ANNALS OF HEPATOLOGY

Volume 4 *Number* 3 *July-September* 2005

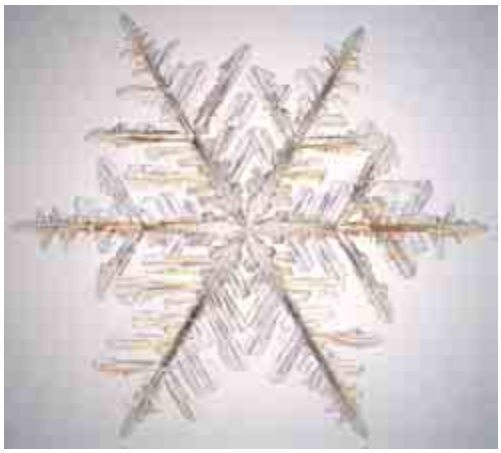
Artículo:

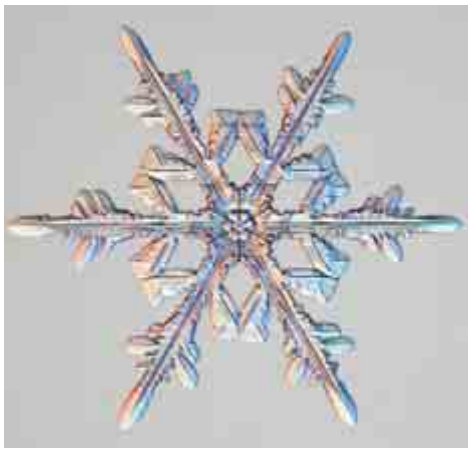
Abstracts of the first meeting on cryobiology

Annals of Hepatology 2005; 4(3): July-September: 210-229

Annals of Hepatology

UNESCO CHAIR IN CRYOBIOLOGY
LABORATORIO DE INVESTIGACIONES EN CRIOBIOLOGÍA
UNR ROSARIO ARGENTINA





The First Congress on Cryobiology in Medical Sciences in Latin America was held under the auspices of the UNESCO Chair in Cryobiology, Cathedral of National University of Rosario, Argentine on May 3'5th, 2005. The congress was organized by Professors Joaquin Rodriguez, Edgardo Guibert and María Mamprin from the joined Laboratory of Investigations in Cryobiological Sciences and with the major collaboration of Professor Eduardo Ceccarelli from the Institute of Molecular and Cellular Biology of Rosario. The meeting was held in the lecture rooms of the University with support from staff in the university. The importance of the occasion was confirmed by the presence of the Rector of UNR, Professor Ricardo Suarez, and the University Secretary of Science and Technology, Dr Cristina Vidal at the opening ceremony.

The aim of the Congress was to engender a multidisciplinary approach and understanding of fundamental aspects of cryobiology, which plays an increasingly important role in many areas of medicine and biotechnology. This aim was supported by the range of topics presented, which included basic principles of cryobiology, considerations of thermodynamic changes in the solidification of ice in biological samples, medical applications of low temperature storage of organs in the liquid state, cold adaptation responses in bacteria, and cryopreservation of cells. The participation was both national within the states of Argentina, and international with colleagues from Uruguay, Mexico and United Kingdom. The congress included a mini course on technical aspects of the use of cryogenics and automated cell cooling machines delivered by representatives from commercial organizations, who also contributed important financial support to the meeting. Registrants were provided with certification of attendance.

The responses from the attendees was very positive and all reported that they appreciated the multidisciplinary format and focus on technical perspectives. The continuing work of the UNR Cathedral of the UNESCO Chair in Cryobiology will be to develop an e-mail contact list and discussion forum to expand joint education and research in the field.

Also, the bases for the realization of the second workshop in Cryobiology Applied to Medical sciences were established during the conclusions of the meeting.

Organizers: *Dr. Edgardo Guibert*
Dr. Joaquin Rodríguez
Dra. María Mamprin
Dr. Eduardo Ceccarelli

Annals
of
Hepatology

Program

Tuesday, May 3, 2005

09:00-10:30 Inscription / Accreditations

10:30 **Opening Ceremony**

11:00-12:30 **Conference:** Dr. Barry J Fuller: " Introduction to Cryobiology and the UNESCO Chair".

12:30-14:30 Lunch break

14:30-15:30 **Conference:** Prof. Nahum Méndez-Sánchez: "Hepatocyte Transplantation for Acute and Chronic Liver Diseases".

15:30-16:00 Interval

16:00-17:00 **Conference:** Dr. Barry J. Fuller: "Liver preservation at the Royal Free Hospital. Clinical practice and current research projects".

20:00 Cocktail

Wednesday, May 4

09:30 **Simposium**: Criopreservation of tissues. Methods to evaluate viability post-preservation.
Moderator: Dra. María E. Mamprin
* Ing. Blas Melissari: Biomechanical assays to evaluate cripreserved tissues.
* Dr. Joaquin V. Rodriguez: ¿What is the meaning of cellular viability?
* Dr. Norberto Baumgartner: Viability Studies in Cryopreserved Human Heart Valves.

11:30 **Conference:** Dra. Cecilia Mansilla: Molecular Mechanisms of Low Temperature Sensing in Bacteria

12:45 Lunch Break

14:30 **Oral presentations**

1. S-Nitrosoglutathione Added To The University Of Wisconsin Solution Prevents Morphological Alteration On Rat Livers. Alejandra Quintana, Joaquín Rodriguez y Edgardo Guibert.
2. Polarographic Measurement Of Oxygen Content In Cold Storage Solutions For Isolated Cells. María Soledad Llarrull, Angel Scandizzi, Edgardo Guibert and Joaquin Rodríguez.
3. The Urea Cycle Enzymes Activity And Its Gene Expression In Rat Hepatocytes Are Not Affected By Cold Storage In University Of Wisconsin Solution. Luciana Almada, Cristina Bellarosa, Pablo Giraudi, María Mamprín, María Mediavilla, Edgardo Guibert, Claudio Tiribelli and Joaquín Rodríguez.
4. The Heme Oxygenase System And Organ Preservation : Studies Towards Protection In A Kidney Model. Barry J Fuller, Liz Balogun, Colin Green and Roberto Motterlini.

16:00-17:00 **Conference:** Ing. Blas Melissari: Thermodynamic Analysis Of The Solidification Processes For Cryopreservation Of Biological Tissues.

17:00-18:00 **Conference:** Dr. Jorge Genovese: "Stem cells, cellular therapy and tissue engineering".

18:30-19:30 **Open questions to experts**: Dr. Barry Fuller, Prof. Nahum Méndez-Sánchez, Ing. Blas Melissari, Dr. Edgardo E Guibert, Dr. Norberto Baumgartner, Dra. Cecilia Mansilla, Dra. María E. Mamprin and Dr. Joaquín V. Rodríguez.

Thursday, May 5
Minicourses

09:30 Why to use Apple in science?. Mariano Turinetto, Presentation of Toolkit-Apple, Argentina.

10:15 Cryopreservation of biological samples. Gonzalo Briner, Microlat Argentina.

11:00 How to handle cryogenics materials. Bioch. Guillermo Quiroga, L'Air Liquid, Rosario, Argentine.

12:00 Lunch Break

14:30 Validation of cryogenics filtres. Dr. Nelson Sturz, AGA, Rosario, Argentine.

16:00 Finals Conclusion.

21:00 Closing Ceremony and Dinner.

Annals of Hepatology 2005; 4(3): July-September: 212-220

Annals of Hepatology

CONFERENCES

An Introduction to Cryobiology and the UNESCO Chair in Cryobiology

Barry J. Fuller PhD; DSc[1]
[1] Royal Free & University College Medical School. London NW3 2QG, UK

Cryobiology is the science of effects of low temperatures on living cells, tissues and organisms. Although the study of cold in biology and medicine has a long history, the modern science, which is covered by the term 'cryobiology', has only been in existence for some 50 years. Methods of storage of living cells in a state of 'suspended animation' outside the body has become a key-stone practice in the development of many modern clinical therapies, including organ and tissue transplantation, infertility treatment and oncology. During this time, some of the underlying principles of the quantitative molecular and biophysical aspects of cryobiology have been clarified. In the applied and clinical sciences, low temperatures have been used across two broad categories, either as hypothermic storage in the liquid state, or as cryopreservation at deep sub-zero temperatures. During hypothermic storage, the main aim has been to try and counteract the biochemical changes and loss f homeostasis in cells which have been removed from the body and cooled. In such situations, hypoxia is a frequent additional, but not mandatory, factor. The multiple linked processes which control the intracellular environment and energy balance become disrupted, leading to an accumulation of intracellular changes which eventually become irreparable. The main thrust of work in this area has been the development of preservation solutions providing supportive balances of ions, osmotic agents, buffers and cryoprotective factors which can prolong viability in the cold.

Cryopreservation requires overcoming additional problems as the ice transition temperature is passed. Water is the universal biocompatible solvent but also possesses unique properties for stability of living cells. The phase transition of water to ice is the most profound challenge for cell survival. The thermodynamics of dilute aqueous solutions dictate how cells and tissues respond to the freezing process. Current concepts of nucleation, ice crystal growth and solute exclusion from the ice lattice will be discussed to illustrate what cells must negotiate to avoid lethal damage, and the role of cryoprotectants (the essential biocompatible 'antifreezes') in enhancing recovery. Models exist to predict how water and solutes move across cell membranes before and during freezing, or how nucleation events will proceed. Cryoprotectants have both positive and negative effects on cell function depending on the kinetics of exposure, the osmotic stresses involved in permeation into and out of the intracellular compartment, and potential chemical toxicities. The concept of tolerable osmotic excursion of cell volume will be discussed, along with the evidence for a 'pseudo-glassy' state for cells during traditional cryopreservation. This will be compared to the recent interest in promoting glassy states in the whole sample using high cryoprotectant concentrations and vitrification protocols, outlining the advantages and drawbacks of each approach. Additional methods for controlling ice nucleation have a role to play here, and a brief outline of current technologies will be given. Finally, issues of safety and stability of cryopreserved samples will be discussed.

Alongside the expanding global applications of cryobiology, it has been recognized that continuing research and training in low temperature sciences need to be maintained at a high scientific level and in a format available to all countries. For this reason, UNESCO choose to establish a Chair in Cryobiology at the Institute for Cryobiology and Cryomedicine in Kharkov, Ukraine. During the soviet era, this became the largest such Institute in the world. The basic aims of the UNESCO Chair have been to establish a centre of knowledge in the field, to promote scientific exchanges between scientists working on the problem, and assist postgraduate training in cryobiology.

Hepatocyte transplantation for acute and chronic liver diseases

Nahum Méndez-Sánchez;[1] Norberto C. Chávez-Tapia;[1] Misael Uribe[1]
[1] Departments of Biomedical Research, Gastroenterology & Liver Unit, Medica Sur Clinic & Foundation, Puente de Piedra 150, Col. Toriello Guerra, Mexico City, Mexico. Phone: (+525) 55606-6222, ext. 4215 Fax: (+525) 55666-4031 and 55606-1651; E-mail: nmendez@medicasur.org.mx

Abstract

Over three decades of research in experimental animals and several clinical trials have brought us to the threshold of hepatocyte transplantation for the treatment of acute and chronic liver failure, and inherited metabolic disorders. However, more extensive clinical studies and routine clinical application are hampered by the shortage of good quality of donor cells. To overcome these hurdles, current research has fo-

cused on the search for alternatives to adult primary hepatocytes, such as liver cell progenitors, fetal hepatoblasts, embryonic, bone marrow or umbilical cord blood stem cells and conditionally immortalized hepatocytes. Cross-species hepatocyte transplantation is also being explored. It is hoped that ongoing research will permit the application of hepatocyte transplantation to the treatment of a wide array of liver diseases.

Key words: hepatocyte, transplantation, liver failure, liver diseases

Epidemiology

In 1998, chronic liver disease (CLD) was classified as the tenth most frequent cause of death in the United States according to the national vital statistics report.[1] Excessive alcohol intake and viral hepatitis infections are thought to be two important causes.

In Mexico, in 2000, chronic liver disease was the fourth leading cause of death. More importantly, it was the second leading cause of death in people aged between 35 and 55 years.[2,4] Furthermore the trends in mortality rates for liver cirrhosis between 1955 and 1990 have been analyzed for 38 countries (two from North America, six from Latin America, five from Asia, 23 from Europe, and Australia and New Zealand) on the basis of official death certification data derived from the World Health Organization database. Chile and Mexico had exceedingly high rates (around 60/100,000 males and 15/100,000 females in the late 1980s), while in Canada, the United States, and Latin American countries that provided data, cirrhosis death rates were between 5 and 17/100,000 males and 3 and 5/100,000 females over the same calendar period. The pattern of trends was, however, similar in all American countries, with some increase between the 1950s and the 1970s, and declines thereafter. A similar trend was observed in Japanese males, whose rate was 13.6 in 1990. Conversely, cirrhosis mortality declined steadily from 8.0 to 4.6 in Japanese females. Appreciable downward trends were observed in Hong Kong and Singapore, whereas mortality increased in Thailand. In Europe, in the late 1950s, the highest rates were registered in Portugal (33.6/100,000 males and 14.6/100,000 females), followed by France (31.8/100,000 males and 14.1/100,000 females), Austria, Italy, Spain, and Germany. Most of these countries, however, after some further rise up to the 1970s, showed reversal of the trends over most recent years. Thus, in the late 1980s or early 1990s, only Austria, Italy, and Portugal had cirrhosis mortality around 30/100,000 males and 10/100,000 females. Britain, Ireland, and Nordic countries started from much lower values (2 to 4/100,000 males), but showed some, although discontinuous, upward trend.[5]

Physiology and Clinical Aspects

Liver is an important organ with complex functions, including gluconeogenesis, synthesis of blood proteins, amino acid metabolism, urea synthesis, lipid metabolism, drug detoxification, waste removal, and immune and hormonal modulation.[6]

Many people suffer from liver diseases, especially severe hepatitis, most of the cases give rise to widespread hepatic necrosis with little hepatocyte regeneration. Currently, the only available treatment is liver transplantation. However, liver transplantation faces acute shortages of donors worldwide and the patients treated with liver transplantation are subjected to the lifetime risks of graft rejection and immunosuppression.[7,8] The liver has a remarkable capacity for regeneration. But a minimum critical mass of hepatocyte is required to support homeostasis while regeneration progresses after liver damage. Without this critical mass, liver failure supervenes and regeneration is impaired.[9]

Hepatocyte transplantation

In view of this, many investigators have evaluated transplantation of isolated liver cells as a less invasive alternative to whole organ transplantation or as a “bridge” while awaiting the availability of a donor liver.[10] In contrast to intact livers, hepatocytes could be cryopreserved for immediate availability in emergencies.[11] Since the recipient liver remains intact, the metabolic risk of transplant rejection is minimized and the possibility of subsequent orthotopic liver transplantation or liver-directed gene therapy remains open. This minimally invasive procedure requires minimal or no hospitalization, which should lower the cost of the procedure and permit earlier treatment of inherited or acquired liver disorders, thereby reducing complications of the diseases. Studies on laboratory animals over the last three decades and recent clinical trials indicate the usefulness of liver cell transplantation in the treatment of metabolic liver diseases and as a bridge for patients with liver failure awaiting transplantation. Safety and feasibility of this approach have been demonstrated. However, widespread application of liver cell transplantation has been tantalizingly slow, principally because of the shortage of usable primary human hepatocytes, which is, at this time, even more severe than the shortage of transplantable organs. It is anticipated, therefore, that in the coming years, investigators will focus on identifying alternatives to adult primary hepatocytes for transplantation and methods for inducing selective proliferation of the transplanted cells. A brief discussion of the current issues in liver cell transplantation follows.

Clinical studies

Acute liver failure: It has been reported clinical study in patients with acute liver failure showed that injection

of human fetal liver cells into the peritoneal cavity resulted in a small but statistically significant improvement in overall survival, compared with age-matched controls, particularly in patients with grade 3 hepatic coma.[12] In later studies, hepatocyte transplantation was used primarily to "bridge" patients with acute liver failure awaiting the availability of a donor liver.[13,14] In general, 10^7 to 10^{10} allogeneic hepatocytes from adult cadaver livers were infused into the splenic artery or the portal vein. There are isolated reports of improvement in serum ammonia levels, prothrombin time, level of encephalopathy, cerebral perfusion pressure and cardiovascular stability. Complications included sepsis and hepatocyte embolization into the pulmonary circulation, and transient, reversible hemodynamic instability.[15]

Chronic liver failure: In most patients with liver cirrhosis, the cirrhotic nodules contain hepatocytes in large enough numbers that could have been expected to support metabolism at a relatively normal level. However, hepatocytes present in cirrhotic nodules are dysfunctional because of abnormalities of the hepatic architecture. Based on this concept, investigators have transplanted hepatocytes recovered from segments of the cirrhotic livers of patients and transplanted them by injection into the splenic pulp, splenic artery, splenic vein or portal vein.[15,16] Although the injections were tolerated well and there was some evidence of improvement in encephalopathy, protein synthesis and renal function, the ultimate clinical outcome was not altered significantly. In retrospect, the results were not surprising because most of these patients had received hepatocyte transplantation through the splenic artery.[17]

Liver-based inherited metabolic diseases: As discussed above, attempts to treat familial hypercholesterolemia (LDL receptor deficiency) by ex vivo gene therapy did not result in therapeutically significant reduction of serum cholesterol levels.[18] However, these studies demonstrated the safety and feasibiliry of hepatocyte transplantation. Subsequently, other investigators have transplanted allogeneic hepatocytes into the liver bed to correct ornithine transcarbamylase (OTC) deficiency, alpha-1-antitrypsin deficiency, glycogen storage disease type Ia, infantile Refsum disease and Crigler-Najjar syndrome type 1.[19-24] Hepatocyte transplantation resulted in transient correction of hepatic OTC deficiency.[21,22] Long-term improvement in glucose metabolism was reported after hepatocyte transplantation in an adult patient with glycogen storage disease type Ia.[23] Direct evidence of survival and function of transplanted human hepatocytes was obtained in a 10 year old patient with Crigler-Najjar syndrome type I (UGT1A1 deficiency), in whom serum bilirubin levels were reduced to 50% of pretransplant levels, and 5% of hepatic UGT1A1 activity was reconstituted following a single session of hepatocyte transplantation. However, the metabolic correction was not sufficient to eliminate the need for phototherapy. Therefore, although the bile contained bilirubin glucuronides two and a half years after hepatocyte transplantation, indicating persistence of the transplanted hepatocytes, the patient ultimately underwent successful auxiliary liver transplantation.[20] Recently, a 4 year old patient with infantile Refsum disease received hepatocyte transplantation, which led to partial clearance of abnormal bile acids; with pipecholic acid being reduced to 60% of pre-transplantation levels. The child was able to stand and walk 6 months after hepatocyte transplantation.[24]

Future Research

Since primary hepatocytes from adult human liver cannot be expanded greatly in culture without genetic modification, reseach has focused on the use of fetal hepatoblast/hepatocytes, liver stem/progenitor cells isolated from adult liver, embryonic or umbilical cord blood stem cells and hepatocytes conditionally immortalized by gene transfer. Studies are also underway to explore xenogenic hepatocytes for transplantation. Although concerns about hyperacute xenograft rejection have not been addressed fully, current data indicate that cirrhotic animals may tolerate xenogenic hepatocytes. Providing proliferative advantage to transplanted cells by manipulations of the host liver is an active area of current research. Since long-term immunosuppression is associated with significant risk of toxic injury, genetic manipulation of donor hepatocytes to induce immune ignorance in the host or tolerance to allogeneic or xenogenic hepatocytes is another area of active research.

References

1. Martin JA, Smith BL, Mathews TJ, Ventura SJ. Births and deaths: preliminary data for 1998. In: *National Center for Health Statistics*. National Vital Statistics Report. Vol. 47, No. 25. Hyattsville, MD: National Center for Health Statistics, 1998.
2. Secretaría de Salud M. *Base de datos de información sobre mortalidad* in http://www.salud.gob.mx/apps/htdocs/estadisticas/publicaciones/Mortalidad2000.zip. Access: 3th january 2005.
3. Méndez-Sánchez N, Aguilar-Ramirez JR, Reyes A, et al. Etiology of liver cirrhosis in Mexico. *Ann Hepatol* 2004 Jan-Mar; 3: 30-3.
4. Méndez-Sánchez N, Villa AR, Chavez-Tapia NC, Ponciano-Rodriguez G, Almeda-Valdes P, Gonzalez D, Uribe M. Trends in liver disease prevalence in Mexico from 2005 to 2050 through mortality data. *Ann Hepatol* 2005; 4: 52-55.
5. La Vecchia C, Levi F, Lucchini F, Franceschi S, Negri E. Worldwide patterns and trends in mortality from liver cirrhosis, 1955 to 1990. *Ann Epidemiol* 1994; 4: 480-6.
6. Lee SW, Wang X, Chowdhury NR, Roy-Chowdhury J. Hepatocyte transplantation: state of the art and strategies for overcoming existing hurdles. *Ann Hepatol* 2004; 3: 48-53.
7. Shaw BW Jr. Auxiliary liver transplantation for acute liver failure. *Liver Transpl Surg* 1995; 1: 194-200.
8. Everhart JE, Lombardero M, Detre KM, Zetterman PK, Wiesner RH, Lake JR. Increased waiting time for liver trans-

plantation results in higher mortality. *Transplantation* 1997; 64: 1300-1306.
9. Court FG, Wemyss-Holden SA, Dennison AR, Maddern GJ. Bioartificial liver support devices: historical perspectives. *ANZ J Surg* 2003; 73: 739-748.
10. United Network for Organ Sharing *(http://www.unos.org)*.
11. Moshage HJ, Rijntjes PJ, Hafkenscheid JC, Roelofs HM, Yap SH. Primary culture of cryopreserved adult human hepatocytes on homologous extracellular matrix and the influence of monocytic products on albumin synthesis. *J Hepatol* 1988, 7: 34-44.
12. Habibullah CM, Syed IH, Qamar A, Taher-Uz Z. Human fetal hepatocyte transplantation in patients with fulminant hepatic failure. *Transplantation* 1994; 58: 951-952.
13. Strom SC, Fisher RA, Thompson MT, Sanyal AJ, Cole PE, Ham JM, Posner MP. Hepatocyte transplantation as a bridge to orthotopic liver transplantation in terminal liver failure. Transplantation 1997; 63: 559-569.
14. Bilir BM, Guinette D, Karrer F, Kumpe DA, Krysl J, Stephens J, et al. Hepatocyte transplantation in acute liver failure. *Liver Transplantation* 2000; 6: 32-40.
15. Mito M, Kusano M. Hepatocyte transplantation in man. *Cell Transplantation* 1993, 2: 65-74.
16. Mito M, Kusano M, Ohnishi T, Saito T, Ebata H. Hepatocellular transplantation. *Gastroenterol Jpn* 1978; 13: 480-490.
17. Strom SC, Roy Chowdhury J, Fox IJ. Hepatocyte transplantation for the treatment of human disease. *Semin Liver Dis* 1999; 19: 39-48.
18. Grossman M, Raper SE, Kozarsky K, Stein EA, Engelhardt JF, Muller D, et al. Successful ex vivo gene therapy directed to liver in a patient with familial hypercholesterolemia. *Nat Genet* 1994; 6: 335-341.
19. Strom SC, Roy Chowdhury J, Fox IJ. Hepatocyte transplantation for the treatment of human disease. *Semin Liver Dis* 1999; 19: 39-48.
20. Fox IJ, Roy Chowdhury JR, Kaufmann SS, Goertzen TC, Chowdhury NR, Warkentin PI, et al. Treatment of Crigler-Najjar syndrome type 1 with hepatocyte transplantation. *N Eng J Med* 1998; 338: 1422-1426.
21. Horslen SP, McCowan TC, Goertzen TC, Warkentin PI, Cai HB, Strom SC, Fox IJ. Isolated hepatocyte transplantation in an infant with a severe urea cycle disorder. *Pediatrics* 2003; 111(6 Pt 1): 1262-7.
22. Reyes J, Rubenstein WS, Mieles L, Strom SC, Towbin RB, Trucco M, et al. The use of cultured hepatocyte infusion via the portal vein for the treatment of ornithine transcarbamoylase deficiency by transplantation of enzymatically competent ABO/Rh- matched cells [abstract]. *Hepatology* 1996; 24: 308A.
23. Muraca M, Gerunda G, Neri D, Vilei MT, Granato A, Feltracco P, et al. Hepatocyte transplantation as a treatment for glycogen storage disease type 1a. *Lancet* 2002; 359(9303): 317-318.
24. Sokal EM, Smets F, Bourgois A, Van Maldergem L, Buts JP, Reding R, et al. Hepatocyte transplantation in a 4-year-old girl with peroxisomal biogenesis disease: technique, safety, and metabolic follow-up. *Transplantation* 2003; 76: 735-73.

Liver Preservation: Clinical and Research Aspects of the Royal Free Hospital Programme

Barry J. Fuller[1]
[1] University Department of Surgery & Liver Transplant Programme
Royal Free Hospital, London NW3 2QG, UK

Liver transplantation was first practiced in the United Kingdom in Cambridge and Kings College Hospital in the late 1970s. The Royal Free Hospital began a pilot programme in 1981. The technique moved forward to be accepted as a valid clinical procedure for end-stage liver disease in that decade, and the Royal Free programme was established as one of the UK supra-regional liver transplant units by 1987. There are currently 7 designated units across the UK, geographically located to cover the entire population.

Without the ability to harvest and preserve organs in a good functional state, organ transplantation would not be possible as the extensive activity seen to-day. The UK network (as in most other countries) has been established largely on donation from heart-beating, brain dead cadavers. Most organ donors are multi-organ donors, and techniques for simultaneous cold preservation of the important abdominal and thoracic organs have been developed. The service depends upon good ethical practices and equity of sharing of organs, which are organized and monitored centrally through UK Transplant and serviced by a network of donor and recipient co-ordinators. Each of the 7 centres acts as a multi-organ donor harvest team, providing full cover at any time. The current techniques, based on *in situ* cold flush and preservation with University of Wisconsin solution are similar in all the centres. Organ distribution is directed by a combination of patient need, best match, and cold ischaemic time. If there are patients in fulminant hepatic failure, they can be registered as 'super-urgent' through UK Transplant, and take priority.

Routine cold ischaemic times in the clinical programme are 12 – 14h, and organ preservation generally remains an important area for further research. Some prospective trials on modifications of the clinical protocol have been made, which will be discussed. Descriptions will also be given on current research on preservation and hypoxic injury within the transplant programme.

Molecular Mechanisms of Low Temperature Sensing in Bacteria

María C. Mansilla;[1] Daniela Albanesi;[1] Larisa E. Cybulski;[1] Diego de Mendoza[1]
[1] Institute of Molecular and Cellular Biology of Rosario, CONICET-National University of Rosario. Suipacha 531. S2002LKR Rosario, Argentina

Abstract

Both prokaryotes and eukaryotes respond to a decrease in temperature with the expression of a specific subset of proteins. We are investigating how Bacillus subtilis cells sense and transduce low-temperature signals to adjust its gene expression. One important step has been accomplished in the dissection of a novel pathway for the adjustment of unsaturated fatty acid synthesis in B. subtilis, termed the Des pathway. It responds to a decrease in growth temperature by enhancing the expression of the des gene, coding for an acyl-lipid desaturase. The Des pathway is uniquely and stringently regulated by a two-component system composed of a membrane-associated kinase, DesK, and a soluble transcriptional activator, DesR. The temperature sensing ability of the DesK protein is regulated by the extent of disorder within the membrane lipid bilayer. In this work, we present the mechanism by which the sensor protein DesK controls the signal decay of its cognate partner, DesR, and how this response regulator activates transcription of its target promoter. The results of these analysis will be presented and discussed in the context of transcriptional regulation of membrane fluidity homeostasis.

Key words: Cold sensor, membrane lipid fluidity, signal transduction, acyl-lipid desaturase.

Introduction

Poikilothermic organisms are exposed to frequent changes in thermal environmental conditions and their survival depends on their ability to acclimate to such changes. As the selective barrier between living cells and their environment, the plasma membrane plays a key role in cell viability. It has been established that normal cell function requires membrane lipid bilayers that are largely fluid at physiological temperatures. However, at lower temperatures, membrane lipid bilayers undergo a reversible change of state from a fluid (disordered), to a nonfluid (ordered) array of the fatty acyl chains. The temperature at the midpoint of this transition, called the transition temperature, is a function of membrane lipid composition and, in organisms deficient in cholesterol, mainly depends on the fatty acid composition of the membrane lipids. The mechanism of regulation in all examined cases seems to occur via the incorporation of proportionally more unsaturated fatty acids (UFAs) (or other low-melting point fatty acids) as the temperature decreases. This means that cells must process temperature signals to adjust enzyme activities or to activate unique genes necessaries to adapt the membranes to the new temperature. The question arises, how do cells sense a change in temperature and adjust the fluidity of the membrane lipid bilayer accordingly?

Although a large body of information concerning cold-shock induced genes has been gathered, studies on temperature regulation have not yet clearly identified the key regulatory factor(s) responsible for thermosensing and signal transduction at low temperatures. We will discuss the basic features of thermal regulation of membrane lipid fluidity in *Bacillus subtilis*, one of the Nature´s best studied organisms, that in recent years has become the principal paradigm for studies of the cold-shock response in gram-positive bacteria.

Results and discussion

Bacillus cells respond to a decrease in ambient growth temperature by increasing the proportion of low-melting-point fatty acids of membrane lipids. This can be accomplished by increasing the proportion of anteiso-branched fatty acids or by desaturating the fatty acids. To explore the molecular mechanism of cold-induction of UFAs synthesis and how a change in growth temperature regulates the expression of the *Bacillus* desaturase, our research group decided to study this phenomena in *B. subtilis*, which is an excellent experimental model because its general experimental tractability.

B. subtilis contains a sole desaturase, encoded by the *des* gene.[1] The *B. subtilis* desaturase (Δ5-Des) catalyzes the introduction of a *cis*-double bond at the Δ5 position of a wide range of saturated fatty acids.[2] Δ5-Des is a polytopic membrane-bound desaturase containing a tripartite motif of His, essential for the catalysis, located on the cytoplasmic side of the membrane.[3] In *B. subtilis* the transcription of *des* gene increases in response to a decrease in temperature.[4] Targeted mutagenesis led to the identification of a histidine kinase (DesK) and a response regulator (DesR) that are involved in the increased expression of *des* in response to low temperatures.[5] The *B. subtilis* DesK protein features a highly hydrophobic N-terminal segment, that define the sensor domain, and a long cytoplasmic C-terminal tail harbouring the histidine predicted to be the site of autophosphorylation (His 188). *In vitro* experiments showed that the purified C-terminal domain

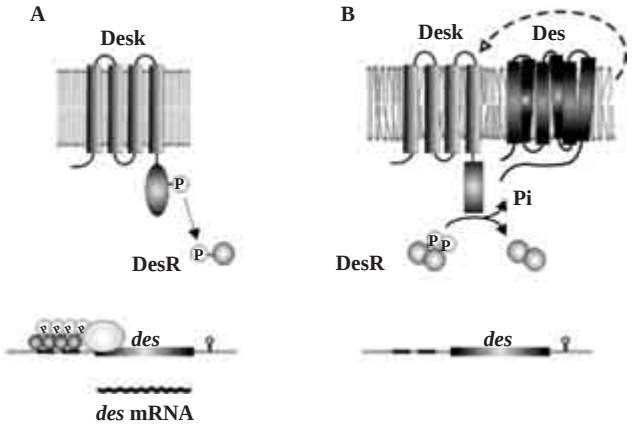


Figure 1. Model for the signal transduction pathway leading to membrane fluidity optimization in *B. subtilis*.
(A) A kinase dominant state of DesK predominates upon an increase in the proportion of ordered membrane lipids. DesR-P interacts with the *des* promoter and RNA polymerase, resulting in transcriptional activation of *des*. (B) Δ5-Des desaturates the acyl chains of membrane phospholipids. The decrease in membrane lipids order favors the phosphatase-dominant state of DesK. DesR dephosphorylation of results in decreased transcription of the *des* gene.

of DesK (DesKC) undergoes autophosphorylation in the presence of ATP.[6] Autophosphorylated DesKC transfers the phosphoryl group to the effector protein DesR (DesR-P), and also posses phosphatase activitiy on DesR-P.[6] However, DesKC does not function as a phosphatase *in vivo*, implying that the truncated protein might be locked in a kinase-dominant state.[6] Thus, the transmembrane segments of DesK are essential to sense changes in membrane fluidity and for regulating the ratio of kinase to phosphatase activities of the cytoplasmic C-terminal domain. Phosphorylation of the regulatory domain of dimeric DesR promotes, in a cooperative fashion, the hierarchical occupation of two adjacent, non identical, DesR-P DNA binding sites, so that there is a shift in the equilibrium toward the tetrameric active form of the response regulator.[7] This results in the recruitment of RNA polymerase to the *des* promoter and activation of *des* transcription, as demonstrated by *in vitro* transcription experiments.[7] Limiting the supply of isoleucine dramatically reduces the amount of anteiso-branched-chain fatty acids of plasma membrane lipids, resulting in decreased membrane fluidity. Growth of cells in the absence of isoleucine results in activation of *des* transcription at 37ºC using a DesK/DesR-dependent mechanism.[8] Thus, a decrease in the content of membrane isoleucine-derived fatty acids at constant temperature mimics a drop in growth temperature, and both stimuli can induce UFAs synthesis.

We propose a model to explain the signal transduction pathway controlling the low-temperature induction of Δ5-Des *(Figure 1)*. We envisage that one or more of the transmembrane segments of DesK could sense a change in the ordering of the acyl chains of membrane phospholipids and transmit this information to the cytoplasmic domain of the sensor kinase. This would result in adjustment of the ratio of kinase to phosphatase activities of this bifunctional enzime. When lipids are ordered DesK autophosphorylates, and subsequently the phosphoryl group is transferred to the cytoplasmic response regulator DesR *(Figure 1A)*. Phosphorylation of DesR promotes binding of the DesR-P dimers to P*des* and this favours DesR-P tetramerization. Tetrameric DesR-P would occupy a site centered 52 bp upstream of the *des* transcription start point, allowing DesR-P to interact specifically with RNA polymerase, to turn on *des* transcription. Transcription of *des* results in the synthesis of Δ5-Des, which introduces double bonds in the acyl chains of membrane lipids *(Figure 1B)*. These newly synthesized UFAs decrease the phase transition temperature of the phospholipids, favouring the phosphatase activity of DesK, resulting in hydrolysis of DesR-P. The unphosphorylated regulator is unable to bind to P*des* and, as a consequence, *des* transcription is turned off. This metabolic pathway, termed the Des pathway, therefore generates a regulatory loop that optimizes membrane fluidity.

References

1. Aguilar PS, Cronan JE Jr, de Mendoza D. A *Bacillus subtilis* gene induced by cold shock encodes a membrane phospholipid desaturase. *J Bacteriol* 1998; 180: 2194-2200.
2. Altabe SG, Aguilar PS, Caballero GM, de Mendoza D. The *Bacillus subtilis* acyl lipid desaturase is a Δ5 desaturase. *J Bacteriol* 2003; 185: 3228-31.
3. Díaz AR, Mansilla MC, Vila AJ, de Mendoza D. Membrane topology of the acyl-lipid desaturase from *Bacillus subtilis*. *J Biol Chem* 2002; 277: 48099-106.
4. Aguilar PS, López P, de Mendoza D. Transcriptional control of the low-temperature-inducible *des* gene, encoding the Δ5 desaturase of *Bacillus subtilis.* J Bacteriol 1999; 181: 7028-7033.
5. Aguilar PS, Hernandez-Arriaga AM, Cybulski LE, Erazo AC, de Mendoza D. Molecular basis of thermosensing: a two-component signal transduction thermometer in *Bacillus subtilis*. *EMBO J* 2001; 20: 1681-1691.
6. Albanesi D, Mansilla MC, de Mendoza D. The membrane fluidity sensor DesK of *Bacillus subtilis* controls the signal decay of its cognate response regulator. *J Bacteriol* 2004; 186: 2655-2663.
7. Cybulski LE, del Solar G, Craig PO, Espinosa M, de Mendoza D. *Bacillus subtilis* DesR functions as a phosphorylation-activated switch to control membrane lipid fluidity. *J Biol Chem* 2004; 279: 39340-7.
8. Cybulski LE, Albanesi D, Mansilla MC, Altabe S, Aguilar PS, de Mendoza D. Mechanism of membrane fluidity optimization: isothermal control of the *Bacillus subtilis* acyl-lipid desaturase. *Mol Microbiol* 2002; 45: 1379-1388.

Biomechanical testing of cryopreserved tissues

Blas Melissari Costa
Laboratorio de Biomecánica. Instituto de Ensayo de Materiales. Facultad de Ingeniería de la Universidad de la República. Montevideo. Uruguay

The Laboratory of Biomechanics of the Testing of Materials Institute of the Uruguayan Engineering School has ongoing biomechanical research for the last 25 years. First about fixators employed in osteosynthesis and now also on the characterization of biological tissues.
A multidisciplinary group with physicians, chemists and statistical and mechanical engineers was integrated for that purpose.
Research of biological tissues is carried out together with the National Organs and Tissues Bank.
All materials are provided from cadaver donors.
The objective is the biomechanical evaluation of tissues to be used as allografts and the improvement of preservation methods.
Elastic properties are determined for example in compression, tensile and bending tests.
Sample extraction and preparation, equipments, testing procedures and some results for tendons and bones are detailed.
Evaluation of fresh and cryopreserved vascular tissues is described and conclusions about their biomechanical difference between them are drawn.

Key words: Cryopreservation, Biomechanics, Testing, Vascular tissues.

Thermodynamic analysis of the solidification processes for cryopreservation of biological tissues

Blas Melissari Costa
Laboratorio de Biomecánica. Instituto de Ensayo de Materiales. Facultad de Ingeniería de la Universidad de la República. Montevideo. Uruguay

Nucleation of a cooling liquid occurs when atomic aggregates reach critical size that assures decreasing free energy as the crystal grows. Thermodynamic analysis allows to understand the ways to control the process. Evaluation of crystallized fraction in a freezing process is described as an example of biomedical application. For that purpose, 4 cm^3 samples with different mixtures of RPMI 1640 and DMSO cryopreservant, were frozen in a programmed cooling chamber at a cooling rate of 1ºC/min. The temperatures of the chamber and the probe were plotted against time. Characteristic curves with different crystallization zones were obtained.
The area of that zone beyond the base line determines the Relative Crystallization Index (RCI) of the solution. The results were compared with usual methods of the National Organs and Tissues Bank of Uruguay. With 10 % DMSO and VALPA 799 Program, the BNOT has obtained good quality cryopreserved cardiac valves and elastic arteries.

Key words: Cryopreservation, Solidification, Crystallization.

Viability Studies in Cryopreserved Human Heart Valves

Norberto Baumgartner[1]
[1] Valvular and Vascular Heart Banking. ¨San Juan de Dios¨ Hospital. 27 and 70 street. (1900) La Plata City. Argentina.

Introduction

Grafts derived form humans are called homografts. Valvular and vascular homograft use began in 1962 for surgical purposes, with the experiences of Donald Ross.[1] Surgical treatment of endocarditis was benefited by the homograft use and progressively,[2,3] it started to take a predominant place in the surgical selection of valvular prosthesis, with a wider spectrum of indications that reserved in early stages. This modification in the selection was based on the change in the processing and valvular homograft cryopreservation techniques.

Until 1976, fresh valvular homografts were kept at 4°C. Later, O'Brien implemented cryopreservation tech-

niques to control freezing, together with sterilization procedures with antibiotics.[4-7]

Duration was measured by the freedom from reoperation. This factor limited the indication of fresh homografts and scientist preferred mechanical prosthesis or bioprosthesis. However, long-term monitoring experiences of cryopreservated valvular homograft recently published/showed very encouraging results, such as freedom from reoperation in 10 years in almost 100% of the population.[3,4] This was a new stimulus for the use of homologue materials whose benefits were based on the achievement of a higher index of endocarditis cure, freedom from endocarditis –with respect to other prosthesis-, on better hemodynamic results (lower transvascular gradient) and freedom from anticoagulation and freedom for reoperation. The socio-economic situation in the developing countries with a population with lack of periodic medical control, anticoagulation, dental vigilance constitute another aspect to select the valvular homografts. In the valvular homograft donor selection, there exists a disparity of criteria as regards the acceptance according to the age groups for the different banks or international associations of tissue banks.

The quality of the valvular homografts observed during the processing, dissection and cryopreservation and for its later surgical use, could be related to other variables of cardiovascular epidemiological significance which would determine the subcategorization risk of the donors to be evaluated and viability testing would define their aptitude for medical use.

Surgical use of homografts valves

Indications for use include replacement of the aortic, pulmonary, mitral and tricuspid valve, and others.[2,4] Advantages of homografts use for cardiac valve replacement include low risk of thromboembolism, low transvalvular gradient, absence of haemolysis, lack ofring/cuff of graft support, very good haemodynamic performance and higher resistance to endocarditis compared to all the other valves.[2,3,7,8,11]

Late morbidity with the use of homografts as opposed to mechanical prosthetic valves in a 4 year follow-up study, thromboembolic events and valve endocarditis were more frequent in patients with mechanical valves[2]. The disadvantages are a more demanding surgical technique, progressive degeneration of the homograft, and the limited capacity of the homografts banks.[11] The tissue engineered valves could be an alternative solution and may improve the long term results with homografts valves.

Viability of the homografts

Many factors can influence the durability of a homograft valves as age donors, weight donor, etc.. Heart donors show a much higher level of viability than non-beating-heart donors.[12]

Leaflet cellularity may be influencing in the viability at the time of collection.

A longer exposure time with the antibiotic solution for sterilization produce a significantly lower viability.[12,13]

The homografts valves viable at the time of cryopreservation have a much lower level of structural deterioration than non-viable valves.

The cryopreservation involves a complex process by the preservation of biological systems at low temperatures. The most important advantage over other preservation methods is the inhibitory effects on the chemical and physical processes which allow for longer storage time.

During freezing processing the cells and tissue matrix can be injured by the formation of the ice in the extracellular environment and inside the cell, and by tissue damage in the matrix, but it is mostly related to structural damage.

Maintenance of structural and mechanical characteristics of tissue is necessary for appropriate performance of the heart valves.

To realize successful cryopreservation, it depends of the optimal freezing and the use of cryoprotectants. The cryoprotectants are substances that give protection in the intra or extracellular environment. In the heart valves cryopreservation we utilize Dimethylsulfoxide (Me_2SO). Dimethylsulfoxide can avoid the amount of ice formed and dehydration during freezing processing.[12]

Viability of the heart valves

Viability is a complex process of cells preservation, structural and mechanical properties following storage. Long term survival of the heart valves it depend.

Viability tests are a series of biological measurements that can be performed and characterize the in vivo cellular and structural function.[12-14]

The viability tests proposed are:

Cell membrane Integrity

- Light Microscopy
- Fluorescence Microscopy: acridin orange –AO- and propidium iodide –PI-
- Electron Microscopy .
- Cell proliferation.

Measurements of metabolism

- tritiated (^3H) proline, collagenase activity (electrophoresis).

Mechanical evaluation

- longitudinal traction tests

Perform a viability assay protocol is very important for evaluate viability in the laboratory vs. long term survival of the homografts heart valves determined by clinical studies.

The viability tests is a group of studies that allow to evaluate the quality processes in the tissue banking activities and warrant the improve results of the heart valves cryopreserved.

References

1. Ross D. N. Replacement of the aortic valve. *Lancet* 1962; 2: 487.
2. McGiffin D, Galbraith AJ, O'Brien M, Geoffrey J, McLachlan, Naftel D, Adams P, Reddy S, Lesley E. An analysis of valve re-remplacement after aortic valve replacement with biologic devices. *J Thorac Cardiovasc Surg* 1997; 113: 311-8.
3. Vaturi M, Aravot D, Ben-Gal T, Shapira Y, Adler Y, Sahar G, Vidne B, Sagie A. Natural history of left-sided valves after heart trasplantation. *Transplantation Proceedings* 2000; 32: 735-736.
4. Ross DN. Replacement of aortic and mitral valves with a pulmonary autograft. *Lancet* 1969; 2: 956.
5. O'Brien MF, Stafford EG, Gardner MAH, Pohlner PG, Mc Giffin DC. A comparison of aortic valve replacement with viable cryopreserved and fresh allograft valves, with a note on chromosomal studies. *J Thoracic Cardiovasc Surg* 1987; 94: 812-823.
6. Pomar JL, Martinez A, Agusti E, Mestres CA. Nuevas Técnicas de Preservación y una mayor logística consolidan los homoinjertos valvulares. Organització Catalana de Trasplantements. Servei Catalá de la Salut. Societat Catalana de Trasplantement. *Revista Trasplantement* 1997; 1: 12-14.
7. Aguirre A, Baumgartner N, Guerrero E, Mengassini M, Menna M, Soratti C. Eficacia de las técnicas de esterilización con antibióticos en el procesamiento de tejidos valvulares. *X Revista Enfermedades infecciosas y microbiología* 2001; 21: Supl.
8. Langley Stephen M, McGuirk SP, Chaudrhry MA, Livesey SA, Ross JK, Monro JL. Twenty-year follow-up of aortic valve replacement with sterilized homografts in 200 patients. *J Thoracic Cardiovasc Surg* 1999; Suppl 1: 28-34.
9. Maselli D, Pizio R, Lantieri PB, Di Bella I, Gasperis C. Left ventricular mass reduction after aortic valve replacement: Homografts, Stentless and Stented valves. Ann Thorac Surg 1999; 67: 966-71.
10. Niwaya K, Knott Craig CJ, Santangelo K, Lane MM, Chandrasekaran K, Elkin RC. Advantage of autograft and homograft valve replacement for complex aortic valve endocarditis. *Ann Thorac Surg* 1999; 67: 1603-8.
11. Favaloro R, Alvarez M, Stutzbach P. Banco de Homoinjertos de la Fundación Favaloro, 7 años de trabajo. Revista Novedades. Fundación Favaloro; *Trim* 2001; 4: 10-14.
12. Strong M. Cryopreservation of cells and tissues. In: Radiation and tissue banking. G.O. Phillps et al, eds. World Scientific, Singapore, pp 103-4.
13. Arminger Lois C. Viability studies of human valves prepared for use as allografts. *Ann Thorac Surg* 1995; 60: S118-21.
14. Fischlein T, Schutz A, Haushofer M, et al. Immunologic reaction and viability of cryopreserved homografts. *Ann Thorac Surg* 1995; 60: S122-6.
15. Massahi K, Yukata M, Takashi T, Takaki H et al. Collagen syntesis and collagenase activity of the cryopreserved heart valves. *J Thorac Cradiovasc Surg* 2001; 122: 706-11.

Annals
of
Hepatology

ABSTRACTS

S-Nitrosoglutathione added to the University of Wisconsin solution prevents morphological alteration on rat livers

Alejandra Quintana;[1] Joaquín Rodriguez;[1] Edgardo Guibert[2]

[1] Departamento de Fisiología; [2] Departamento de Ciencias Biológicas.

Facultad de Ciencias Bioquímicas y Farmacéuticas, Universidad Nacional de Rosario, Suipacha 531-S2002LRK Rosario, Argentina

Abstract: Livers cold preserved (48 HS-0ºC) in University of Wisconsin solution (UW) suffered morphological injuries due to cold and ischemia. The most important alterations were: denudation of the sinusoidal lining cell; presence of blebs; loose of parenchymal cells glycogen content; disruption of collagen and reticulin networks. Reperfusion aggravated these damages by adding perivenous vacuolation. The addition of 100 µM S-nitrosoglutathion, e (GSNO) as a Nitric Oxide donor (NO), to UW solution before reperfusion time, prevented most of the morphological damages mentioned above. NO had apparently no effect on glycogen content storages while sinusoidal cells detachment, blebs and vacuolation diminished substantially. Collagen and reticulin networks appeared more organized. In conclusion, the addition of 100 µM GSNO to UW solution improves rat liver morphology during cold preservation/reperfusion.

Key words: UW solution; cold preservation; S-nitrosoglutathione; morphological injuries.

1. Introduction: Rat livers cold preserved in UW solution during variable time period, suffered severe morphological injuries that could alter hepatic function.[1] Reperfusion performed after preservation, aggravates the damages, mainly because of oxygen-derived free radicals generation.[2] One important effect of cold ischemia on livers is the denudation of the sinusoidal lining cells.[3] In such condition, swelling and disruption of the sinusoidal lining induced microcirculatory disturbances during reperfusion.[4] Microcirculatory blood flow is modulated by vasoactive substances, such as nitric oxide (NO) and endothelins.[1] S-nitrosoglutathione (GSNO) which is an S-nitrosothiol[5] is a NO donor. Since abnormalities in microcirculation could play a primary role in the pathogenesis of the graft nonfunction,[6] this could be prevented by adding vasodilators to the UW solution. Direct effect of cold preservation/reperfusion on liver can be studied with the Isolated Perfused Rat Liver model (IPRL). The purpose of this work was to study the potential benefit of GSNO, added to the UW solution during 48 hours of cold ischemia, to prevent morphological alterations, using the IPRL model.

2. Materials and methods: *Animals.* Adult male Wistar rats weighing 250-350 g were used in all experiments. Animals had free access to standard rat chow and tap water, and were not fasted before surgery. The experiments described in this report were conducted according to international regulations. *Chemicals*. S-nitroso-glutathione (GSNO) was prepared according to the method of Hart.[7,8]

Solutions. The composition of UW solution and perfusate Krebs-Henseleit - BSA used in this study were described previously.[9]

Hepatectomy and cold storage and Liver reperfusion. These techniques were described previously.9 *Experimental groups*. Five groups were compared in this study: **I**) normal controls, livers from Wistar rats neither preserved nor reperfused ($\mathbf{I}_{NC}$) (n = 6); **II**) controls of reperfusion, where hepatectomy was immediately followed by the organ perfusion during 60 min ($\mathbf{II}_{RC}$) (n = 6); **III**); livers maintained 48 hs in UW ($\mathbf{III}_{P48}$) (n = 6); IV) livers preserved 48 hs in UW + 100 µM GSNO ($\mathbf{IV}_{PGSNO100}$) (n= 6). Groups III and IV were reperfused in IPRL system 60 min.

Tissue Processing. Five liver biopsies were taken from each experimental group. Tissues were fixed in 4 % PBS buffered formalin (pH = 7,40) and embedded in paraffin. Sections were cut at 5 µm thick and stained appropriately for further analysis: **a**. parenchymal and nonparenchymal cell morphology with Hematoxylin and Eosin (H.E.). **b**. liver extracellular matrix observations with: **I**) conventional Picrosirius Red stain to study collagen network, **II**) Gordon-Sweets' Silver Impregnation Method to study reticulin network. **c**. glycongen content with Periodic Acid Schiff (PAS) reaction.

3. Results and discussion: Results are presented in the following figure.

Morphological alteration seen in groups $\mathbf{II}_{RC}$ and $\mathbf{III}_{P48}$ were: denudation of the sinusoidal lining cells; hepatocyte swelling; presence of blebs; loose of hepatocyte glycogen content; disruption of collagen and reticulin networks and perivenous vacuolation. The addition of 100 µM GSNO to UW solution before preservation, prevented most of the morphological damages mentioned above. NO had no effect on glycogen content while sinusoidal cells detachment, blebs and vacuolation, diminished substantially. Collagen and reticulin networks appeared more organized.

Hypothermia, necessary to slow down liver metabolic activities, produces histological damages on rat livers preserved (48 HS – 0ºC). After long terms of ischemic cold storage, reoxigenation during reperfusion causes an incre-

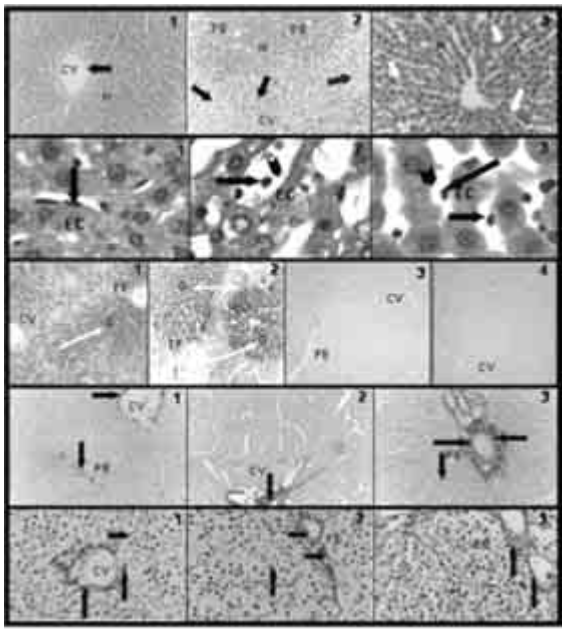


Figure A. H-E. 1) II_{RC}. Vacuolation (arrow); swollen hepatocyte (H); central vein (CV). **2) III_{P48}.** Vacuolation (arrows); swollen hepatocyte (H); portal spaces (PE). **3) $IV_{PGSNO100}$.** Edothelial cells inside sinusoids lumen (arrows); swollen hepatocytes (H). **Figure B. H-E. 1) I_{NC}.** Endothelial cells (EC) with normal shape (arrow). **2) III_{P48}.** Endothelial cell inside sinusoidal lumen (arrow) connected to extracellular matrix with a thin rest of citoplasm (arrow head), vacuolation (V). **3) $IV_{PGSNO100}$.** Swollen endothelial cells still attached to extracellular matrix (arrows).; bleb (arrow head). **Figure C. PAS. 1) I_{NC}.** Glycogen (G) is distributed within the hole parenchyma (arrow). **2) II_{RC}.** Glycogen (G) is distributed heterogeneously within the parenchyma (arrows). **3) III_{P48} and 4) $IV_{PGSNO100}$.** The parenchyma is depleted of glycogen. **Figure D. Picrosirius Red. 1) II_{RC}.** Disorganized collagen network (arrows)**. 2) III_{P48}.** Collagen network completed disorganized (arrow). **3) $IV_{PGSNO100}$.** Organized collagen network (arrows). **E) Gordon-Sweets. 1) II_{RC} and 2) $III_{P48.}$** Disorganized reticulin network (arrows). **3) $IV_{PGSNO100}$.** Organized and abundant fibers of reticulin (arrows).

ment of superoxide anion concentration producing superoxide-mediated cytotoxicity.[10] This could explain the extended vacuolated areas seen around central veins in group **III_{P48}**. To improve hepatic morphology during cold preservation, GSNO was added. According to the results, 100 µM GSNO proved to be effective preserving rat hepatic parenchyma since it reduced cell vacuolation and endothelial cell detachment. It also maintained collagen and reticulin networks organized with a good amount of fibers. However, it was not efficient to prevent glycongen content loose. The efficacy of 100 µM could be a consequence of a satisfactory NO concentration reached in UW solution that allowed an adequate intrahepatic vascular dilatation that reduced portal resistance during reperfusion. Changes in collagen and reticulin networks can be assigned to protease action during preservation. GSNO could prevent their activation and in this way, the components of extracellular matrix remained organized after reperfusion. In conclusion, 100 µM GSNO added to UW solution prevents most of the morphological injuries on rat livers cold preserved (48 HS – 0ºC) and then reperfused.

Acknowledgments

The authors acknowledge Histotechnologist Alejandra Inés Martinez for her excellent technical assistance. This work was supported by the Agencia Nacional de Promoción Científica y Tecnológica (ANPCyT), República Argentina. PICT-03-14492.

References

1. Koeppel TA, Gebhard MM, Otto, Post E. Inhibition of Nitric Oxide Synthesis in Ischaemia/Reperfusion of the Rat Liver is followed by Impairment of Hepatic Microvascular Blood Flow. *J Hepatol* l997; 27: 163-169.
2. Blankenstejn JD, Terpstra OT. Liver Preservation: The Past and the Future. *Hepatology* 1991; 13: 1235-1250.
3. Clavien PA. Sinusoidal Endothelial Cell Injury During Hepatic Preservation and Reperfusion. *Hepatology* 1998; 28(2): 281-285.
4. Geriach JC, Hentschel F, Spatkowski G, Zeilinger K, Smith MD, Neuhaus P. Cell Detachment During Sinusoidal Reperfusion After Liver Preservation: An *in vitro* Model. *Transplantation* 1997; 64: 907-912.
5. Kubes P, Granger DN. Nitric Oxide Modulates Microvascular Permeability. *Am J Physiol* 1992; 262: H611-H615.
6. Churchill TA, Green CJ, Fuller BJ. Protective Properties of Amino Acids in Liver Preservation: Effects of Glycine and a Combination of Amino Acids on Anaerobic Metabolism and Energetics. *J Hepatol* 1995; 23: 720-726.
7. Hart TW. Some Observation Concerning S-nitroso and S-phenylsufonyl Derivatives of L-cysteine and glutathione". *Tetrahedron Lett* l985; 26: 2013-2016.
8. Quintana AB, Joaquin V. Rodríguez, Angel L. Scandizzi, Edgardo E. Guibert. The benefit of adding Sodium Nitroprusside (NPNa) or S-nitrosoglutathion (GSNO) to the University of Wisconsin Solution (UW) to prevent morphological alterations durin cold preservation/reperfusion of rat livers. *Annals of Hepatology* 2003; 2(2): 84-91.
9. Rodriguez J, Guibert E, Quintana A, Scandizzi A, Almada L. The Role of Sodium Nitroprusside in the Improvement of Rat Liver Preservation in University of Wisconsin Solution. A Study in the Isolated Perfused Liver Model. *J Surg Res* 1999; 87: 201-208.
10. Huet PM, Kassissia I, Semret M. In: Ballet, F. and Thurman, R. G., Eds. Perfused liver. *Clinical and Basic Applications*. London: John Libbery 1991: 33-42.

Polarographic measurement of oxygen content in cold storage solutions for isolated cells

María Soledad Llarrull;[1] Angel Scandizzi;[1] Edgardo Guibert;[2] Joaquín Rodríguez[1]*

[1] *Farmacología,* [2] *Biología Molecular, Facultad de Ciencias Bioquímica y Farmacéuticas, Universidad Nacional de Rosario, Suipacha 531, S2002 LRK Rosario, Argentina, * E-mail: jrodrig@fbioyf.unr.edu.ar*

Abstract: A simple polarographic assay was used for the measurement of O_2 concentrations in University of Wisconsin solution (UW) at 5°C. This work describes the time course evolution of O_2 content in UW solution containing hepatocytes preserved up to 72 hours at 0°C. Our results show that during cell preservation in UW solution bubbled with N_2 gas anoxia is not achieved and, under the current procedures, the O_2 concentration adopts similar values whether UW solution is bubbled with air or N_2. Therefore, more stringent procedures are necessary to control the O_2 concentration during cold storage as this might affect the overall quality of preservation procedures.

Key words: Oxygen, cold preservation, UW, hepatocytes.

1. Introduction: Little is known about the effect of the oxygen content of cold storage solutions for cell preservation on the overall quality of preservation protocols. Oxygen polarography is the standard technique for measuring the O_2 content of biological media and many polarographic methods have been developed for the determination of O_2 concentrations. As the operating characteristics of most oxygen sensors depend markedly on temperature, it is advised to establish them at the onset of measurements. Therefore, the specific aims of this study were: 1- to study the operating characteristics of our polarographic oxygen sensor at low temperatures, 2- to find a suitable and accurate polarographic method for measuring O_2 concentrations at low temperatures, 3- to investigate the oxygen content changes in gassed preservation solutions (UW+N_2, UW+air) during cold storage and 4- to correlate this information with cell viability during the cold storage period.

2. Materials and methods: Experimental procedures. There were two parts of the study. The aims of part I were: 1-to characterize the sensor behavior at 5ºC by investigating the linearity and the rate of the response and 2- to find a suitable and accurate method for measuring O_2 concentrations at 5ºC. The purpose of part II was to study the oxygen content changes in gassed preservation solutions (UW+ N_2, UW+air) during cold storage. To do this, the following experiments were performed: a- 50 mL screw cup polycarbonate tubes containing UW solution equilibrated with N_2 or air were left at 0ºC up to 72 hs. b- Hepatocytes (30.10^6 cells in 10 mL UW solution equilibrated with N_2 or air) were allowed to settle to the bottom of the 50 mL screw cup polycarbonate tubes, and left undisturbed at 0ºC up to 72 hs. Daily aliquots of the solutions and suspensions were removed to evaluate the evolution of the oxygen content (in a and b) and viability (LDH release in b).

Instrumentation: The oxygen concentration was measured with YSI Model 5300 Biological Oxygen Monitor, (Yellow Springs, Ohio, USA) equipped with a Clark–type sensor (YSI 5331 oxygen probe, Yellow Springs, Ohio, USA) attached to a thermoregulated glass reaction vessel of 2.6 mL. The chamber was isolated from contact with the atmosphere by a close fitting cup that has a central hole for introducing samples. The oxygen signal was registered by a two-channel chart recorder (Rikadenki R-102, Rikadenki, Japan) on a paper running at 0.5 cm/min. The stirring rate was standard for all experiments (1000 rpm).

Part I. Sensor characterization at 5 ºC: A gas-phase calibration curve was constructed by filling the chamber with humidified gasses of different O_2 content. The gasses used were: pure N_2, a mixture with 0.08 % O_2, air and pure O_2. These values were corrected for the water content of each phase. The sensor response time was measured as follows: the sensor chamber was filled with air-saturated water, and the sensor signal recorded. Then sodium ditionite was added to the chamber and the O_2 consumption recorded. The resulting data was fit to the following exponential equation: $\mathbf{O_2(t) = O_2(0)\,[M_f\, e^{-1/\tau f} + M_s\, e^{-1/\tau s}]}$ and the time constants τ_f and τ_s were determined.

Polarographic measurements of O_2 concentrations: O_2 concentrations were measured by the physical method described by Rasmussen.[1] Our procedure was somewhat different. First, the sensor signal was calibrated as described.[2] The chamber was then filled with low-oxygen destilled water and once the signal stabilized, 300 µL of air-equilibrated water was injected to the chamber. The signal increment was recorded until stabilization at a new final value occurred. The procedure was repeated to complete a series of n additions, refilling the chamber with low-oxygen water before each addition. Finally, a series of additions of the test solution was carried out. Water was degassed by boiling, sonication and bubbling with N_2. Water and the test solution were equilibrated by bubbling with humidified air at the same temperature and barometric pressure. The oxygen concentration in the test solution was calculated as: $\mathbf{C_{O2}\,(\mu M.O) = (S_S / S_W)\,.\,S^o\,.\,(B\text{-}p_W)}$. The quantities $\mathbf{S_S}$ and $\mathbf{S_W}$ were calculated for each addition of test solution and water respectively according to: $\mathbf{S = S_I + (S_F - S_I)\,.\,V_C / V_A.\,S_I}$ is the signal value before the addition, $\mathbf{S_F}$: signal value after the addition, V_C: volume of the sensor chamber, $\mathbf{V_A}$: volume of the added aliquot, Sº: oxygen solubility in pure water (µM.O/kPa), **B**: barometric pressure and $\mathbf{p_W}$: water pressure at the operation temperature. $\mathbf{S^o}$ and $\mathbf{p_W}$ were obtained from Rasmussen.[1] The accuracy of this method was evaluated by measuring the oxygen content of three solutions A, B and E of known O_2 concentrations.[1]

Part II. Animals: Male Wistar rats weighing 250-300 g were used in all experiments. The rats were allowed access to a standard laboratory diet and water ad libitum freely prior to the experiment and received care in compliance with international regulations.

Hepatocyte Isolation. The method was described previously.[3]

Hepatocyte cold preservation. The method was described previously.[3] UW solution was equilibrated by bubbling with humidified gasses (N_2 and air) for 20 minutes.

Statistical Analysis. Results are presented as the mean ± SD and the number of sample additions and cell preparations analyzed are indicated in each case. Statistical significance of the differences was assessed by Student´s *t* test. A p value less than 0.05 was considered statistically significant (Statgraphics, Statistical Graphics System, USA).

3. Results and discussion: Sensor characterization at 5 ºC. By plotting the sensor output signal *vs* % O_2 of the gas phase a linear fitting was obtained (slope of 1.008 measured % O_2/actual % O_2; n = 5, r = 1), indicating that the electrode response was linear in the range from 0 % O_2 (0 mmHg) to 99.13 % O_2 (749.9 mmHg). The time constant τ_f was 5.46 ± 1.58 s (n = 5) and a 90 % response time of 14.54 ± 3.849 s was calculated.

Accuracy of the polarographic measurements of oxygen concentration using the modified physical method. *Table I.* compares O_2 concentrations in solutions A, B and E.

Table I. Comparison of the measured and published O_2 concentration in solutions A, B and E. (A) KCl (75 mM), phosphate (7.5 mM), $MgCl_2$ (5 mM), Hepes (25 mM), pH 7.4; (B) sucrose (150 mM), phosphate (7.5 mM), $MgCl_2$ (5 mM), Hepes (25 mM), pH 7.4; (E) mannitol (225 mM), sucrose (75 mM), Tris (20 mM), phosphate (10 mM), EDTA (0.5 mM), pH 7.35

Solution	T (ºC)	Measured O_2 concentration (μM.O)	Published O_2 concentration (μM.O)
A	23	511.4 ± 5.4 (5)	515.6 ± 1.2 (7)
B	23	511.4 ± 9.7 (5)	500.4 ± 1.2 (7)
E	25	447.3 ± 41.9 (5)	467.9 ± 1.2 (7)

The statistical analysis of these results indicates that there is no significant difference between the measured and known O_2 concentrations for solutions A and E. The difference is marginally significant for the case of solution B.

Time dependent changes in O_2 concentration in UW solution during hypothermic storage of rat hepatocytes. Figure 1 shows the evolution of the O_2 content in UW solution during 72 hs of cold storage of isolated hepatocytes at 0 ºC. Resuspension of freshly isolated hepatocytes in UW+N_2 lead to oxygenation of the media and the O_2 concentration in both cold storage media (UW+N_2 and UW+air) reached similar values during the preservation period. Based on these results, no differences in cell viability due to the O_2 content of preservation media should be expected. Accordingly, the LDH release did not present significant differences under both conditions. We may conclude that more stringent preservation conditions will be necessary in order to keep anoxia during cold storage and to test if the gas atmosphere of preservation solutions affects the quality of preservation.

Acknowledgements

This work was founded by Agencia Nacional de Promoción Científica y Tecnológica (ANPCyT), PICT-03-14492, BID 1201 OC/AR.

References

1. Rasmussen HN, Rasmussen UF. Oxygen solubilities of media used in electrochemical respiration measurements. *Analytical Biochemistry* 2003: 319: 105-113.
2. Walker D. The use of the oxygen electrode and fluorescence probes in simple measurements of photosynthesis. Robert Hill Institute, University of Sheffield, 1990.
3. Mamprin ME, Guibert EE, Rodriguez JV. Glutathione synthesis during the rewarming of rat hepatocytes preserved in University of Wisconsin (UW) solution. *Cryobiology* 2001; 43: 310-315.

The urea cycle enzymes activity and its gene expression in rat hepatocytes are not affected by cold storage in University of Wisconsin solution

Luciana Almada;[1] Cristina Bellarosa;[2] Pablo Giraudi;[1] María Mamprín;[1] María Mediavilla;[1] Edgardo Guibert;[3] Claudio Tiribelli;[2] Joaquín Rodríguez[1]

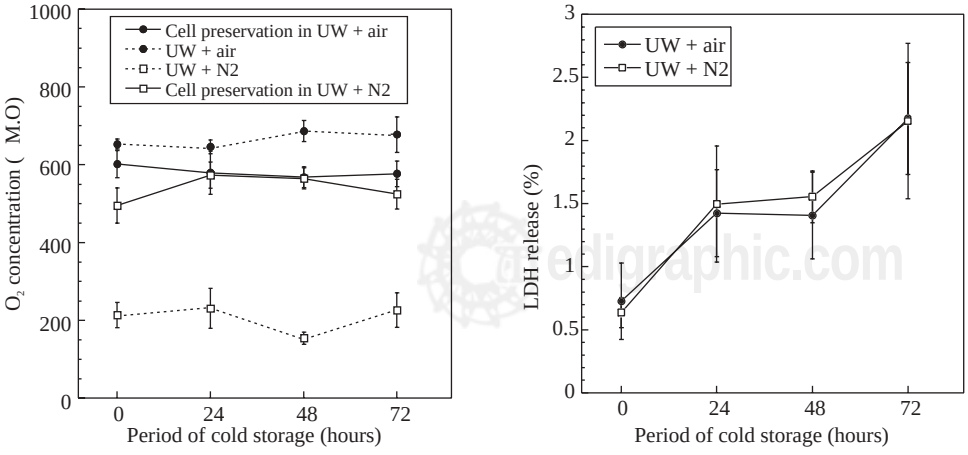


Figure 1. O_2 concentration in UW solution and viability of cold preserved cells during 72 hours of cold storage. Each concentration value represents the mean ± SD of 10 additions, n = 2 cell preparations.

[1] Farmacología y [3]Biología Molecular, Facultad de Ciencias Bioquímicas y Farmacéuticas, Universidad Nacional de Rosario, Suipacha 531-S2002 LRK Rosario, Argentina
[2] Centro Studi Fegato, AREA Science Park, Basovizza SS 14, Km 163,5. 34012 Trieste, Italia

Abstract: The Urea Cycle (UC) is the main pathway of ammonium removal. A deficiency in any of the six classical enzymes of the pathway causes an Urea Cycle Disorder. Hepatocellular transplantation is one of the techniques applicable to treat this disorder. In the present work, we investigated the activities and the relative expression levels of two of the UC enzymes: Carbamyl Phosphate Synthetase I (CPS1) and Ornithine Transcarbamylase (OTC), in isolated hepatocytes preserved up to 120 hr in UW solution at 0ºC, and during the rewarming of these suspensions. During preservation, CPSI showed differences in both parameters measured respect to time 0. OTC remained unchanged in this step. At the end of the rewarming, CPSI and OTC showed values of enzymatic activity and relative mRNA level comparable with the control. Confirming this results, we found that hepatocytes cold preserved up to 120 hr in UW solution showed no difference in their ability to remove a concentration ammonium load respect to freshly isolated hepatocytes. These data indicated that cold preservation of rat hepatocytes up to 120 hr in UW solution followed by rewarming maintain UC enzymes with a behavior similar to freshly isolated hepatocytes.

Key words: UW, hepatocytes, cold preservation, CPSI, OTC.

1. Introduction: The Urea Cycle (UC) is the only metabolic pathway capable of disposing excess of nitrogen. It converts nitrogen derived from dietary intake and from breakdown of endogenous protein into urea, which is excreted from the body. Although other tissues express some UC enzymes, only the hepatocyte has the full metabolic capability of detoxify ammonia to urea and, an alteration of some of its enzymes can be seen in the Urea Cycle Disorder (UCD). UCDs can be treated in the long term by correction of the enzymatic defect in hepatocytes. Isolated hepatocyte transplantation is a technique applicable to achieving this therapeutic effect. The aim of the present study was to analyze if the storage of hepatocytes up to 120 hs at 0ºC in UW solution could modify a) the relative expression level, b) the activity of carbamyl phosphate synthetase I (CPSI), and ornithine transcarbamylase, (OTC), and finally, c) to investigate the ammonium detoxification efficiency of rat hepatocyte suspensions after cold preservation.

2. Materials and methods: *Animals.* Male Wistar rats weighing 250-300 g were used in all experiments. Rats were allowed access to standard laboratory diet and water *ad libitum* freely, prior to the experiment and received care in compliance with international regulations.

Hepatocyte isolation. Hepatocyes were isolated by collagenase perfusion as it was described previously.[1]

Hepatocyte cold preservation. Freshy isolated hepatocytes were cold preserved in UW solution. The method was described previously.[2] The hepathocyte suspensions ($1.2.10^7$ cells in 40 mL UW solution) were allowed to settle to the bottom of the 50 mL screw cup polycarbonate tubes and left undisturbed at 0°C up to 120 hs.

Hepatocyte rewarming. After the period of cold storage, the hepatocytes were washed twice with a rinse solution developed in our laboratory (2) and sedimented (50 g, 3 min) in warm Krebs-Henseleit resuspension (KHR) media (114 mmol/L NaCl, 25 mmol/L $NaHCO_3$, 4.8 mmol/L KCl, 1.5 mmol/L $CaCl_2$, 10 mmol/L hepes, 5 mmol/L fructose, 5 mmol/L glucose, 1 mmol/L allopurinol, 3 mmol/L glycine and 1% BSA; pH 7.20). Hepatocytes ($2\text{-}3.10^6$ cells/mL) were then incubated (120 min, 37°C, $2\text{-}3\times10^6$ cells/mL) in KHR media under carbogen atmosphere in a Dubnoff metabolic shaker.

Experimental protocol. To analyze the mRNA levels of the genes and the activity of the respective proteins during 1) the preservation period, at time 0, 72 and 120 hours of storage, hepatocytes were resuspended in the storage solution and aliquots were removed and centrifuged. Determinations were made on the sedimented cells; 2) the rewarming period, 72 or 120 hours after cold storage in the preservation solution, cells were rewarmed in KHR solution. Samples of the suspension were removed at 0 and 60 min.; and 3) to study the ammonium detoxification ability of hepatocytes, after the cold storage, cells were rinsed and rewarmed in KHR in presence of 0.2 mM NH_4Cl overload. At 0 and 60 min, samples were removed for the measurement of ammonium extracellular concentration. Suspensions of freshly isolated hepatocytes were used as control.

RNA isolation, reverse transcription, and quantitative PCR. Total RNA was extracted using the Tri Reagent™ (Sigma) according to the manufacturer's instructions. Single-strand cDNA was obtained from 1 µg of purified RNA using the iScriptTM cDNA Synthesis Kit (BIORAD), according to manufacturer's instructions. Real-time PCR was performed using SYBR Green stain (iQ™ SYBR Green Supermix) (BIORAD). The results were normalized to β-actin (endogenous control) and the amount of each sample was determined as relative expression versus one of the samples chosen as reference (in this case the control sample). *Table I* shows the primers designed for each gene expression analysis

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'

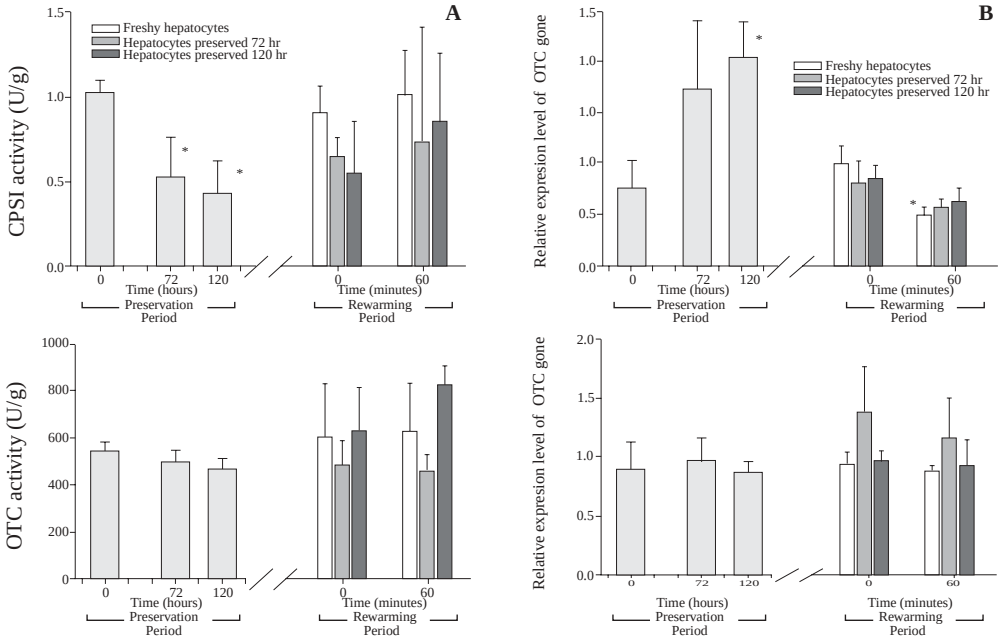


Figure 1. Panel A: CPSI and OTC activity during the cold storage and rewarming period.

Panel B: CPSI and OTC relative expression level corresponding to the preservation and rewarming period.

* Different from t=0

Determination of CPSI and OTC activities. Activities of CPSI were determined using a rapid colorimeric assay described by Pierson.[3] OTC activity was measured as the rate of citrulline formation from ornithine and carbamyl phosphate.[4]

Determination of extra-cellular ammonium concentration. Ammonium was determined enzymatically according with the method by van Anken et al.[5] Ammonium Removal Efficiency (ARE) was calculated from the measured values of ammonium concentration as follows: $ARE=[(C_o - C_t) / C_o] \times 100$, where C_o 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 best efficiency of ammonium removal.[6]

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.

3. Results and discussion: 1. C*old preservation period*. As shown in *figure 1* panel A and B, during the cold storage at 0ºC, the enzyme activity and the relative mRNA level of CPSI gene changed respect to the values obtained at time 0. On the contrary, there was not statistical difference in both parameters measured for the OTC enzyme. This behavior could be associated with the fact that the rate-limiting step of the UC is realized by CPSI.

2. Rewarming period. *Figure 1*, panel A and B, demonstrate that both OTC and CPSI showed no statistical difference respect to the control group. These results indicate that independently of the cold storage time, cell suspensions could finish the rewarming period with similar values of activity and mRNA level related to freshly isolated hepatocytes. These later findings are in line with the observation that the ARE of preserved hepatocytes did not show statistical differences irrespective of the preservation time (freshly hepatocytes: ARE =18.04 ± 10.77 %; hepatocytes preserved 72 hr: ARE = 23.1 ± 12.64%; hepatocytes preserved 120 hr: ARE = 29.23 ± 7.5%).

These results show that under our preservation conditions and with the analysis performed after rewarming step, the UC enzymes are preserved, and also, the cells maintain the capacity to detoxify ammonia, as compared with freshly isolated hepatocytes.

Acknowledgements

This work was funded by Agencia Nacional de Promoción Científica y Tecnológica (ANPCyT), PICT-03-14492, BID 1201 OC/AR and Ministero degli Affari Esteri, Rep. Italia, Prot. 269/8/0085082 and Prot. 269/P/0093044.

References

1. 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: 269-274.
2. Mamprin ME, Rodriguez 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: 270-276.
3. Pierson DL. A rapid colorimetric assay for Carbamyl Phosphate Synthetase I. *Journal of Biochemical and Biophysical Methods* 1980; 3: 31-37.
4. Ceriotti G. Ornithine Carbamoyltransferase. In: "Methods in Enzymatic Analyse". Bergmeyer H.U. Editors: Verlag Chemie, Weinheim/Bergsts, Second Edition. 1974; 2: 691-698.
5. van Anken HC, Schiphorst ME. A kinetic determination of ammonia in plasma. *Clin Chim Acta* 1974; 56: 151-157.

6. 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: 245-253.

The heme oxygenase system and organ preservation: Studies towards protection in a Kidney model

Barry J Fuller; Liz Balogun; Colin Green; Roberto Motterlini
University Department of Surgery & Liver Transplant Programme, Royal Free Hospital, London NW3 2QG & Northwick Park Institute for Medical Research, Watford Road, Harrow HA1 3UJ, UK

One way to improve cold preservation of organs for transplantation may be to maximize the cells' natural ability for repair via the stress response. A variety of stress proteins are expressed by most mammalian cells in response to damage such as encountered in hypoxia, free radical exposure, and toxic heavy metals. One member of the stress protein family is the heme oxygenase system. HO-1 is the inducible form of the enzyme, which possesses a range of cytoprotective effects, generated through the end products of the enzyme activity (biliverdin, carbon monoxide (CO), and iron). We have been interested in attempting to improve cold preservation by induction of HO-1 in a rabbit kidney model. Various methods for induction of HO-1, and the time course of stability during cold preservation have been investigated. Prior induction of HO-1 using hemin as an inducing agent provided protection against cold preservation injury in the rabbit transplant model during 24h cold hypoxia, but not during 48h cold hypoxia. However, hemin was itself found also to cause kidney damage, and thus a search was made for other inducers of HO-1 in using isolated kidney cells. We have also moved towards investigating the direct effects of CO during cold preservation. The limitations and possibilities for these approaches in organ preservation will be discussed.

What is the meaning of cellular viability?

Pablo J. Giruadi;[1] Joaquín V. Rodríguez[1]
[1] Farmacología, Departamento de Ciencias Fisiológicas, Facultad de Ciencias Bioquímicas y Farmacéuticas, Universidad Nacional de Rosario. jrodrig@fbioyf.unr.edu.ar

The application of hypothermic/cryopreservation protocols to living cells and tissues are now widely used in areas of biotechnology and medicine, where the cells or tissues are from mammalian origin (i.e., hepatocytes). The main purpose of these methodologies is to provide a viable and functional cell/tissue for manipulation or transplantation. Therefore it is very important to know the functional cell responses to low temperature preservation protocols and how these may affect the overall outcome. From this requirement has arisen the need to assess "cell viability" which, because it is rather general term that should express the quality of the preserved tissue.

Viability, as was defined by David Pegg,[1] "is the ability of a treated sample to exhibit a specific function or functions, expressed as a proportion of the same function exhibited by the same sample before treatment or an identical fresh sample". Here, we take account of some important considerations as was clearly pointed out by Muldrew and McGann:[1]

- Viability is not synonymous with life
- Should not use absolute measures as an index of via bility (always normalize respect to control values)
- Viability indices are specific to the damaging mechanism as well as to the biological sample and the measured function (a particular sample may have more than one viability index)
- The function that must be maintained *in vivo* is the ideal function to measure for a viability index
- Viability assays on cell populations can be affected by the loss of cells from the sample or the loss of function from same or all of the cells in a sample and by the degraded function in cells of the sample

In the field of cryobiology/low temperature preservation the use and interpretation of viability assays for cells as hepatocytes arises from the fact that they are used in three different situations:

1. Viability assays are used to determine the quality of cells immediately after isolation as a form of quality control.
2. The same measurements are used to assess the effect of the cryopreservation/hypothermic preservation protocol during the cold storage period.
3. The viability measures are used to determine the quality of cells after cold storage during the rewarming period and those which depend upon cold exposure but can only be detected after return to normothermic temperatures. The aim in this case is to demonstrate as far as possible all the functions required to maintain cell function.

The ideal assay for cell viability is one which would be easy and rapid to perform, which can be used in the three differents situations described above, which reflects a vital cell function and which would be sensitive to even minor alterations of such function. Also, the viability tests should be predictive of the quality of preserved cell/tis-

sue. As other have pointed out[2] there is no single assay that fulfils all these criteria. The use of a combination of viability assays can facilitate to evaluate how the preservation protocol affects viability.

Various techniques have been used in an attempt to assess quickly and reproducibly the numbers of hepatocytes which survive to cold preservation protocols. These tests are based on: a) permeability properties of plasma membrane, b) functional competence of cells, c) morphological appearance of cells, d) reproductive performance of cells and e) primary culture of cells.[2-5]

In this study, we are using several plasma membrane integrity tests as expression of cell viability. Tests of plasma membrane integrity are the most widely used to estimate cellular viability because they are simpler to perform, faster and less expensive than metabolic test such as ATP/ADP ratio or stimulation of respiration by succinate, etc. These permeability tests include trypan blue exclusion (TBE), lactate dehydrogenase retention (LDH) and fluorescent dyes such as propidium iodide (PI) that upon membrane damage bind to nucleic acids and become highly fluorescent.[3,6]

As example, we describe a simple assay for measurement of cell viability using the fluorescent dye PI and a cell lysis agent, the assay is a modification of the one previously described by Gores *et. al*.[7] and we compare it with two classical methods (TBE and LDH retention). This method,[8] based on dye exclusion utilizes the fact that dead cells lose the ability to exclude membrane impermeable dyes due to membrane damage. PI is a membrane impermeable dye that binds to nucleic acids, whereby its fluorescence is enhanced. Therefore, the fluorescence of cells incubated with PI can be used to quantify the nucleic acids accessible by the dye which correlates with the number of dead cells in the sample. After permeabilization of the plasma membranes with digitonin, the PI fluorescence correlates with the total number of cells (dead and live or viable), the ratio of the two fluorescence measurements can be used as an indicator of the percentage of dead cells in the sample. The accuracy of the proposed assay for determining cell viability was assessed by comparing the results obtained by PI method with those of TBE and LDH, we made this comparison because the assays tested are based on permeability properties of the plasma membrane. To evaluate whether the PI assay is useful for checking cold preservation and rewarming conditions of cells, the hepatocyte viability under different conditions of preservation and rewarming were measured. The results were compared with those obtained by TBE and LDH assays. As was demonstrated in *Figure 1*, the cell viability values estimated by PI and TBE assays correlated well; on the contrary, LDH assay estimated viability at levels greater than PI and TBE tests. This fact could be due to: (i) a non-specific retention of LDH activity inside the permeabilized cells, or (ii) losses of the extracellular LDH activity during the isolation and purification of the cell suspension.

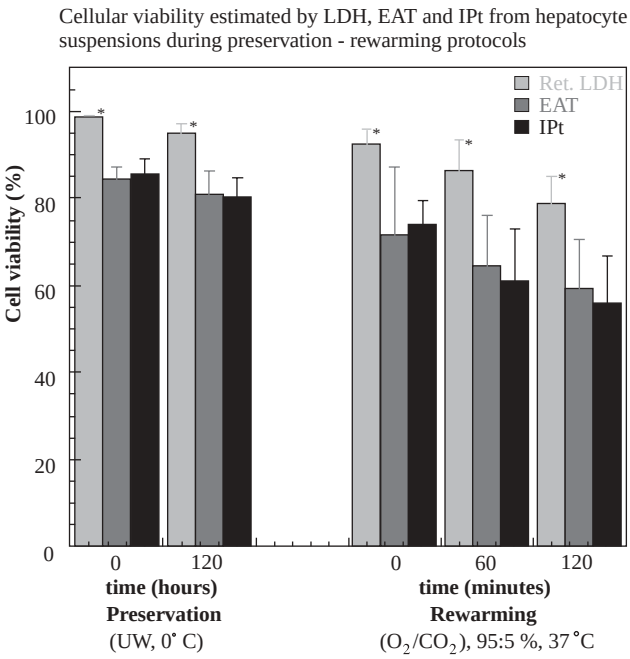


Figure 1. Time course of cellular viability of cold preserved isolated hepatocytes during 120 hours and then rewarmed during 120 min. Hepatocytes were incubated as was described in.[8] The cell viability was assessed by PI, TBE and LDH tests. Values are expressed as means ± SD (error bars) of samples obtained of three preparations. * statistical different from PI and TBE tests.

Enzyme retention or leakage and vital dye staining are additional criteria of plasma membrane integrity that are simpler to perform, faster and less expensive than other methods. TBE by the hepatocyte preparation has become the most widespread technique for viability comparative purposes. However, the main disadvantage of this method includes the subjectivity characteristic of visual cell counting procedures and the necessity to control and standardize, the pH, dye concentration and the exposition time to dye carefully.[2] Therefore a procedure based on fluorometry which is simpler to perform would be advantageous in terms of rapidity, accuracy and less subjective than cell counting procedures.

The general assessment of cellular viability by different tests such as TBE, LDH release or PI fluorescence is restricted to the assessment of plasma membrane integrity. These assays are appropriate to used in the three situations previously described, but this test should always be accompanied by the microscopic assessment of cell morphology and complemented with a test of functional competence of cells as maintenance of ATP levels,[9] respiratory activity[10] or MTT colorimetric assay.[11]

Acknowledgements

This work was founded by Agencia Nacional de Promoción Científica y Tecnológica (ANPCyT), PICT-03-14492, BID 1201 OC/AR.

References

1. Muldrew K, McGann LE. Cryobiology - A Short Course. *http://www.ucalgary.ca/~kmuldrew/cryo_course/*.
2. Baur H, Kasperek S, Pfaff E. Criteria of viability of isolated liver cells. *Hoppe-Seyler's Zeitschrift fur Physiologische Chemie* 1975; 356: 827-838.
3. Becker B, Clapper J, Harkins KR, Olson J. *In situ* screening assay for cell viability using a dimeric cyanine nucleic acid stain. *Anal Biochem* 1994; 221: 78-84.
4. Fry JR, Hammond AH. Assessment of the functional integrity of hepatocytes: a brief review. *ATLA 21* 1993: 324-329.
5. Pazo JA, Rodriguez ME, Vega F, DE La Cruz L, Guibert EE, Mediavilla MG, Furno G, Rodriguez JV. Primary culture of rat hepatocytes after cold storage in the University of Wisconsin SOLUTION: A tool to study the effects of hypothermic preservation. *Cell Preservation Technology* 2003; 1: 189-199.
6. Jones KH, Senft JF. An improved method to determine cell viability by simultaneous staining with fluorescein diacetate-propidium iodide. *J Histochem* 1985; 33: 77-79.
7. Gores GJ, Nieminen AL, Fleishman KE, Dawson TL, Herman B, Lemasters JJ. Extracellular acidosis delays onset of cell death in ATP-depleted hepatocytes. *Am J Physiol* 1988; 255: C315-C322.
8. Giraudi PJ, Almada LL, Mamprin ME, Guibert EE, Furno G, Rodriguez JV. The assessment of viability in isolated rat hepatocytes subjected to cold or subzero non-freezing preservation protocols using a propidium iodide modified test. *CryoLetters* 2005; 26: 169-184.
9. Page R, Stowell KM, Hardman MJ, Kitson KE. The assessment of viability in isolated rat hepatocytes. *Anal Biochem* 1992; 200: 171-175.
10. Kravchenco LP, Petrenko AYu, Somov AYu, Grischenko VI, Fuller BJ. Respiratory activity of isolated rat hepatocytes following cold storage and subsequent rewarming: A comparison of sucrose-based and University of Wisconsin solutions. *Cryobiology* 2000; 42: 218-221.
11. Schiller CD, Kainz A, Mynett K, Gescher A. Assessment of viability of hepatocytes in suspension using the MTT assay. *Toxicology In Vitro* 1992; 6: 575-578.