Transpl Int (2002) 15: 233–239
DOI 10.1007/s00147-002-0400-6

ORIGINAL ARTICLE

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Intracellular Ca^{2+} regulation in hepatocytes under experimental transplantation conditions

Received: 18 May 2000
Revised: 20 June 2001
Accepted: 21 January 2002
Published online: 4 April 2002

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Abstract Under transplant conditions excessive accumulation of intracellular calcium ($[Ca^{2+}]_i$) is considered to be a mediator of cell injury during ischaemia and re-oxygenation. To clarify this consideration as well as the necessity of calcium-free preservation solutions, we used a well-known in-vitro model. Furthermore, a new application to mimic clinical situation was established, and we evaluated the correlation between $[Ca^{2+}]_i$ change and cell survival in monolayers of isolated rat hepatocytes undergoing cold hypoxia in defined solutions and during re-oxygenation. $[Ca^{2+}]_i$ was measured in single cells by ratio imaging of Fura-2 fluorescence after various periods of cold hypoxia (ischaemia phase) in different preservation solutions [UW, HTK, EC and Krebs–Henseleit buffer (KH)] and following warm normoxic reperfusion (re-oxygenation phase) with KH. Cell survival was measured simultaneously by trypan-blue exclusion. Cell survival decreased, depending on preservation solution and preservation time. The partially tremendous $[Ca^{2+}]_i$ change under cold hypoxia did not correlate with the change in cell survival. For example, UW-stored cells showed a $[Ca^{2+}]_i$ loss from 280 nM to 56 nM, compared with KH-stored cells with a $[Ca^{2+}]_i$ increase of up to 445 nM. Our results indicate that $[Ca^{2+}]_i$ plays only a minor role in the pathomechanisms of hypoxic and re-oxygenation hepatocellular injury.

Keywords Fura-2-AM · Hepatocytes · Hypoxia · Intracellular $[Ca^{2+}]_i$ · Re-oxygenation

Introduction

Organ conservation under cold conditions and in special preservation solutions is common in clinical transplantation procedures. Hypothermia and special solutions are applied to diminish cellular injury caused by oxygen deprivation, energy loss and disturbances in ion homeostasis. Intracellular calcium ($[Ca^{2+}]_i$) homeostasis disturbance especially, i.e. $[Ca^{2+}]_i$ increase under cold and hypoxic conditions, is considered to be responsible for cellular injury [1, 6, 18, 21, 23, 28]. Calcium is one of the principal intracellular regulative ions. Both the Ca^{2+}-calmodulin complex and free-Ca^{2+} activate and regulate a great number of enzyme systems. Elevated Ca^{2+} leads to activation of hydrolytic enzymes (such as phospholipase A_2 and C, some endonucleases, proteases and protein kinases), to expression of several immediate-early genes [28] and to possible formation of cytoplasmic blebs, and exerts a number of destabilising effects on the cytoskeleton involving actin, tubulin and other proteins [13, 19].

The unregulated $[Ca^{2+}]_i$ increase under cold hypoxia is primarily caused by impaired calcium transport across the endoplasmic reticulum, mitochondria and plasma membranes due to ATP-depletion [1, 7, 20]. Gasbarrini et al. [7] postulated an initial increase of Ca^{2+} from intracellular sources and a subsequent massive increase from extracellular sources under hypoxic conditions. To

prevent this massive increase of $[Ca^{2+}]_i$, the common preservation solutions are nearly Ca^{2+} free. However, the once widely accepted view, that increased $[Ca^{2+}]_i$ is the major factor causing lethal cell injury during hypoxia, is increasingly being questioned [3, 7, 12, 16, 19].

To help to clarify this, we analysed the relationship between $[Ca^{2+}]_i$ change and cell survival under cold hypoxia in defined preservation solutions and after warm normoxic perfusion (re-oxygenation phase). To compare the effects of high or low extracellular Ca^{2+} we applied a further solution for preservation, namely Krebs Henseleit buffer (KH). KH is an 'extracellular' buffer with a high Ca^{2+} content, in contrast to the standard solutions. It has not been used for organ preservation in the past.

The present study was carried out with isolated rat hepatocytes, which have proven to be very useful in the study of liver function in vitro [2, 22, 24, 31, 32]. We monitored the $[Ca^{2+}]_i$ change continuously and non-invasively with fluorescence microscopic ratiometry during cold hypoxia and re-oxygenation. Simultaneously, cell survival was measured by trypan-blue exclusion.

Materials and methods

Materials, chemicals and solutions

Nigericin, pluronic F127 and Fura-2 AM were purchased from Molecular Probes (Eugene, Oregon, USA). The L15-Leibovitz medium and supplements were purchased from Biochrom (Berlin, Germany). UW was purchased from Dupont Pharma (Netherlands), EC from Fresenius (Bad Homburg, Germany) and HTK from Köhler (Alsbach, Germany). Components of these preservation solutions are shown in Table 1. All other chemicals were from Sigma (Deisenhofen, Germany). Stock solutions of Fura-2 AM (5 mM in dry dimethylsulphoxide) and of nigericin (10 mM in dimethylformamide/ethanol 3:1) were prepared in aliquots and preserved at –20 °C until use. Modified KH (Table 1) was used as a medium in the re-oxygenation phase, for incubation of untreated reference cells and as a preservation solution under cold hypoxia. The Ca^{2+} calibration buffer contained 120 mM KCl, 10 mM NaCl, 1 mM $MgSO_4$ and 10 mM HEPES. For high Ca^{2+} solution the calibration buffer contained an additional 10 mM $CaCl_2$. For Ca^{2+}-free solution, the calibration buffer contained 10 mM EGTA. The pH was adjusted to 7.35 with Tris-base or Tris-HCl.

Isolation and incubation of hepatocytes

Hepatocytes were isolated by collagenase perfusion from livers of male Wistar rats (140–160 g) as described previously [26] and resuspended at 8×10^6 cells/ml. Hepatocytes were cultured in L15-Leibovitz medium supplemented with $NaHCO_3$ (14.28 mM), L-glutamine (2 mM), FCS (5% v/v), BSA (0.1% v/v) and gentamicin (0.1%). The cells were cultured in Petri dishes on 18-mm square glass coverslips coated with collagen A, at 37 °C in 5% CO_2 for 16 h. The animals were housed in temperature-controlled animal quarters under a 12-h light–dark cycle with free access to tap water and standard rat chow until use. All animal experiments were approved by the Animal Care Committee of Tübingen and conform to the National Institute of Health guidelines.

To simulate transplant conditions, we incubated the hepatocytes in the different preservation solutions at 4 °C for 2, 4 and 6 h in special gas chambers. Hypoxia was obtained by equilibrating the

Table 1. Composition (mmol l^{-1}) of the solutions. UW, HTK and EC are common preservation solutions with low Na^+ and different buffering systems. There are additional cell protective components in UW and HTK. KH is a common extracellular buffer for hepatocytes not used in organ preservation, with high Na^+ and low K^+. Equilibration depended on the buffering system. pH of solutions was measured after each equilibration (values are means ± SD from $n = 10$)

Solution	KH	EC		HTK		UW	
Na^+	143.6		10		15		30
K^+	5.9		115		10		125
Cl^-	127		15		50		–
Mg^{2+}	1.18		–		4		5
Ca^{2+}	2,5		–		0.015		–
HCO_3^-	25		10		–		–
SO_4^{2-}	1.2		–		–		5
$H_2PO_4^-$	1.23		15		–		25
HPO_4^-	–		42.5		–		–
HEPES	20		–		–		–
Supplements	–	Glucose	198	HistidineHCl/		Lactobinate	100
				Histidine	180/18	Raffinose	30
				Mannitol	30	HES	50 g/l
				Tryptophan	2	Allopurinol	1
				Ketoglutarate	1	Adenosine	5
						Glutathione	3
						Insulin	40 U/l
						Dexamethasone	8 mg/l
						Penicillin G	200000 U/l
Hypoxic equilibration	95%N_2, 5%CO_2	95%N_2, 5%CO_2	100%N_2	100%N_2			
Normoxic equilibration	75%N_2, 20%O_2, 5%CO_2	–	–	–			
pH [4 °C]	7.2 (±0.05)	7.2 (±0.02)		7.2 (±0.02)		7.35 (±0.03)	

solutions with 95% N_2 and 5% CO_2 (EC, KH) or 100% N_2 (UW, HTK), respectively. The pH of each solution was measured after equilibration; data are shown in Table 1. Re-oxygenation was established by perfusing the preserved hepatocytes with warm normoxic KH (saturated with a 75% N_2, 20% O_2 and 5% CO_2 mixture; at 25 °C).

Determination of cell survival

Hepatocyte viability was measured by trypan-blue exclusion. Cells with uptake of trypan blue in the nucleus or the cytoplasm were considered damaged. Trypan blue (0.08% w/v) was added to the different preservation solutions 5 min before the end of each preservation phase, and cell survival was measured subsequently. After the preservation period, cells were reperfused with warm normoxic KH and stored therein for 30 min. Five minutes before the end of this phase, trypan blue was added again to measure cell survival. On the underside of each coverslip three representative areas with about 300 cells were marked for observing the trypan-blue uptake throughout the experiment. Cell viability in the marked areas before cold hypoxia was taken as 100%. The percentage viability was calculated by trypan-blue exclusion for each area, and the average of cell survival was recorded.

Determination of $[Ca^{2+}]_i$ in single hepatocytes

The cells were loaded for 45 min with 5 μM Fura-2 AM and 0.02% (w/v) pluronic F127 in KH/L-15 medium (3:1) at 37 °C, 5% CO_2. After being dye-loaded the cells were either used for control measurements or were preserved in defined preservation solutions under cold hypoxic conditions. After preservation the cells were mounted in the absence of O_2 in a cold perfusion chamber on the stage of an epifluorescence microscope (Nikon, Japan). The chamber was filled with the appropriate hypoxic and cold solutions. The re-oxygenation phase was introduced by perfusion of the cells with warm normoxic KH. $[Ca^{2+}]_i$ was measured continuously during the last 5 min of cold hypoxia and during the following 45 min of re-oxygenation, by means of digital imaging fluorescence microscopy (ICMS; Hamamatsu Photonics, Shizuoka, Japan). Fluorescence images of Fura-2-loaded hepatocytes were obtained at excitation wavelengths of 340 nm and 380 nm with an emission wavelength of 510 nm. The images were recorded every 20 s for 60 min. The $[Ca^{2+}]_i$ values were calculated from ratio images of fluorescence (340/380 nm) after background subtraction. Free $[Ca^{2+}]_i$ was determined by the method of Grynkiewicz et al. [10], applying the following equation:

$$[Ca^{2+}]_i = K_d([R - R_{min}]/[R_{max} - R])B$$

K_d is the dissociation constant for the Fura-2-calcium complex; B is the ratio of fluorescence intensity at 380 nm in the absence of Ca^{2+} to a saturated concentration of Ca^{2+}; R represents the Fura-2 fluorescence intensity in the single cell at 340-nm excitation divided by that at 380-nm excitation. R_{min} and R_{max} are the minimum and maximum ratios of the dye fluorescence intensities for Ca^{2+}-free (min) and Ca^{2+}-saturated (max) dye. Kd, B, Rmin and Rmax are obtained from fluorescence standard curves established using calibration buffers with various free Ca^{2+} concentrations as described below. The fluorescence spectra of Fura-2 free and bound forms were calibrated using EGTA-Ca buffers according to the mass equation law:

$$[Ca^{2+}]_{free} = K_dEGTA \cdot [CaEGTA]/[K_dEGTA]$$

The conditional dissociation constant of the EGTA-Ca complex was confirmed to be 150.5 nM under our experimental conditions. Fluorescence standard curves were obtained in vivo with the high Ca^{2+} or Ca^{2+}-free calibration buffer (= EGTA-Ca buffer) as well as with defined free Ca^{2+} concentrations and the Ca^{2+} ionophore 4-bromo A-23187. With the data obtained, the dissociation constant (K_d) of Fura-2 for Ca^{2+} was calculated to be 132 nM.

Because the K_d is also dependent on temperature, osmolarity and pH, we determined fluorescence intensity changes of Fura-2-loaded cells by re-warming them from 4 °C to 37 °C. This temperature change reflects the phase between cold hypoxia and warm re-oxygenation. There was a constant fluorescence from 4 °C to 25 °C, but thereafter, a continuous loss of fluorescence intensity could be observed (data not shown). This loss of fluorescence intensity was also confirmed by Kawanishi [15]. Taking this phenomenon into consideration, we re-warmed the cells only to 25 °C and measured the control cells also at 25 °C. Because the osmolarity of the different preservation solutions was nearly identical, this factor could be neglected. The effect of pH changes on Fura-2 within the physiological range (pH6.75–7.4) is negligible and has no relevant influence on either the spectra of the Ca^{2+} free or bound species, or on the effective Ca^{2+} dissociation constant [10].

Statistics

All data represent at least nine experiments derived from three different cell preparations and are expressed as mean ± standard deviation (SD). $[Ca^{2+}]_i$ measurements were analysed by Dunnett's test (variance analysis). A probability of < 0.05 was considered statistically significant. The cell survival rate was expressed in linear regression. The linear regression slopes of the groups were compared and assessed for significance, by the paired *t*-test.

Results

$[Ca^{2+}]_i$ change after 2, 4 and 6 h of cold hypoxia in different preservation solutions and during the following re-oxygenation

Cells stored in UW, HTK and EC showed a time-dependent loss of intracellular calcium ions, in contrast to KH-stored cells which showed a $[Ca^{2+}]_i$ increase, dependent on duration of cold hypoxia (Fig. 1). After different preservation times the cells were re-oxygenated with warm (25 °C) normoxic KH, which led, in all cells, to regeneration of an approximately physiological $[Ca^{2+}]_i$ level (Fig. 2). Physiological $[Ca^{2+}]_i$ in control cells was 280 (±45) nM.

$[Ca^{2+}]_i$ change in EC-stored cells after cold hypoxia and re-oxygenation

Figures 1 and 2 show the changes in $[Ca^{2+}]_i$ in EC-stored cells after cold hypoxia and re-oxygenation, respectively.

Because of the extreme cell death under EC storage, only the 2- and 4-h values were determined. The $[Ca^{2+}]_i$ decreased in the first 2 h of cold hypoxia to 81 (±35) nM. The following re-oxygenation increased the $[Ca^{2+}]_i$ to 156 (±25) nM. After 4 h of cold hypoxia the $[Ca^{2+}]_i$ decreased to 122 (±71) nM and increased after re-oxygenation to 291 (±49) nM.

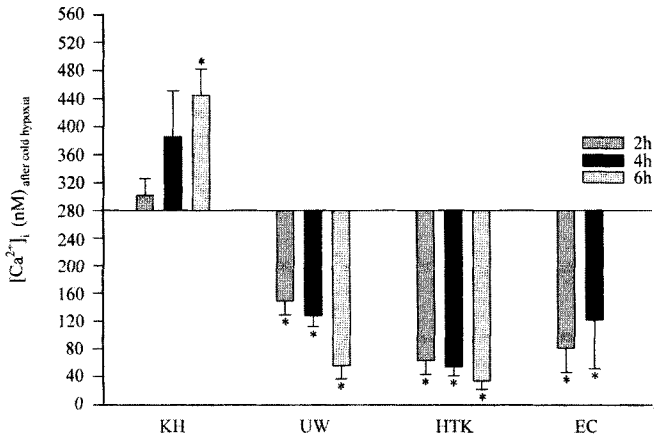

Fig. 1. $[Ca^{2+}]_i$ after cold hypoxia in different preservation solutions. *The basal line* is the physiological $[Ca^{2+}]_i$ of non-treated (control) cells under warm normoxic conditions. The $[Ca^{2+}]_i$ change after 2, 4 and 6 h of cold hypoxia in KH, UW, HTK and EC is shown in the graph. Cells stored in the standard clinical preservation solutions showed a time-dependent loss of $[Ca^{2+}]_i$, in contrast to KH-stored cells, which showed a time-dependent increase. The data after 6 h of preservation in EC are missing because of the extreme cell death thereby. Significance (*) compared with control was evaluated in all solutions

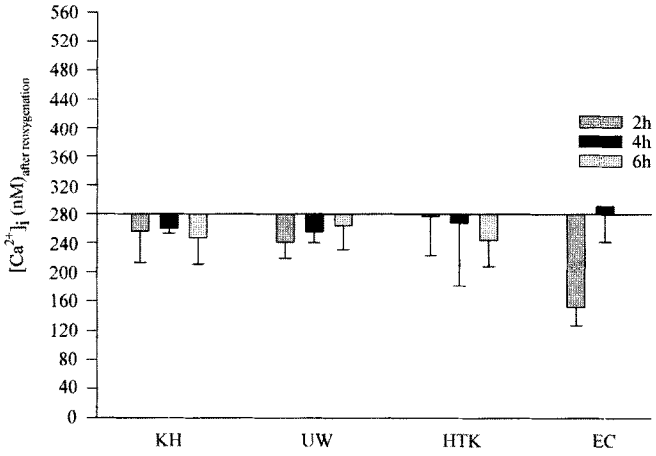

Fig. 2. $[Ca^{2+}]_i$ after re-oxygenation. The graph represents the $[Ca^{2+}]_i$ change after reperfusion with KH subsequent to 2, 4 and 6 h of cold hypoxia. The re-oxygenation led, in all cells, to regeneration of an approximately physiological $[Ca^{2+}]_i$ level

$[Ca^{2+}]_i$ change in HTK-stored cells after cold hypoxia and re-oxygenation

Figures 1 and 2 show the changes in $[Ca^{2+}]_i$ in HTK-stored cells after cold hypoxia and re-oxygenation, respectively.

In particular, cells stored in HTK showed a very strong intracellular calcium loss. After only 2 h of cold hypoxia the $[Ca^{2+}]_i$ dropped to 63 (±20) nM, but almost returned to the control level after re-oxygenation (278 (±54) nM). Further cold preservation with hypoxia of 4 and 6 h decreased the $[Ca^{2+}]_i$ to 54 (±13) nM and to 34 (±12) nM, while subsequent re-oxygenation increased the $[Ca^{2+}]_i$ to 268 (±87) nM and 244 (±87) nM, respectively.

$[Ca^{2+}]_i$ change in UW-stored cells after cold hypoxia and re-oxygenation

Figures 1 and 2 show the changes in $[Ca^{2+}]_i$ in UW-stored cells after cold hypoxia and re-oxygenation, respectively.

Also under UW storage, cells suffered a continuous, time-dependent $[Ca^{2+}]_i$ loss. After 2, 4 and 6 h of cold hypoxia, $[Ca^{2+}]_i$ was 147 (±20) nM, 128 (±16) nM and 56 (±19) nM, respectively. The subsequent re-oxygenation increased the $[Ca^{2+}]_i$ to 241 (±22) nM, 256 (±15) nM and 264 (±33) nM, respectively.

$[Ca^{2+}]_i$ change in KH-stored cells after cold hypoxia and re-oxygenation

Figures 1 and 2 show the changes in $[Ca^{2+}]_i$ in KH-stored cells after cold hypoxia and re-oxygenation, respectively.

In contrast to the standard storage solutions, KH-stored cells showed a time-dependent $[Ca^{2+}]_i$ increase. After 2, 4 and 6 h of cold hypoxia the $[Ca^{2+}]_i$ increased to 302 (●24) nM, 385 (±66) nM and 445 (±37) nM, respectively. The subsequent re-oxygenation led to $[Ca^{2+}]_i$ decrease to near control levels: 256 (±43) nM, 260 (±7) nM and 247 (±36) nM, respectively.

Cell survival after cold hypoxia in different storage solutions and after 30 minutes of subsequent re-oxygenation

Figures 3 and 4 show cell survival after cold hypoxia in different storage solutions, and after 30 min of subsequent re-oxygenation, respectively. The cell survival depended on preservation solution and preservation time.

After re-oxygenation continuous cell death was observed, with the same dependence on preservation solution and duration as under cold hypoxia.

After 6 h of cold hypoxia in UW, cell survival decreased to 91% (±1.25), and after subsequent re-oxygenation, further to 82% (±2.46). Under KH preservation 81% (±2.80) of the cells survived after 6 h and after re-oxygenation cell survival decreased to 72% (±3.42). Similar results were observed in HTK-preserved cells, where survival was 83% (±2.35) after 6 h of cold hypoxia, and 65% (±6.16) after subsequent re-oxygenation. EC preservation led to pronounced cell death, with only 33% (±5.23) cell survival after 6 h of

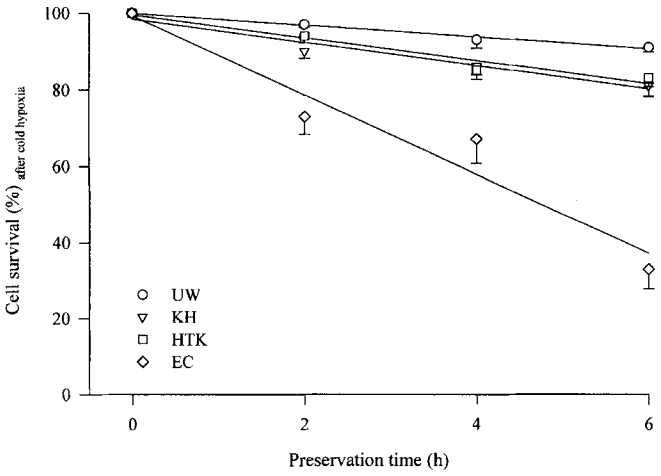

Fig. 3. Cell survival of hepatocytes dependent on preservation solution and duration. Cell survival rate decreased in all cells depending on preservation time. EC-preserved cells especially, showed dramatic cell death

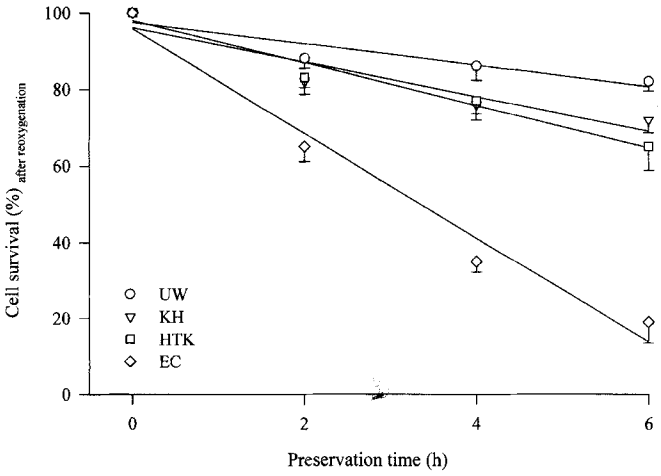

Fig. 4. Cell survival after re-oxygenation undergoing 2, 4 and 6 h of cold hypoxia. The viability was determined 30 min after re-oxygenation with warm normoxic KH. The cells showed the same time-dependent but increasing cell death after re-oxygenation as did cells under cold hypoxia. EC-preserved cells especially, underwent a high cell-death rate

cold hypoxia, and after re-oxygenation, with only 19% (± 5.57) survival. Cell survival (expressed in linear regression) after cold hypoxia and re-oxygenation in EC-preserved cells was significantly lower ($P < 0.001$) than that in HTK, KH and UW. Cell survival in KH and HTK was also significantly lower ($P < 0.05$) than in UW.

Discussion

The standard clinical preservation solutions (EC, HTK and UW) not only prevented a $[Ca^{2+}]_i$ increase, but also led to a massive, time-dependent $[Ca^{2+}]_i$ loss during cold hypoxia. In contrast, the Ca^{2+}-containing KH solution led to a massive, time-dependent $[Ca^{2+}]_i$ increase during cold hypoxia. This $[Ca^{2+}]_i$ change indicates an unregulated Ca^{2+} flux, which is dependent on the Ca^{2+} gradient. The gradient between high extracellular calcium concentration and the 10,000-fold lower intracellular calcium concentration is maintained by the low permeability to Ca^{2+} of the plasma membrane and by an active calcium transport system which requires energy. The energy loss caused by ATP-depletion under hypoxia probably led to this passive, unregulated Ca^{2+} flux. This would be in agreement with other workgroups [8, 11], who showed that the addition of Ca^{2+} antagonists, such as verapamil, nifedipine, nisoldipine or diltiazem, could not prevent the increase in $[Ca^{2+}]_i$ under cold hypoxic conditions. An additional explanation by Gasbarrini and co-workers [9] for this passive Ca^{2+} influx under cold hypoxia in KH is activation of the Na^+-Ca^{2+} antiporter in the reverse mode evoked by a low Na^+ potential caused by the increased intracellular Na^+. In solutions without Ca^{2+} and low Na^+, as in standard clinical preservation solutions, the mechanisms are vice versa and the Ca^{2+} efflux, also from intracellular stores, continues while the Ca^{2+} influx is abolished.

The role of intracellular calcium concentrations under cold hypoxia and their influence on cell damage remains controversial [4, 5, 25, 27]. Many hydrolytic enzymes, especially the phospholipase A2, one of those principally responsible for cell damage, are activated by $[Ca^{2+}]_i$ increase but inactivated by low pH_i. For prevention of $[Ca^{2+}]_i$ the common preservation solutions are Ca^{2+}-free, but questions arise concerning the necessity for this, or whether it is even beneficial. Our experiments demonstrated that cells stored in HTK and KH showed nearly the same survival rates despite their discrepant $[Ca^{2+}]_i$ during preservation. This may indicate that the cell injury caused by $[Ca^{2+}]_i$ increase and the resulting activation of hydrolytic enzymes are inhibited in their activity. It is well known that low temperature, ATP loss and pHi decrease during cold hypoxia also prevent activity of the described Ca^{2+}-activated enzymes, and in the case of KH-stored cells the negative effect of the $[Ca^{2+}]_i$ increase seemed to be neutralised in the process.

Furthermore, the cell survival did not suggest any correlation between $[Ca^{2+}]_i$ increase or decrease under cold hypoxia or after re-oxygenation. Both UW and EC solutions are nearly Ca^{2+} free but induce very different degrees of preservation effectiveness. UW-stored cells showed the highest cell survival rate, in great contrast to EC-stored cells with an extremely high cell death rate. Concerning the cell survival rate, similar results were observed by Viebahn et al. [30, 31]. The quality of UW for preservation is well known and is attributed to its osmotic benefits and its particular protective substances.

The deficiency of EC storage in hepatocytes is attributed in part to its high glucose content. Because glucose is the preferred substrate for anaerobic glycolysis the increased lactate production leads to an increase of intracellular H^+ concentration [14].

Studies in our laboratory showed that, especially, the intracellular pH stability under cold hypoxia was associated with high cell survival and that extreme pH loss led to extreme cell death [33]. In contrast, the wide range of $[Ca^{2+}]_i$ under cold hypoxia measured in our experiments did not influence cell survival under transplant conditions.

Despite our experimental results we should not neglect the fact that some workgroups reported better results in preservation solutions containing 0.2–0.8 mM Ca^{2+} [17, 29]. They explained that the protective effect of the extracellular calcium content is related to suppression of lipid peroxidation caused by a reduction of glutathione depletion during cold preservation [29]. Furthermore McAnulty [17] postulated the preservation of mitochondrial function from the use of perfusate with 0.5 mM Ca^{2+}. In conclusion, our data indicate a rather minor role for the $[Ca^{2+}]_i$ change under cold hypoxia in the pathomechanisms of cellular injury under transplant conditions. This new application of the hepatocyte model enables us to create primary experiments and, furthermore, to reduce unnecessary animal experiments. Nevertheless, we have to validate these primary results with an animal model, i.e. orthotopic rat liver transplantation.

References

1. Ar'Rajab A, Ahren B, Bengmark S (1991) Improved liver preservation for transplantation due to calcium channel blockade. Transplantation 51:965–967
2. Bissell DM, Hammaker LE, Meyer UA (1973) Parenchymal cells from adult rat liver in nonproliferating monolayer culture. I. Functional studies. J Cell Biol 59:722–734
3. Brecht M, Groot de H (1994) Veränderung der cytosolischen Ca^{2+}-, Mg^{2+}-, H^+-, Na^+- und K^+-Konzentrationen in kultivierten Hepatozyten unter hypoxischen Bedingungen. Zentralbl Chir 119:341–346
4. Cho PW, Miescher EA, Clemens MG (1990) Calcium-free reperfusion prevents mitochondrial calcium accumulation but exacerbates injury. Circ Shock 32:43–53
5. Cohen AJ, Burczynski FJ, Rosser BG, Lipschitz J, Minuk GY (2000) The effects of various organ preservation solutions on hepatocyte membrane potentials, intracellular calcium concentrations, and outcome following liver transplantation. Am J Surg 179:154–160
6. Fitzpatrick DB, Karmazyn M (1984) Comparative effects of calcium channel blocking agents and varying extracellular calcium concentration on hypoxia/reoxygenation and ischemia/reperfusion-induced cardiac injury. J Pharmacol Exp Ther 228:761–768
7. Gasbarrini A, Borle AB, Farghali H, Caraceni P, Fagiuoli S, Azzarone A, Starzl TE, Van Thiel DH (1992) Relationship between intracellular adenosine triphosphate, cytosolic-free calcium and cytotoxicity in hepatocytes exposed to anoxia/reoxygenation. Transplant Proc 24:2814–2815
8. Gasbarrini A, Borle AB, Van Thiel DH (1993) Ca^{2+} antagonist do not protect isolated perfused rat hepatocytes from anoxic injury. Biochim Biophys Acta 1177:1–7
9. Gasbarrini A, Caraceni P, Farghali H, Van Thiel DH, Borle AB (1994) Effects of high and low pH on $Ca^{2+}{}_i$ and on cell injury evoked by anoxia in perfused rat hepatocytes. Biochim Biophys Acta 1220:277–285
10. Grynkiewicz G, Poenie M, Tsien RY (1985) A new generation of Ca^{2+} indicators with greatly improved fluorescence properties. J Biol Chem 260:3440–3450
11. Haddad P, Cabrillac JC, Piche D, Musallam L, Huet PM (1999) Changes in intracellular calcium induced by acute hypothermia in parenchymal, endothelial, and Kupffer cells of the rat liver. Cryobiology 39:69–79
12. Herman B, Gores GJ, Nieminen A-L, Kawanishi T, Harman A, Lemaster JJ (1990) Calcium and pH in anoxic and toxic injury. Toxicology 21/2:127–148
13. Jewell SA, Bellomo G, Thor H, Orrenius S (1982) Bleb formation in hepatocytes during drug metabolism is caused by disturbances in thiol and calcium ion homeostasis. Science 217:1257–1258
14. Katz J, McGarry JD (1984) The glucose paradox. Is glucose a substrate for liver metabolism? J Clin Invest 74:1901–1909
15. Kawanishi T, Blank LM, Harootunian AT, Smith MT, Tsien RY (1989) Ca^{2+} oscillations induced by hormonal stimulation of individual Fura-2-loaded hepatocytes. J Biol Chem 264:12859–12866
16. Lemasters JJ, Thurman RG (1997) Reperfusion injury after liver preservation for transplantation. Annu Rev Pharmacol Toxicol 37:327–338
17. McAnulty JF, Ploeg RJ, Southard JH, Belzer FO (1989) Successful five-day perfusion preservation of the canine kidney. Transplantation 47:37–41
18. Murata M, Monden M, Umeshita K, Nakano H, Kanai T, Gotoh M, Mori T (1994) Role of intracellular calcium in superoxide-induced hepatocyte injury. Hepatology 19:1223–1228
19. Nieminen AL, Gores GJ, Wray BE, Tanaka Y, Herman B, Lemasters JJ (1988) Calcium dependence of bleb formation and cell death in hepatocytes. Cell Calcium 9:237–246
20. Nishikawa Y, Ukida M, Matsuro R, Omori N, Tsuji T (1994) Ca^{2+} influx initiates death of hepatocytes injured by activation of complement. Liver 14:200–205
21. Opie LH (1989) Reperfusion injury and its pharmacologic modification. Circulation 80:1049–1062
22. Orrenius S, Thor H, Rajs J, Berggren M (1976) Isolated rat hepatocytes as an experimental tool in the study of cell injury. Effect of anoxia. Forensic Sci 8:255–263

23. Pastorino JG, Snyder JW, Hoek JB, Farber JL (1995) Ca^{2+} depletion prevents anoxic death of hepatocytes by inhibiting mitochondrial permeability transition. Am J Physiol 268:C676–685
24. Renner EL, Lake JR, Scharschmidt BF, Zimmerli B, Meier PJ (1989) Rat hepatocytes exhibit basolateral Na^+/HCO_3^- cotransport. J Clin Invest 83:1225–1235
25. Schanne FAX, Kane AB, Young EE, Farber JL (1979) Calcium dependence of toxic cell death: a final common pathway. Science 206:700–702
26. Seglen PO (1973) Preparation of rat liver cells. Exp Cell Res 82:391–398
27. Smith MT, Thor H, Orrenius S (1981) Toxic injury to isolated hepatocytes is not dependent on extracellular calcium. Science 213:1257–1259
28. Trump BF, Berezesky IK (1995) Calcium-mediated cell injury and cell death. FASEB J 9:219–228
29. Umeshita K, Monden M, Fujimori T, Sakai H, Gotoh M, Okamura J, Mori T (1988) Extracellular calcium protects cultured rat hepatocytes from injury caused by hypothermic preservation. Cryobiology 25:102–109
30. Viebahn R, De Groot H, Lauchart W, Becker HD (1991) Primary hepatocyte cultures as a model of experimental study of liver preservation. Langenbecks Arch Chir 376:268–272
31. Viebahn R, De Groot H, Lauchart W, Becker HD (1991) Liver cell preservation studies using hepatocyte cultures. Experimental and clinical liver transplantation 111–118
32. Viebahn R, Metzdorf B, De Groot H, Lauchart W (1993) Stimulation of hypoxic and preservation injury in human hepatocyte cultures: influence of FK506 and cyclosporine. Transplant Proc 25:2691–2692
33. Weinlich M, Baumstark C, Viebahn R, Starlinger M (1995) Conservation of intracellular pH after cold ischemia in rat hepatocytes: effect of different preservation solutions. Gastroenterology 108:A1197