 100 ml/min. The digestion solution was composed of 66.7 mM NaCl, 6.7 mM KCl, 4.8 mM $CaCl_2$, 67.1 mM HEPES, 0.3% (w/v) BSA and 0.007% (w/v) Liberase™ RH, 100,000 IU/L penicillin-G, 40 mg/L gentamycine, 100 mg/L vancomycin, 2 mg/L fluconazol, and set to pH 7.6 with NaOH. Hereafter, the liver was perfused with 200 ml of an ice-cold Hanks' buffer solution composed of 136.9 mM NaCl, 5.4 mM KCl, 0.8 mM $MgSO_4$, 0.3 mM Na_2HPO_4, 0.4 mM KH_2PO_4, 6.7 mM HEPES, 5.0 mM D-glucose, 0.3% (w/v) BSA, 100,000 IU/L penicillin-G, 40 mg/L gentamycine, 100 mg/L vancomycin, and 2 mg/L fluconazol, which was set to pH 7.4 with NaOH. The capsula of the liver was then opened and the digested parenchyma was collected on ice after filtration through surgical gauze. The cell suspension was diluted with ice-cold Hanks' buffer solution and washed three times through centrifugation at 4°C and $50 \times g$ for 3 min followed by resuspension in Hanks' buffer solution. Cell counts were determined after the third centrifugation. Finally, a last centrifugation was performed, after which the cells were resuspended in hypothermic preservation solutions or culture medium.

Table 1. Composition of Preservation Solutions

	Celsior	HTK	UW
Adenosine	—	—	5
Allopurinol	—	—	1
Calcium	0.245	0.015	—
Chloride	28	50	—
Dexamethasone (mg/L)	—	—	8
Glutamate	20	—	—
Gluthatione	3	—	3
Histidine	30	198	—
Hydroxy-ethyl-starch (g/L)	—	—	50
Insulin (U/L)	—	—	100
Lactobionate	80	—	100
Magnesium	13	4	5
Mannitol	60	30	—
Ketoglutarate	—	1	—
Penicillin (U/L)	—	—	200
Phosphate	—	—	25
Potassium	15	9	120
Raffinose	—	—	30
Sodium	100	15	30
Sulphate	—	—	5
Tryptophane	—	2	—
pH	7.3	7.3	7.3
Osmolality (mOsm/L)	320	310	320

Solutions are in mM, unless otherwise stated.

Cell Preservation and Culture

Hepatocytes were hypothermically (4°C) stored in either phosphate-buffered saline (PBS; NPBI, Emmer-Compascuum, The Netherlands), HTK (Custodiol™, Köhler Chemie GmbH, Alsbach, Germany), UW solution (ViaSpan™, Du Pont Pharmaceuticals, Wilmington, DE), or Celsior (IMTIX, Amstelveen, The Netherlands). The composition of the preservation solutions is shown in Table 1. Penicillin-G (100,000 UI/L) was added to all four preservation solutions, and cells were stored in sterile tubes containing 10 ml preservation solution at a concentration of 10^6 cells/ml. Cells were stored in the different preservation solutions at 4°C for 24 and 48 h.

Cells were cultured in 24- and 96-well culture plates (Corning Costar, Badhoevedorp, The Netherlands). The culture medium consisted of Williams' medium E, supplemented with 10% (v/v) heat-inactivated FBS, 2 mM glutamine, 50 µg/L dexamethasone, 1 IU/L insulin, 100,000 U/L penicillin, 100 mg/L streptomycin, and 0.25 mg/L fungizone. Cells were maintained at 37°C in a humidified atmosphere of 5% CO_2 in air.

Trypan Blue Exclusion

After cell isolation and hypothermic storage for 24 and 48 h, a viability count was performed using trypan blue. For this, 100 µl cell suspension (1×10^6 cells/ml) was centrifuged at $50 \times g$ and 4°C for 3 min. The cell pellet was resuspended in 100 µl culture medium and subsequently mixed with 100 µl (0.4% w/v) trypan blue in 0.9% (w/v) NaCl. The cell suspension was then transferred to a counting chamber and cell numbers and viability were determined under a microscope.

LDH Leakage

LDH leakage was determined by measuring LDH activity in supernatant and within cells after either hypothermic preservation or cell culture. LDH activity was spectrophotometrically determined at the Laboratory of Clinical Chemistry using Technicon reagent and an automatic analyzer (Hitachi 747).

For isolated and hypothermically stored cells, 0.8 ml of the cell suspension (1×10^6 cells/ml) was centrifuged at $50 \times g$ and 4°C for 3 min. The supernatant was collected for determination of LDH activity in the preservation solution. The cell pellet was dissolved in 0.8 ml cell lysis buffer of the histone-associated DNA fragment kit. The lysed cells were then centrifuged at $200 \times g$ and 4°C for 10 min, after which the supernatants were collected for determination of LDH activity.

For cultured cells, 2×100 µl cell suspension (1×10^6 cells/ml) of freshly isolated or hypothermically preserved cells was added to 2 wells of a 96-well culture

plate and cultured for 24 h. Hereafter, 2×100 µl culture medium were collected, pooled, and diluted with 800 µl PBS, after which LDH activity of the culture medium was determined. Subsequently, 100 µl of the cell lysis buffer of the histone-associated DNA fragments kit was added to the cells in the 96-well culture plate. After 5-min incubation at 4°C, the 100 µl lysates were collected, pooled, and diluted with 800 µl PBS for determination of LDH activity in cultured cells.

MTT Test

The ability of the cells to transform the tetrazolium salt MTT into formazan was assessed using the Cell Proliferation Kit I from Roche (Almere, The Netherlands). The test was performed according to the instructions of the manufacturer in 96-well culture plates and 50,000 cells in 100 µl culture medium per well. The test was performed on freshly isolated cells, on cells that had been hypothermically preserved for 24 and 48 h, and on cells that had been cultured for 24 h after cell isolation or hypothermic preservation. Total incubation time was 3 h.

Low Molecular Weight DNA Determination

Histone-associated low molecular weight (LMW) DNA fragments were determined using the Cell Death Detection ELISAplus assay of Roche (Almere, The Netherlands). The assay was performed according to the instructions of the manufacturer.

For isolated and hypothermically stored cells, 1.0 ml of the cell suspension (1×10^6 cells/ml) was centrifuged at $50 \times g$ and 4°C for 3 min. The supernatant was collected for determination of histone-associated LMW DNA fragments in the preservation solution. The cell pellet was dissolved in 1.0 ml cell lysis buffer, followed by centrifugation at $200 \times g$ and 4°C for 10 min. Hereafter, the supernatant was collected for determination of histone-associated LMW DNA fragments in isolated or preserved cells.

For cultured cells, 2×20 µl cell suspension (1×10^6 cells/ml in culture medium) of freshly isolated or hypothermically preserved cells was added to 2 wells of a 96-well culture plate containing 80 µl culture medium. The cells were cultured for 24 h. Hereafter, 2×100 µl culture medium was collected for determination of histone-associated LMW DNA fragments in culture medium. Subsequently, 100 µl of the cell lysis buffer was added to each well of the 96-well culture plate. After 5-min incubation at 4°C, the 100 µl cell lysates were collected for determination of histone-associated LMW DNA fragments in the cells.

Caspase-3 Activity

Caspase-3 activity was determined using a fluorometric immunosorbent enzyme assay (Caspase-3 Activity Assay, Roche, Almere, The Netherlands). The test was performed according to the instructions of the manufacturer using 2×10^6 cells. Caspase-3 activity was determined in cells directly after isolation or hypothermic preservation and after subsequent culture for 4 h at 37°C in culture medium.

Statistics

Results are reported as means ± SEM. Data were analyzed using GraphPad Prism software (San Diego, CA). Analysis of variance was used to compare the experimental groups. When overall effects were significantly different ($p < 0.05$), Dunnett's multiple comparison analysis was used to compare cell fractions after 24- and 48-h hypothermic preservation to freshly isolated cells. Tukey's multiple comparison analysis was used to compare the results of different storage solutions after the same preservation period.

RESULTS

As shown in Table 2, cell viability was significantly reduced after 24 and 48 h of hypothermic storage in PBS, HTK, UW, and Celsior solutions, when compared with freshly isolated hepatocytes. Cell numbers also decreased during 24- and 48-h hypothermic preservation (Table 2). This decrease in cell number was significant after 24-h hypothermic preservation in UW solution and 48-h hypothermic preservation in all solutions. Cell viability and numbers were the highest for hepatocytes stored in Celsior solution for both 24 and 48 h. Significant differences were observed for cell viability of cells that had been stored for 48 h in HTK and Celsior solutions.

Table 2. Cell Viability and Cell Numbers of Hepatocytes After 24- and 48-h Hypothermic (4°C) Preservation in PBS, HTK, UW, and Celsior Solutions Based on Trypan Blue Exclusion

Preservation Time	Solution			
	PBS	HTK	UW	Celsior
Viability (% of total)				
24 h	20 ± 5*	16 ± 7*	27 ± 10*	35 ± 10*
48 h	4 ± 3*	2 ± 2*†	11 ± 3*	13 ± 8*
Cell number ($\times 10^6$)				
24 h	6.4 ± 1.0	7.5 ± 1.3	5.1 ± 1.3*†	9.1 ± 1.9
48 h	5.0 ± 1.6*	5.4 ± 1.5*	4.1 ± 0.8*	4.6 ± 1.1*

Cell viability of freshly isolated cells was 79 ± 3 (mean ± SEM, $n = 10$). At zero time, 10×10^6 cells were added to 10 ml of each preservation solution. Results are means ± SEM of 7–8 experiments.

*Significantly different from viability or cell number at zero time ($p < 0.05$).

†Significantly different from viability or cell numbers after storage in Celsior solution at the same time period ($p < 0.05$).

Similar results were obtained when measuring LDH leakage from hypothermically preserved hepatocytes, as shown in Figure 1. The percentage of released LDH was more than 50% after 24 h of hypothermic storage in PBS, HTK, UW, and Celsior solutions, and was the lowest for cells that were preserved in Celsior solution. At this time point, LDH release of cells stored in Celsior was significantly lower than of cells stored in PBS. After 48-h hypothermic preservation, the percentage of released LDH was further increased and similar for all storage solutions (Fig. 1).

The ability of the hepatocytes to reduce MTT was significantly decreased after hypothermic preservation in PBS, HTK, UW, and Celsior solutions (Fig. 2). After 24-h hypothermic preservation, the MTT reducing activity was 28 ± 6%, 27 ± 4%, 47 ± 12%, and 49 ± 14% of the activity at day 0 for PBS, HTK, UW, and Celsior solutions, respectively (means ± SEM, $n = 7$). After 48-h hypothermic preservation, the ability of the cells to reduce MTT was 6 ± 3%, 6 ± 3%, 16 ± 7%, and 18 ± 9% of the activity at day 0 for PBS, HTK, UW, and Celsior solutions, respectively ($n = 7$). No significant differences in MTT reducing activity were observed between cells stored in PBS, HTK, UW, or Celsior solution, but the highest activities were measured in cells stored in UW and Celsior solutions for 24- and 48-h preservation.

As shown in Figure 3, the amount of histone-associated LMW DNA fragments in the preservation solution and hypothermically preserved cells relative to freshly isolated cells was slightly increased after 24- and 48-h storage. However, this relative increase in the amount of histone-associated LMW DNA fragments was not significantly different from the amount of histone-associated LMW DNA fragments of cells directly after the isolation ($p = 0.20$ and 0.26 for 24- and 48-h preservation periods, respectively).

Caspase-3 activity in freshly isolated cells was 0.05 ± 0.03 μM/h ($n = 4$). This is very low in comparison to a positive control of camptothecin-treated U937 cells, which

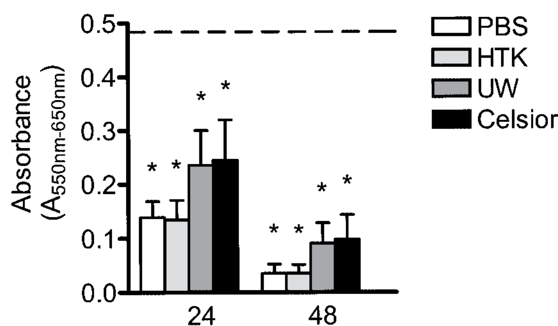

Figure 2. MTT reduction in hypothermically preserved hepatocytes as determined by measuring the absorbance at 550 and 650 nm wavelengths. Hepatocytes were preserved for 24 and 48 h in PBS, HTK, UW, and Celsior solutions and subsequently incubated with MTT in culture medium for 3 h at 37°C. The $A_{(550nm-650nm)}$ after 3-h incubation with MTT in culture medium at 37°C of freshly isolated cells was 0.488 ± 0.063 (means ± SEM, $n = 7$) as indicated by the dashed line. Bars represent means ± SEM of 7 experiments. *Significantly different from the MTT reduction of freshly isolated cells ($p < 0.05$).

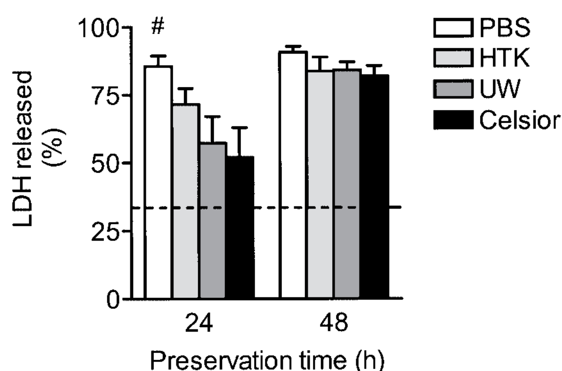

Figure 1. LDH leakage from hypothermically preserved hepatocytes. The percentage of LDH activity that was released was calculated from the LDH activity in cells and preservation solution after 24- and 48-h storage in PBS, HTK, UW, and Celsior solutions. The percentage released LDH activity of freshly isolated cells was 34 ± 5% (mean ± SEM, $n = 6$) as indicated by the horizontal dashed line. Bars represent means ± SEM of 4–5 experiments. #Significantly different from the LDH released after storage in Celsior solution at the same preservation period ($p < 0.05$).

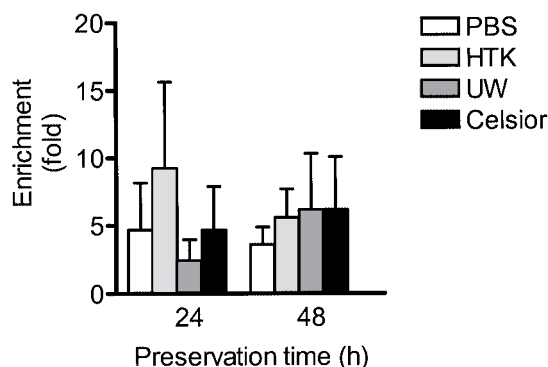

Figure 3. Histone-associated LMW DNA fragments of hypothermically preserved hepatocytes. Hepatocytes were preserved for 24 and 48 h in PBS, HTK, UW, and Celsior solutions, after which the amount of histone-associated LMW DNA was determined in both the preservation solution and cell pellet. Hypothermia-induced enrichment of histone-associated LMW DNA fragments was calculated through dividing the sum of the optical density values from the preservation solutions and the cells by the optical density value of cells directly after isolation. Bars represent means ± SEM of 6 experiments.

Table 3. Caspase-3 Activity (µM/h) in Hepatocytes After Hypothermic Preservation in PBS, HTK, UW, and Celsior Solutions and Subsequent Cultivation

Preservation Time	Solution			
	PBS	HTK	UW	Celsior
Hypothermic preservation (4°C, 24 and 28 h)				
24 h	0.05 ± 0.03	0.01 ± 0.01	0.04 ± 0.02	0.01 ± 0.01
48 h	0.06 ± 0.04	0.04 ± 0.02	0.10 ± 0.07	0.03 ± 0.01
Cultivation (37°C, 4 h)				
24 h	0.07 ± 0.05	0.13 ± 0.11	0.18 ± 0.14	0.16 ± 0.13
48 h	0.03 ± 0.01	0.09 ± 0.06	0.11 ± 0.07	0.11 ± 0.07

Caspase-3 activity of freshly isolated cells was 0.05 ± 0.03 µM/h (mean ± SEM, $n = 4$) and 0.14 ± 0.11 (mean ± SEM, $n = 3$) after subsequent cultivation of the freshly isolated cells at 37°C. Data are means ± SEM of 3–4 experiments. No significant differences were observed.

was 3.06 ± 1.20 µM/h ($n = 4$). As shown in Table 3, caspase-3 activity in hypothermically stored cells was not significantly increased when compared with freshly isolated cells. Moreover, no differences were observed between cellular caspase-3 activity of hepatocytes after storage in PBS, HTK, UW, or Celsior solution.

Hepatocytes were cultured at 37°C after 24- and 48-h hypothermic preservation to simulate normothermic reperfusion. After 24-h culturing of hypothermically preserved cells, average LDH release was more than 75%, as shown in Figure 4. When compared with LDH release of freshly isolated cells that had also been cultured for 24 h, the increase in LDH release was significant after 48-h hypothermic preservation and normothermic culturing (Fig. 4). No differences were observed between LDH release of cells that had been preserved in PBS, HTK, UW, or Celsior solution.

Culturing of freshly isolated cells at 37°C slightly decreased the MTT reducing activity of the cells. After 24 h, MTT reducing activity was 84 ± 16% of the activity of freshly isolated cells ($n = 6$). Hypothermic preservation for 24 and 48 h and subsequent normothermic culturing for 24 h, however, significantly decreased the ability of the cells to reduce MTT, as shown in Figure 5. The MTT reduction after 24-h hypothermic preservation in PBS, HTK, UW, and Celsior solutions and subse-

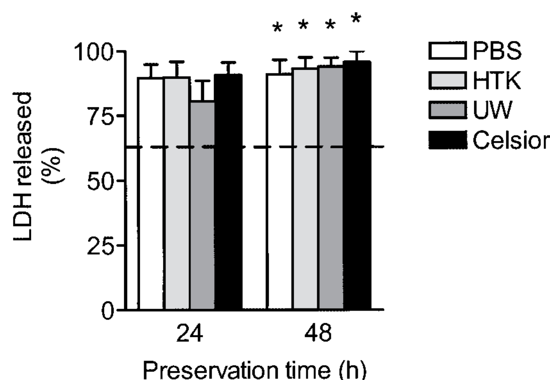

Figure 4. LDH release during 24-h culturing of hypothermically preserved hepatocytes. The percentage of released LDH activity was calculated from the LDH activity in cultured cells and culture medium after the cells had been stored for 24 and 48 h in PBS, HTK, UW, and Celsior solutions and subsequently cultured for 24 h. The percentage released LDH activity of freshly isolated and subsequently cultured cells was 64 ± 14% (mean ± SEM, $n = 5$) as indicated by the horizontal dashed line. Bars represent means ± SEM of 4–5 experiments. *Significantly different from freshly isolated and cultured cells ($p < 0.05$).

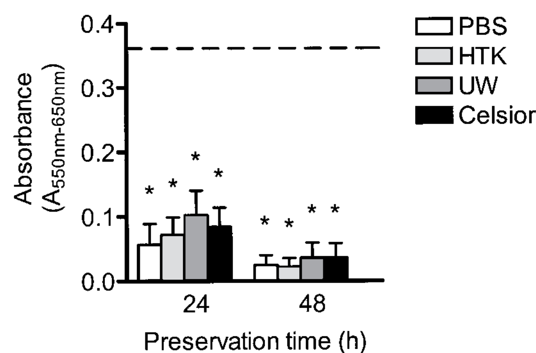

Figure 5. MTT reduction in hypothermically preserved and subsequently cultured hepatocytes as determined by measuring the absorbance at 550 and 650 nm wavelengths. Hepatocytes were preserved for 24 and 48 h in PBS, HTK, UW, and Celsior solutions and subsequently cultured at 37°C for 24 h, after which the cells were incubated with MTT in culture medium for 3 h at 37°C. The $A_{(550nm-650nm)}$ of freshly isolated cells after 24-h culturing at 37°C followed by 3-h incubation with MTT in culture medium at 37°C was 0.370 ± 0.046 (means ± SEM, $n = 6$) as indicated by the dashed line. Bars represent means ± SEM of 7 experiments. *Significantly different from the MTT reduction of freshly isolated cells ($p < 0.05$).

quent culturing for 24 h was decreased to 27 ± 14%, 42 ± 13%, 40 ± 15%, and 34 ± 16%, respectively, of cell activity of freshly isolated and cultured cells ($n = 7$). After 48-h hypothermic preservation and subsequent normothermic culturing for 24 h, MTT reduction activity of the hepatocytes was strongly decreased for all preservation solutions (Fig. 5). No significant differences in MTT reducing activity were observed between cultured cells from different preservation solutions.

The amount of histone-associated LMW DNA fragments of freshly isolated cells after 24-h culturing at 37°C was increased by 39 ± 25-fold ($n = 6$) relative to the amount of histone-associated LMW DNA fragments in cells directly after the isolation. Hypothermic preservation of hepatocytes prior to normothermic culturing also increased the formation of histone-associated LMW DNA fragments relative to the amount of histone-associated LMW DNA fragments of freshly isolated cells, as shown in Figure 6. A significant increase in the amount of histone-associated LMW DNA fragments relative to freshly isolated cells was observed for cells stored in HTK and Celsior solutions after 48-h hypothermic preservation followed by 24-h normothermic culturing (Fig. 6). However, the increase of histone-associated LMW DNA fragments in cells stored in HTK and Celsior solutions after 48-h hypothermic preservation and 24-h normothermic culturing was not significantly different from the amount of histone-associated DNA fragments in cells stored in PBS or UW solution for 48 h and subsequently cultured for 24 h ($p = 0.06$).

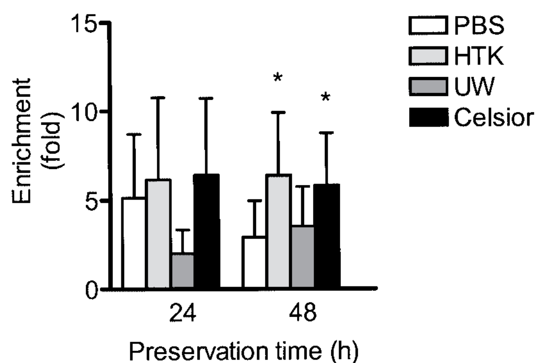

Figure 6. DNA fragmentation in hepatocytes after 24-h culturing of hypothermically preserved cells. Hepatocytes were preserved for 24 and 48 h in PBS, HTK, UW, and Celsior solutions, followed by 24-h culturing at 37°C. Thereafter the amount of histone-associated LMW DNA was determined in both culture medium and cell pellet. Enrichment of histone-associated LMW DNA fragments was calculated through dividing the sum of the optical density values from the preservation solutions and the cells by the optical density value of cells directly after isolation. Bars represent means ± SEM of 6 experiments. *Significantly different from histone-associated LMW DNA levels of freshly isolated cells ($p < 0.05$).

Caspase-3 activity in cells that had been cultured for 4 h directly after isolation was only slightly increased in comparison with freshly isolated cells (0.14 ± 0.11 μM/h vs. 0.05 ± 0.03 μM/h, respectively, $n = 4$, not significantly different). Caspase-3 activity in cells after 24- and 48-h hypothermic preservation and subsequent normothermic culturing for 4 h was similar to the caspase-3 activity of cells directly after isolation and subsequent normothermic culturing for 4 h (Table 3). No significant differences were observed between cellular caspase-3 activity of cells stored in PBS, HTK, UW, or Celsior solution.

DISCUSSION

The aim of this study was 1) to investigate the mechanisms of cell death during hypothermic preservation and subsequent normothermic culturing of hepatocytes and 2) to compare the efficacy of HTK and UW solutions as well as the newly devised hypothermic storage solution Celsior in preventing these processes. Our results indicate that necrosis predominated in isolated and hypothermically preserved hepatocytes. Both membrane integrity and mitochondrial function were impaired after 24- and 48-h hypothermic preservation of isolated hepatocytes. DNA fragmentation was slightly, but not significantly, increased under these conditions, as shown by the enrichment of histone-associated LMW DNA fragments. Caspase-3 activity of hypothermically preserved cells was low and not increased when compared with freshly isolated hepatocytes. Subsequent normothermic culturing of hypothermically preserved cells resulted in a further loss of cell viability. Cell viability of hypothermically preserved and subsequent normothermically cultured hepatocytes was less than 20%, as can be deduced from LDH release and mitochondrial MTT reduction of these cells. The increase in histone-associated LMW DNA fragments during hypothermic preservation in HTK and Celsior solutions and subsequent normothermic culturing of isolated hepatocytes suggests a role of apoptosis in cell death during this simulation of reperfusion. However, caspase-3 activity was not increased in hypothermically preserved and subsequently normothermically cultured cells. Even if we consider a reduction in the number of viable cells of up to 90%, the caspase-3 activity in hypothermically preserved and normothermically cultured hepatocytes did not reach the level of camptothecin-treated U937 cells.

Our study was based on the use of isolated hepatocytes. Hepatocytes have been used in the past to study the mechanisms involved in cell necrosis during hypothermic storage (2,6,8,12,13,20,29,34,36,39–41) and subsequent culturing under normothermic conditions (2,6, 12,21,34,37,41). Moreover, studies investigating the optimal solution for hypothermic preservation of porcine hepatocytes and identifying key components of these so-

lutions have been reported (14,24,27,31,36). The relation between forms of cell death (i.e., necrosis, apoptosis, or a mixed phenotype) and the solution used for hypothermic preservation has not been evaluated yet, but clinical studies have demonstrated the presence of apoptotic hepatocytes in livers after transplantation (1,7,16,22,25,28,32,43).

Our results on cell viability and cell number as well as LDH release and MTT reduction of hypothermically preserved hepatocytes demonstrated a rapid decrease in cell viability of 50–80% during the first 24 h (Figs. 1 and 2), thereby confirming earlier reports on the rate of hepatocyte necrosis during hypothermic preservation (2,29,36). DNA fragmentation was low in these cells and caspase-3 activity could not be detected, suggesting that apoptosis does not occur during hypothermic preservation. Cellular ATP is most likely the key discriminator between necrosis and apoptosis during hypothermic preservation. Sufficient amounts of cellular ATP are a prerequisite for apoptotic cell death (17), but known to be decreased in isolated hepatocytes during hypothermic preservation (13).

Subsequent culturing of hepatocytes after 24-h hypothermic preservation, simulating normothermic reperfusion, results in a further decrease in cell viability, as shown in this and an earlier study (37). In contrast to hypothermically preserved cells, the subsequently normothermically cultured hepatocytes showed signs of DNA fragmentation. However, like for hypothermically preserved cells, we could not detect caspase-3 activation in these cells, indicating that apoptosis does not significantly contribute to cell death after hypothermic preservation for more than 24 h and subsequent normothermic culturing. The presence of DNA fragments in the absence of caspase-3 activation suggestes that the DNA of some of the hypothermically preserved and subsequently normothermically cultured cells was degraded through a caspase-independent pathway. Such event has been described before during necrotic cell death and is attributed to noncaspase proteolytic cascades [for review see (4)].

High levels of histone-associated LMW DNA fragments were also observed in cultured cells that had not been stored at 4°C. In these cells, viability was reduced by only 15% during 24-h culturing, as can be deduced from the mitochondrial MTT reduction of both freshly isolated cells and cells that had been directly cultured after isolation. The high level of LMW DNA fragments in isolated and subsequently cultured hepatocytes is in line with results of Rivera et al. (30), who have shown that up to 45% of all cells in primary gel-entrapped rat hepatocyte cultures are positive for terminal deoxynucleotidyl transferase-mediated dUTP nick-end labeling (TUNEL). However, like for the hypothermically preserved cells and cells that had been cultured after hypothermic preservation, we could not detect increases in caspase-3 activity upon culturing the freshly isolated hepatocytes after isolation. These data are different from the results of Nyberg et al. (26), who reported that the addition of a caspase inhibitor to the culture medium significantly reduces cell death of hepatocytes during the first 24 h of cell culture. Our results, in combination with the data of Nyberg et al. (26), suggest a role of a mixed phenotype of both necrotic and apoptotic cell death in the cultured hepatocytes that needs further investigation.

In this study, we compared three commercially available hypothermic preservation solutions with respect to their efficacy to prevent hepatocyte cell death. UW is the most widely used preservation solution and can be considered the current golden standard in liver graft preservation (35). The Bretschneider's solution HTK forms an alternative to UW solution. Although principle of action and composition of HTK differ substantially from UW solution, both are suitable with comparable results in clinical liver graft preservation (5,11). Celsior is a relatively new preservation solution, which combines the key components of UW and HTK solutions (i.e., lactobionate and glutathione of UW solution and the histidine buffer, chloride content, low potassium concentration and mannitol of HTK solution) (11). Celsior has been tested in clinical settings and has shown to be effective in protecting livers against ischemic tissue degradation (19,23,42). PBS served as a control solution. None of the preservation solutions tested in this study was capable of adequately preventing total cell death after 24-h hypothermic preservation and subsequent 24-h normothermic culturing. Although small, differences between UW, HTK, and Celsior were observed. HTK was found to be the least effective in preventing hypothermia-induced necrosis in these cells when compared with UW and Celsior solutions. These results are in line with observations of Rauen et al. (29), who also reported a superiority of UW solution over HTK solution in preventing hypothermia-induced necrosis of isolated rat hepatocytes. Based on cell viability and cell numbers as well as LDH release, Celsior was most effective in preventing hypothermia-induced necrosis when compared with HTK and UW solutions. Thus, a combination of the key components of UW and HTK solutions results in a solution that is more effective in preventing hypothermia-induced cell necrosis of isolated hepatocytes than UW and HTK solutions themselves. The formation of histone-associated LMW DNA fragments during hypothermic preservation and subsequent normothermic culturing of hepatocytes tended to be the lowest in UW solution and PBS (Fig. 6). This indicates that all tested hypothermic preservation solutions lack components that effectively prevent DNA fragmentation in isolated hepatocytes and suggests that the compositions of HTK and Celsior solutions might even enhance the formation of histone-associated LMW DNA fragments in these

cells. As a marker of both necrosis and apoptosis we measured the MTT reduction activity of the cells. As MTT reduction is measured after normothermic incubation of cells for 3 h and mitochondrial function is impaired in both cell necrosis and apoptosis (10,15), a decrease in MTT reduction is considered to reflect both cell necrosis and early apoptosis. As shown in Figure 2, MTT reduction of hepatocytes after hypothermic preservation for 24 and 48 h in UW and Celsior solutions was identical.

Our results have implications regarding the use of isolated hepatocytes in cell transplantation and bioartificial liver treatment. In contrast to earlier reports (14,27), we consider hypothermic preservation of porcine hepatocytes for more than 24 h in HTK, UW, or Celsior solution far from ideal for liver cell transplantation or for use in a bioartificial liver based on porcine hepatocytes. The reason is that a high percentage of hypothermically stored hepatocytes, which may appear viable directly after the preservation period, is likely to die directly after transplantation or filling of a bioreactor.

In summary, hypothermic preservation of isolated hepatocytes leads to necrosis and in lesser extent to the formation of low molecular weight DNA fragments. Subsequent normothermic culturing of the hypothermically preserved cells further enhances necrosis and DNA fragmentation. Celsior appears to be most effective in preventing cell membrane damage. Overall, with respect to necrosis and DNA fragmentation of hypothermically preserved cells, UW and Celsior solutions are superior to PBS and HTK solutions in this model of isolated porcine hepatocyte preservation.

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