Primary Hepatocyte Cryopreservation: Enhances Survival and Function

John M. Baust, Meindert Sosef, Alex Fowler, and Mehmet Toner

Center for Engineering in Medicine and Surgical Services, Massachusetts General Hospital and Harvard Medical School, and Shriners Hospital for Children, Boston, MA, 02114.

Abstract

The advances in the fields of bio-engineering and regenerative medicine have led to an increasing demand for successful, efficient means of long-term preservation of isolated hepatocytes for utilization in bioartifical liver devices. Accordingly, we investigated the effect of cryopreservation on hepatocyte function and attempted to improve overall cell viability and function through the utilization of CryoStor as the carrier solution. We hypothesized the use of CryoStor would improve survival through the modulation of the bio-molecular response of the hepatocytes to cryopreservation which would translate into enhanced function.

Our findings represent a significant advance in the successful cryopreservation of primary isolated hepatocytes, which have proven difficult to preserve with high viability and stable long-term function. This study represents the first report on assessment of long-term hepatocellular basal function/challenge response and demonstrates that the improvement in preservation efficacy was not strictly limited to cell viability but translated into the maintenance of functional cells. These results may represent an important step forward to the utilization of cryopreserved hepatocytes in tissue engineering and regenerative medical applications.

Introduction and Background

Major steps have been taken recently in the development of treatments for liver failure including extracorporeal and implantable bioartificial liver (BAL) devices and hepatocyte transplantation. Reports on the utilization of extracorporeal BAL devices have detailed improvements in biochemical and neurological parameters as well as overall survival in several animal models. (1) In inherited metabolic diseases of the liver, hepatocyte transplantation offers another therapeutic option in attempt to restore liver function. (2) For any of these cell-based therapies to fully reach their clinical potential; however, isolated hepatocytes need to be preserved for significant periods of time (months to preferably years) so that they can be appropriately banked and distributed for on-demand utilization.

Cryopreservation represents one tenable option for long-term hepatocytes preservation. Various strategies have been described over the last 25 years for cryopreserving hepatocytes. Although progress has been reported for cultured hepatocytes (3,4), attempts to cryopreserved isolated primary hepatocytes have resulted in limited success. Overall, studies that detail the survival and function of entire populations of cryopreserved isolated hepatocytes indicate low cell recovery and greatly impaired metabolic activity. Recent reports on a new concept in cryopreservation solution design focusing on the use of an intracellular-like, cryopreservation medium, CryoStor, prompted us to evaluate this solution for the cryopreservation of primary hepatocyte. (5,6) Based upon previous findings we hypothesized the utilization of CyyoStor would improve overall hepatocyte viability through a modulation of the cellular biochemical response to the cryopreservation process as well as maintaining improved cellular function in comparison to non-preserved cells.

Methods

Freshly isolated rat hepatocytes were incubated at 37°C in culture media for 20 minutes and frozen (-1°C/min to -80°C) in suspension in either culture media or CryoStor supplemented with 10% (v/v) dimethyl sulfoxide (Media + DMSO, CryoStor CS10, respectively). After storage in liquid nitrogen (up to two months), cells were rapidly thawed and maintained in a double collagen gel culture for 14 days. Serial measurements were made of albumin secretion, urea synthesis, deethylation of ethoxyresorufin (CYT P450 activity) and responsiveness to stimulation with Interleukin-6 (IL-6). Survival of the cells at 1, 3, 7 and 14 days was assessed by total intracellular LDH content. Evaluation of survival and function of the cryopreserved samples was normalized to matched controls and reported as a percentage of control values ± standard deviation.

Results

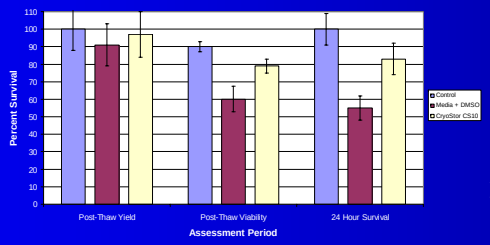

Figure 1. *Primary Hepatocyte Yield, Viability, and 24-Hour Survival Following Cryopreservation.* Hepatocytes cryopreserved in CryoStor CS10 yielded a 32% (overall 20%) improvement in cell viability over the media + DMSO samples and represented only an 11% drop in viability in comparison to controls. Based upon control attachment levels (100%), merely 55% of cells cryopreserved in media + DMSO attached. This represents a significant decline from the assessment of immediate post-thaw viability due to the 90% viability in controls being 100% next day attachment there fore the 55% next day attachment in media + DMSO samples actually represents a mere 49% attachment and viability next day in comparison to the 100% cell yield in controls. Cryopreservation in CryoStor CS10 improved cell attachment over that of media samples by 50% resulting in a final attachment of 75%.

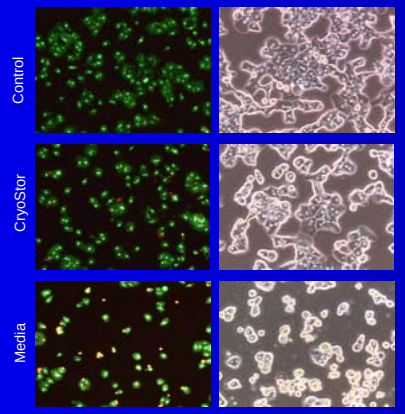

Figure 2. *Morphological Assessment of Hepatocyte Attachment Following Extended Culture.* Morphological assessment by phase contrast microscopy at day 14 revealed a typical monolayer configuration of polygonal cells in both control and CryoStor CS10 samples. In contrast, media samples demonstrated culture morphology characterized by a high density of disintegrating hepatocytes with irregular cell shape. Visualization of cell morphology was performed under phase-contrast microscopy and determination of the viable cell population was performed using Syto13/ Ethidium Bromide staining with visualization under fluorescence microscopy.

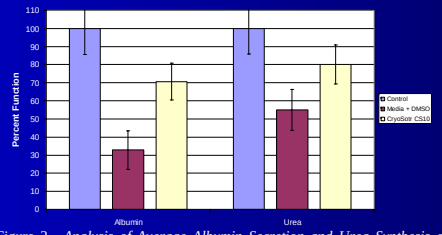

Figure 3. *Analysis of Average Albumin Secretion and Urea Synthesis of Cryopreserved Primary Rat Hepatocytes Following 7 to 14 Days of Culture.* Samples were collected on a daily basis over a 14-day period and analyzed for albumin and urea content. Both albumin excretion and urea synthesis stabilized following the first week of culture, after which a plateau phase was observed in all conditions. Comparison between groups was performed by averaging albumin and urea levels in the plateau phase (day 8 to 14). Evaluation of albumin revealed a reduction in secretion in cryopreserved samples in comparison to controls, yet the CryoStor CS10 hepatocytes yielded improved function over that of the media population (71% vs 33%, P<0.005). Urea synthesis in both the cryopreserved samples was reduced from that of control levels as well (80% and 55% vs. 100%, P<0.01) again with urea synthesis in the CryoStor samples significantly greater than that of the media samples (P=0.003).

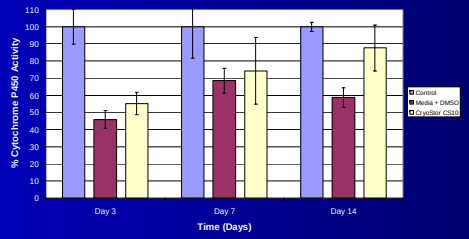

Figure 4. *Analysis of Long Term Detoxification Capasity of Primary Rat Hepatocytes Following Cryopreservation.* Cytochrome P450IA1 isoenzyme activity was assessed with the EROD assay and showed an initial delayed recovery in detoxification function of cryopreserved cells when compared to control (Day 3: CryoStor = 55%; media-cryo = 46%). Following extended culture (14 days) Cytochrome P450 function improved in both the CryoStor and media populations to 88% and 59% of controls, respectively.

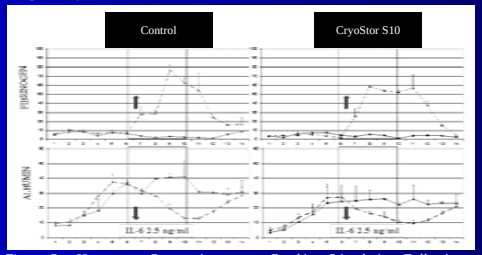

Figure 5. *Hepatocyte Responsiveness to Cytokine Stimulation Following Cryopreservation.* Responsiveness of cryopreserved hepatocytes to cytokine stimulation was evaluated in comparison with controls by measuring rates of fibrinogen and albumin production before, during and after exposure to IL-6, and compared to identical non-stimulated cultures. IL-6 exposure resulted in a strong upregulation of fibrinogen production, with a concurrent decrease in albumin secretion, which was maintained during the exposure period. After removal of IL-6, secretion rates of both albumin and fibrinogen in the CryoStor and control groups returned to typical base line levels.

Discussion

In this study, we evaluated the cryopreservation of isolated primary rat hepatocytes in suspension using the cryopreservation solution, CryoStor as the carrier solution. Critical to the interpretation of the data in this study is that survival and function of the cryopreserved samples were normalized to matched controls and reported as a percentage of control values. Additionally, unlike most prior studies, which focused on immediate or short-term (few days) function, we extended the frame of evaluation to 14 days post-thaw assessment of survival and function.

Cryopreservation of isolated primary hepatocytes, in media+DMSO or CryoStor CS10 resulted in a high cell yield and viability post-thaw in comparison with controls and were cultured overnight to assess viability 24 hours post-thaw. This assessment was performed due to recent reports of the involvement of apoptotic and necrotic cell death following cryopreservation. Assessment of survival at 24 hours revealed, consistent with previous reports, there was continued cell death resulting in a decline in attachment rate in the media and CryoStor samples. Assessment of cellular function revealed albumin production in hepatocytes cryopreserved in media + DMSO was found to be reduced on a per cell basis 42% in comparison to controls. Cryopreservation in CryoStor CS10 resulted in a significant increase (48%) in albumin production over that of media samples. Analysis of urea synthesis and cellular detoxification function by Cytochrome P4501A1 activity revealed there was no significant decline in either function by the cryopreservation process regardless of the preservation regime utilized on a per cell basis.

In addition to basal hepatospecific function, responsiveness of hepatocytes to cytokine stimulation is essential for clinical applications, therefore we tested cryopreserved hepatocyte responsiveness to elevated levels of IL-6. Our data demonstrates that the response of fresh and cryopreserved hepatocytes is similar when stimulated with IL-6 both early in post-thaw culture as well as following culture stabilization.

Conclusions

Our results represent a significant advance in the successful cryopreservation of primary isolated hepatocytes that have been very difficult to preserve with high viability and long-term stable function in the past. This study represents the first report on assessment of long term hepatocellular basal function and challenge response and demonstrated that the improvement in preservation efficacy was not strictly limited to cell viability but translated into the maintenance of viable and functioning cells as well. Taken together these data demonstrated that the effects of the cryopreservation process has a larger effect than merely seen by survival assessment but there was a targeted effect at the physiological and biochemical level resulting in a reduction in albumin production. More importantly, this effect was significantly lessoned by the utilization of CryoStor as the carrier solution. These results may represent an important step forward to the utilization of cryopreserved hepatocytes in tissue engineering and regenerative medical applications such as in bioartificial liver devices.

References

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