



Original Article

Subzero nonfreezing storage of rat hepatocytes using UW solution and 1,4-butanediol. II- functional testing on rewarming and gene expression of urea cycle enzymes

Edgardo E. Guibert;¹ Luciana L. Almada;² María E. Mamprin;² Cristina Bellarosa;³ María Dolores Pizarro;² Claudio Tiribelli;³ Joaquín V. Rodríguez²

Abstract

In the present study we have analyzed the viability and metabolic competence of isolated rat hepatocytes subjected first, to subzero nonfreezing storage (up to 120 h at -4 °C) in modified University of Wisconsin (UW) solution with 8% 1,4-butanediol, and then to a normothermic rewarming step (KHR media, 37 °C, up to 120 min, carbogen atmosphere). Results were compared with hepatocytes stored up to 120 h at 0°C in modified UW solution and with freshly isolated hepatic cells. We have found that only cell suspensions stored in subzero nonfreezing conditions were able to finish the rewarming period with a viability comparable with the control group. Also, we have investigated the enzyme activities and the relative expression at messenger RNAs levels of two of the Urea cycle (UC) enzymes: Carbamyl phosphate synthetase I (CPSI) and ornithine transcarbamylase (OTC), during 60 min of rewarming. Results were

compared with the ammonium removal efficiency of the three groups.

In conclusion: These data indicated that hepatocytes preserved under cold or subzero conditions up to 120 h followed by 60 min of rewarming, maintain UC enzymes at levels similar to freshly isolated hepatocytes, allowing their use in bioartificial liver devices.

Key words: Subzero cold storage, gene expression, urea cycle enzymes.

Introduction

In the last years, the demand of isolated hepatocytes for cellular transplant and bioartificial liver devices has increased. To meet these requirements, various preservation techniques have been investigated. Hypothermic cold preservation in University of Wisconsin (UW) solution¹ is a widespread and well-accepted method for maintaining isolated rat hepatocytes, but only for a period of time shorter than 72 h. Cryopreservation has been suggested as the best technique for long term storage of liver cells, however, cell recovery and viability post-preservation is insufficient.² We previously described a subzero nonfreezing storage protocol that uses UW solution and 8% (W/V) 1,4-butanediol (BDL) as cryoprotectant agent. With this method we could maintained viable and functional isolated rat hepatocytes at -4 °C for up to 120 h, without damage due to ice crystal formation.³

It is important to distinguish between responses which the cell establishes during cooling, and those which depend upon cold storage but can only be detected after return to normothermic temperatures during the rewarming process (KHR media, 37 °C, up to 120 min, carbogen atmosphere). Due to this fact it is necessary to evaluate the viability and metabolic functions of the cold subzero stored hepatocytes during this rewarming process.

The ammonium detoxification is a specific function of hepatocytes that has important influence on the prevention of hepatic encephalopathy in patients with acute or chronic liver failure.⁴ The urea cycle (UC) is the metabol-

¹ Biología Molecular.

² Farmacología, Facultad de Ciencias Bioquímica y Farmacéuticas, Universidad Nacional de Rosario, Argentina.

³ BBCM, Centro Studi Fegato.

University of Trieste, Trieste, Italy.

This work was funded by Agencia Nacional de Promoción Científica y Tecnológica (ANPCyT), PICT-03-14492, BID 1728 OC/AR (Argentina) and PIP5972 from CONICET, Argentina and Progetti Alta Rilevanza, Ministero degli Esteri, Italia, Prot. 269/P/0093044 and prot. 269/P-0114337.

This work was presented in part at the 2nd Workshop of Cryobiology in Medical Sciences, held May 15-17, 2007 in Rosario, Argentina.

Address for correspondence:

Edgardo E. Guibert. Biología Molecular, Dpto. Cs. Biológicas, Fac. de Ciencias Bioquímicas y Farmacéuticas, Universidad Nacional de Rosario. UNESCO Chair in Cryobiology Suipacha 570 (S2002LRK). Rosario, Argentina. Telephone: +54-341-4393400. E-mail: eguibert@fbioyf.unr.edu.ar

Manuscript received and accepted: August 22, 2008 and March 10, 2009.

ic pathway responsible for the ammonium removal. Carbamyl phosphate synthetase I (CPSI; EC 6.3.4.16) and Ornithine transcarbamylase (OTC) catalyze the first and second committed steps of waste nitrogen metabolism in the urea cycle, respectively. In a previous work,⁵ we have investigated the protein activities and the relative messenger RNAs expression levels of CPSI and OTC, in isolated hepatocytes preserved up to 120 h in UW solution at 0 °C, and during the rewarming step.

There are only few reports on the effects of cold storage on gene expression.⁶ In the present study we evaluate whether the subzero nonfreezing storage protocol developed in our laboratory could -1- To sensitize the hepatocytes viability and metabolic functions during the rewarming period, and/or -2- To modify the gene expression of the urea enzymes CPSI and OTC during this step. The Urea cycle enzymes were chosen due to its importance in the maintenance of a transcendental liver function, such as ammonia control and the fact that is a function that it is necessary to maintain in any artificial liver device.

Experimental procedures

Animals. Male Wistar rats weighing 250-300 g were used in all experiments. Rats were allowed free access to standard laboratory diet and water *ad libitum* prior to the experiment and received care in compliance with international regulations. The National Council Committee of Argentine approved animal protocols.

Hepatocyte isolation. Rat hepatocytes were isolated by collagenase perfusion as it was described previously.³⁻⁵ Cell viability was tested by the exclusion of 0.4% trypan blue (TBE) in phosphate-buffered saline. Preparations with a TBE greater than 85% were considered suitable for the experiments.

Hepatocyte cold storage and rewarming. Isolated hepatocytes were rinsed twice and resuspended in freshly prepared cold (0°C) modified UW solution. The composition of the modified UW solution was previously described.⁷ Hepatocytes (120.10⁶ cells in 40 mL UW solution) were allowed to settle to the bottom of a 50 mL screw cup polycarbonate tubes, and left undisturbed at 0°C up to 120 h. After that, the suspensions were washed twice with a rinse solution and sedimented (50 g, 3 min) in warm Krebs-Henseleit resuspension (KHR) media. The hepatocytes were subsequently incubated (120 min, 37 °C, 2-3×10⁶ cells/mL) in KHR media under carbogen atmosphere in a Dubnoff metabolic shaker.

Hepatocyte subzero nonfreezing preservation and rewarming. Freshly isolated hepatocytes were subzero nonfreezing preserved up to 120 h in modified UW solution with 8 % 1,4-BDL (120.10⁶ cells in 40 mL UW solution + 8% 1,4-BDL), as previously described.³ After that cell suspensions were warmed to 0°C and then were washed once with the rinse solution containing 4% of 1,4-BDL and later, twice with rinse solution with no ad-

ditive. Then, the hepatocytes were incubated (120 min, 37 °C, 2-3×10⁶ cells/mL) in KHR media under carbogen atmosphere in a Dubnoff metabolic shaker.

Experimental protocol. Hepatocytes from Wistar rats isolated by collagenase digestion were resuspended in UW solution and divided into the following two groups: 1- subzero nonfreezing stored group (Subzero Storage), and 2- cold stored group (Cold Storage). They were stored for up to 120 h at the temperatures indicated. After that, they were rewarmed for 120 min in KHS media. Suspensions of freshly isolated hepatocytes were used as control. Aliquots of the suspensions were removed at 0, 60, and 120 min to evaluate the viability and metabolic function.

Cell viability measurements

LDH retention. The capacity of the cells to retain macromolecules as Lactate Dehydrogenase (LDH) was determined by measuring the LDH activity in the cell suspension (total activity) and in the supernatant (extracellular LDH).^{8,9} Results were expressed as the percentage of LDH retention by the cells.

Cell viability % = 100 – [100 x (extracellular LDH / total activity)]

PI assay. The capacity of the cells to exclude the fluorescent marker propidium iodide (PI) was established as follows: the cells were incubated with the fluorescent marker and the fluorescence intensity is measured. As previously described,¹⁰ cell viability is expressed as the ratio between the fluorescence originated from non-viable cells that have membrane damage and the fluorescence originated from all cells in the sample.

Evolution of cellular metabolites during the rewarming period

Glutathione assay. Total glutathione-GSH plus GSSG-concentration (GSht) was determined by the enzyme coupled spectrophotometric assay of Tietze,¹¹ as previously described.⁷ Results were expressed as nmol of GSH/10⁶ cells.

ATP assay. ATP concentration was determined by an HPLC technique, as previously described.¹² Results were expressed as nmol of ATP/10⁶ cells.

Glycogen assay. Intracellular glycogen concentration was assessed by the amyloglucosidase enzyme assay, followed by an enzymatic determination of released glucose, technique described by Carr and Neff.¹³ Results were expressed as glycosidic units/10⁶ cells.

MTT assay. The tetrazolium dye 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) is widely used to assess the viability and/or the metabolic state of cells. This colorimetric assay is based on the conversion of the yellow tetrazolium bromide (MTT) to the red formazan derivative by mitochondrial succinate de-

hydrogenase in viable cells. MTT reduction capacity of the suspensions was assessed as was previously described.¹⁴ Results were expressed as OD/10⁶ cells.

Determination of the ammonium removal efficiency. After the preservation period, cells were rinsed and rewarmed in KHR in the presence of ammonium chloride overload (0.2 mM final concentration). Aliquots of the suspension were removed at 0 and 60 min of the rewarming period and centrifuged (13,400g, 15 s). The supernatants were kept under liquid N₂ until the enzymatic determination. Ammonium was determined enzymatically according to the method by van Anken et al.¹⁵ Ammonium removal efficiency (ARE) was calculated from the measured values of ammonium concentration as follows: ARE=[(C₀-C_t)/C₀] \times 100, where C₀ is the ammonium concentration of the medium at t=0, and C_t is the ammonium concentration of the medium after 60 min of incubation. A value of 100 represents the total load - ammonium removal efficiency.¹⁶

RNA isolation, reverse transcription, and quantitative PCR. Total RNA was extracted using the Tri Reagent™ (Sigma Chem. Co. S. Louis, USA) according to the manufacturer's instructions. Reverse transcription and quantitative PCR was made as previously described.³ Table I shows the primers designed for each gene expression analysis.

Determination of CPSI and OTC activities. Activities of CPSI were determined using a rapid colorimetric assay described by Pierson.¹⁷ OTC activity was measured as the rate of citrulline formation from ornithine and carbamyl phosphate.¹⁸

Statistical analysis. Statistical significance of the differences between values was assessed by analysis of variance (ANOVA) followed by Scheffe's multiple range

tests. A p value less than 0.05 were considered statistically significant.

Results

Evolution of cell viability during the rewarming period

Table II shows the cellular viability and the time course evolution of metabolites content after 120 min of the rewarming period, determined in freshly isolated and cold stored hepatocytes. We have found that at the end of rewarming period, cells suspensions subjected to subzero nonfreezing storage showed viabilities and metabolic functions comparable to freshly isolated hepatocytes. It is important to point out that, despite LDH and PI tests are based both on permeability properties of the plasma membrane; in all cases the viability levels estimated by LDH are greater than those valued from PI. We previously reported that this phenomenon could be due to the loss of extracellular LDH activity during the isolation and purification of the cell suspension.⁸

Regarding the glycogen content, we observed that freshly isolated hepatocytes began the rewarming period with the highest values (Controls: 2.19 \pm 0.52, Subzero Stored: 0.15 \pm 0.52, and Cold Stored: 0.09 \pm 0.03 glycosidic units/10⁶ cells). At the end of 90 min. of rewarming step, almost all the glycogen was consumed. However, the control group is the most glycogen consuming group; this behavior could be associated with a greater metabolic capacity of this suspension during the rewarming period.

Gene expression and enzyme activity during rewarming period

When freshly isolated hepatocytes were rewarmed during 60 min, a diminution of approximately 50 % in CPSI transcript levels was observed. No differences were found for OTC gene (Figure 1A). These results suggest that the relative expression level of CPSI gene is susceptible to the normothermic reoxygenation period.

No changes were found between control and cold

Table I. Primers utilized for gene expression analysis.

CPSI:	sense, 5'-ATC TGA GGA AGG AGC TGT CT-3'
	antisense, 5'- AAA ACC ACT TGT CAA TGG AT -3
OTC:	sense, 5'-ATG ACA GAT GCA GTG TTA GC-3'
	antisense, 5'-CAG GAT CTG GAT AGG ATG AT-3'
β-ACTIN:	sense, 5'-CAC TAT CGG CAA TGA GCG GT -3'
	antisense, 5'-ATT TGC GGT GCA CGA TGG A -3'

Table II. Cellular viability at the end of the rewarming step. Cells were stored and rewarmed as was described in Material and Methods. The cell viability was assessed by LDH, PI, GSH, ATP, MTT and ARE tests. Values are expressed as means \pm SD of samples obtained from four preparations.

	Control	Subzero preservation	Col preservation
LDH retention (%)	93.9 \pm 1.6	83.4 \pm 5.5 *	73.7 \pm 5.3 *
PI assay (%)	70.0 \pm 6.0	59.7 \pm 4.8	55.9 \pm 10.9
GSH content (nmol/10 ⁶ cells)	30.3 \pm 4.1	23.8 \pm 4.7	17.1 \pm 1.4 * #
ATP content (nmol/10 ⁶ cells)	7.7 \pm 1.6	5.0 \pm 1.1	2.0 \pm 0.7 * #
MTT assay (OD/10 ⁶ cells)	13.7 \pm 3.7	10.5 \pm 2.5	5.6 \pm 1.3 +
ARE (%)	75.8 \pm 15.5	87.7 \pm 9.6	33.9 \pm 10.4 +

* Statistically different from Control,

Statistically different from Subzero Stored group,

+ Statistically different from the other groups.

preservation groups, neither for $t = 0$ min nor for $t = 60$ min (Figures 1A and C). On the other hand, OTC activity was increased after 60 min of rewarming in cells that were cold preserved 120 h (Figure 1D). After 60 min of rewarming, we found differences in CPSI activity only between the control group and cell suspensions preserved in subzero conditions (Figure 1B). Despite the differences found, after 60 min of rewarming the ARE of preserved hepatocytes did not show statistical differences between them, and in comparison with control group (Controls: ARE = $41.8 \pm 8.7\%$; Subzero Stored: ARE = $52.9 \pm 18.2\%$; Cold Stored: ARE = $27.27 \pm 7.88\%$, $n = 4$). These results show that after 120 h of preservation and 60 min of normothermic rewarming, cells preserved in both conditions maintain a capacity to detoxify ammonia similar to control hepatocytes.

Discussion

Hypothermia decreases the rate of cellular energy-requiring and degradative reactions and is considered to play an important role during prolonged period of ischemia. However, the optimal level of hypothermia during hepatic preservation is controversial. Thus, as it was demonstrated in the joined paper¹⁹ subzero non-freezing temperatures could be reached with the addition of cryoprotectives agents, in this case with 1,4-butanediol.

The objective of the present study was to investigate the hypothesis that cold preservation or subzero nonfreezing storage of hepatocytes maintain the hepatocytes viability and metabolic functions during the rewarming period, and/or and if it is able to maintain the gene expression of the urea enzymes CPSI and OTC during this step.

The obtained results showed: Subzero nonfreezing storage conditions presents some incapacity to retain LDH content as compared with control studies. However, the entire metabolic and functional test (GSH, ATP and GLN content, MTT assay and the capacity to eliminate an overload of NH_4^+) and showed similar behaviour between controls and preserved under nonfreezing conditions. Meanwhile, "conventional" hypothermic preservation showed a wide range of results, meaning that in those conditions (120 h), we are in the limits of preservation time.

Another point that we wanted to probe in this manuscripts was if subzero nonfreezing preservation affects gene expression at mRNA level and the respective proteins activities. To probe this we choose the Urea cycle, the metabolic pathway responsible for the ammonium removal. Carbamyl phosphate synthetase I (CPSI) and Ornithine transcarbamylase (OTC) were analyzed at mRNA level and at protein level. Although hepatocytes suspension subjected to subzero nonfreezing preservation initiated the rewarming step with CPSI and OTC gene relative expression levels statistically reduced in comparison with the control group, they were able to finish this period with values comparable with freshly isolated cells.

Over the years it has been used multiple technologies to provide insights into freeze-responsive gene and protein expression by different animal species, like in example: wood frogs. Initial studies searched for freeze or thaw responsive proteins by using ^{35}S -methionine to label proteins either in vivo after intraperitoneal injection or during in vitro translation of mRNA isolated from control versus frozen frogs. Both studies illustrated freeze-thaw and organ-specific differences in protein biosynthe-

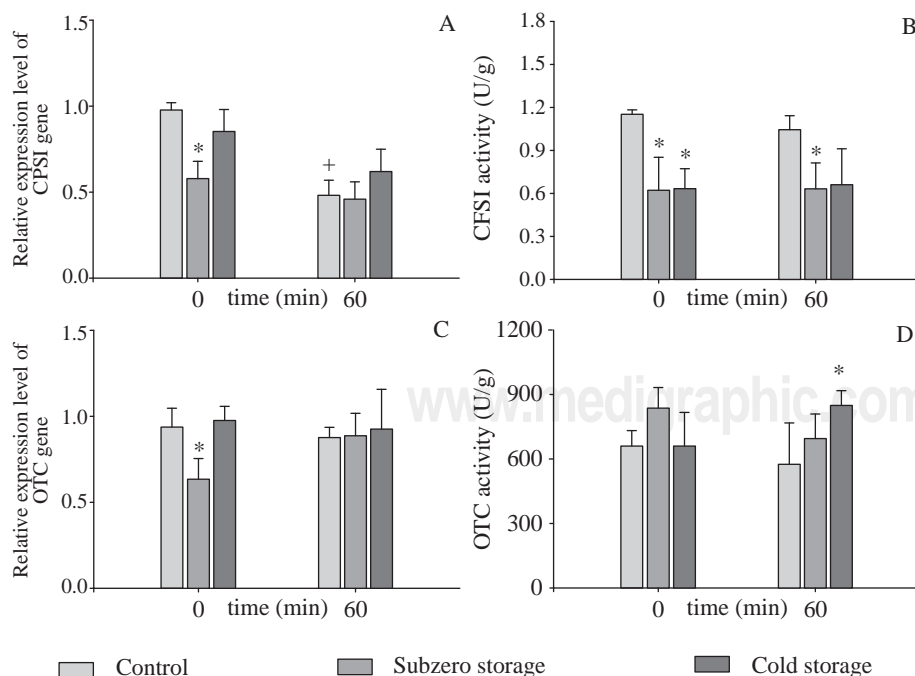


Figure 1. Relative gene expression levels and activities of CPSI (A and B) and OTC (C and D) during the rewarming step (0 and 60 min). The measurements were made in freshly isolated and subzero and cold stored hepatocytes (120 h). Data are expressed as means \pm SD for 3 hepatocyte preparations. * Statistically different from Control, + statistically different from $t = 0$ min

sis in wood frogs with prominent synthesis of some 15–20 kDa proteins, but both were limited the inability to identify individual proteins. Interestingly, major recent advances in proteomics technology including 2- dimensional electrophoresis coupled with LC-MS (liquid chromatography-mass spectrometry) of peptide fragments might now make a return to such studies fruitful.²⁰

The results presented in the current study suggests that the cold subzero stored hepatocytes during 120 h. are able to maintain the viability and metabolic functions after 120 min of rewarming. Also, these data indicates that the hepatocytes cold subzero stored and rewarmed, maintain UC enzymes at levels similar to freshly isolated hepatocytes.

References

1. Belzer FO, Southard JH. Principles of solid-organ preservation by cold storage. *Transplantation* 1988; 45: 673-76.
2. Rijntes PJ, Moshage HJ, Van Gemert PJ, De Waal R, Yap SH. Cryopreservation of adult human hepatocytes. The influence of deep freezing storage on the viability, cell sheeding, survival, fine structure and albumin synthesis in primary cultures. *J Hepatol* 1986; 3: 7-18.
3. Rodríguez JV, Almada L, Guibert EE, Mamprin ME, Scandizzi AL, Secchi M. *Preservación de hepatocitos aislados a temperatura subzero sin congelamiento*. Proceedings of the II Latin American Congress of Artificial Organs and Biomaterial, Belo Horizonte - Brazil, 5-8 December, 2001. M. Pinotti, L. Wykrota and L. Poletto Editors. ARG002.pdf.
4. Zamora-Valdés D, Guibert EE, Rodríguez JV, Uribe M, Méndez-Sánchez N. Hepatocyte transplantation for treating chronic hepatic dysfunction. *Journal of Organ Dysfunction* 2007; 3: 131-36.
5. Almada L, Bellarosa C, Giraudi P, Mamprín M, Mediavilla M, Guibert E, Tiribelli C, Rodríguez J. Gene expression and activity of urea cycle enzymes of rat hepatocytes cold stored up to 120h in University of Wisconsin solution. *Cryobiology* 2006; 52: 393-00.
6. Fuller BJ. Gene expression in response to low temperatures in mammalian cells: a review of current ideas. *CryoLetters* 2003; 24: 95-02.
7. Rodríguez JV, Mamprin ME, Mediavilla MG, Guibert EE. Glutathione movements during cold preservation of rat hepatocytes. *Cryobiology* 1998; 36: 236-44.
8. Mamprin ME, Rodríguez JV, Guibert EE. Glutathione (GSH) content during the rinse and rewarming process of rat hepatocytes preserved in University of Wisconsin solution (UW). *Cryobiology* 2000; 40: 270-76.
9. Mamprin ME, Rodríguez JV, Guibert EE. The importance of pH in resuspension media on viability of hepatocytes preserved in University of Wisconsin solution. *Cell Transplantation* 1995; 4: 269-74.
10. Giraudi PJ, Almada LL, Mamprin ME, Guibert EE, Furno G, Rodríguez JV. The assessment of viability in isolated rat hepatocytes subjected to cold or subzero non-freezing preservation protocols using a propidium iodide modified test. *Cryo-Letters* 2005; 26: 169-84.
11. Tietze F. Enzymatic method for quantitative determination of nanogram amounts of total and oxidized glutathione. Applications to mammalian blood and other tissues. *Anal Biochem* 1969; 27: 502-22.
12. Mamprin ME, Vega F, Rodríguez JV. Adenosine 5'triphosphate transport and accumulation during the cold preservation of rat hepatocytes in University of Wisconsin solution. *World J Gastroenterol* 2005; 11: 1957-64.
13. Carr RS, Neff JM. Quantitative semi-automated enzymatic assay for tissue glycogen. *Comp Biochem Physiol* 1984; 77: 447-49.
14. Hansen MB, Nielsen SE, Berg K. Re-examination and further development of precise and rapid dye method for measuring cell growth/cell kill. *J Immunol Methods* 1989; 119: 203-10.
15. Van Anken HC, Schiphorst ME. A kinetic determination of ammonia in plasma. *Clinica Chimica Acta* 1974; 56: 151-57.
16. Calligaris SD, Almada LL, Guibert EE, Tiribelli C, Rodríguez JV. Ammonium detoxifying activity is maintained after 72 hours of cold preservation of rat hepatocytes in University of Wisconsin (UW) solution. *CryoLetters* 2002; 23: 245-53.
17. Pierson DL. A rapid colorimetric assay for Carbamyl Phosphate Synthetase I. *Journal of Biochemical and Biophysical Methods* 1980; 3: 31-37.
18. Ceriotti G. Ornithine Carbamoyltransferase. In: *Methods in Enzymatic Analyse*. Bergmeyer H.U. Editors: Verlag Chemie, Weinheim/Bergsts, 1974, Second Edition. Vol 2, pp: 691-698.
19. Rodríguez JV, Almada L, Mamprin ME, Guibert EE, Furno G. Subzero nonfreezing storage of rat hepatocytes using modified University of Wisconsin solution (mUW) and 1,4-butanediol. I-effects on cellular metabolites during cold storage. *Ann Hepatol* 2009; 8(1): 57-62
20. Storey KB. Strategies for exploration of freeze responsive gene expression: advances in vertebrate freeze tolerance. *Cryobiology* 2004; 48: 134-145.