Extended Hypothermic Storage of Isolated Human Hepatocytes Utilizing HypoThermosol-FRS

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Abstract

In the *in vitro* drug discovery/toxicity arena, primary hepatocytes serve as the gold standard cellular assay system due to their high sensitivity and diverse functional response. While the demand is high, supply and distribution of hepatocytes remains limited due to several factors including a finite *in vitro* life span. In order to overcome this limitation, we evaluated several commercially available hypothermic preservation media for their abilities to protect isolated human hepatocytes during hypothermic storage. We investigated the temporal limits of these solutions in an attempt to extend storage while retaining a high degree of cell viability and function. Analysis of hepatocytes in the various hypothermic preservation media revealed that following 1-day of storage at 4°C, sample viability ranged from ~5% to ~60% (MF=5%, UW=35%, and FRS= 60%). Extension of the preservation interval to 48 and 72 hours resulted in survival levels <15% in MF and UW whereas 40%(±4%) was achieved using HTS-FRS. Analysis of hepatocyte function demonstrated that along with the increase in viability in the HTS-FRS samples, there was high retention of hepatocellular function within the samples on a per cell basis. These data indicate that hepatocyte storage can be extended 2-3 fold with HTS-FRS while retaining viability and functionality levels at or above those attainable at 24 hours utilizing the current gold standard solution.

Introduction

Each year billions of dollars are spent by the pharmaceutical industry on the discovery and development of new drugs. Research and development at the basic and applied sciences level provide information on compound identification, toxicity, influence on target cell and tissue performance, and downstream effects on other systems. A key component of the R&D process is the utilization of the human hepatocyte *in vitro* model system to facilitate high throughput compound screening.

Currently several companies supply and distribute hepatocytes, yet their ability to meet the demand of the research community is constrained by two key factors. First, there is a limited supply of quality livers, and secondly, once isolated, hepatocytes have a finite life span *in vitro* limiting distribution and utilization. Currently the *ex vivo* usable cold storage life span of a liver is approximately 24 hours with a similar time frame for the preservation of isolated hepatocytes. This finite time frame significantly limits their supply, access, and utility of hepatocytes placing the *in vitro* utilization into the category of "just-in-time" research.

Over the last several years, the preservation sciences have evolved in conjunction with the cell and tissue therapy arena to move living systems from "just-in-time" to "on-demand" utilization. While these ventures have proven highly successful to date, crossover into the basic sciences and drug discovery arena has been limited. As such, in this study we investigated extending the practical preservation interval of hepatocytes beyond the present day 24 hour limit.

Methods

Cell Culture: Human Hepatocytes were isolated and purfied from non-transplantable livers, plated into collagen coated 96-well plates and maintained at 37°C, 10% CO_2 in hepatocyte culture media. For hepatocyte function studies, samples were treated as described above but plating was accomplished in collagen coated 6-well plates.

Hypothermic Storage: Following 24 hours of post-isolation culture, culture media was remove and preservation media was added to each sample (100ul/well for 96-well and 2ml/well for 6-well). Samples were places into 4°C storage for 1, 2, or 3 days in either culture media, Viaspan (UW) or HypoThermosol-FRS (HTS-FRS). Following storage, samples were removed from the cold, preservation media decanted, and fresh culture media was added to each sample well.

Cell Viability: Sample viability was assessed by using the membrane integrity probe, Calcein-AM (Molecular Probes) at 24 hr intervals for 2 days using a fluorescent plate reader (Tecan Spectromax). Fluorescent readings were compared to day-1 37°C control values to obtain "percent sample viability." Fluoresecent Images were obtained from Calcein-AM stained samples using a Zeiss Axiovert 200 fluorescence microscope.

Hepatocyte Function: Hepatocyte function was assessed following removal from the cold and 4 hours recovery culture. To determine enzyme-dependent metabolic function, 1 x 10^6 viable cells from each condition were mixed with a specific probe at the appropiate concentration in a 96-well plate and incubated at 37°C for one hour. Metabolites were extracted with 0.1 ml ice-cold cell lysis solvent and cell debris and protein were removed by centrifugation (1,000 xg, 15 min). Metabolites were analyzed by reverse-phase HPLC analysis. Functional assessment included ECOD (7-ethoxycoumarin O-deethylation), CYP3A4 (testosterone 6b-hydroxylation), FMO (p-tolyl methyl sulfide oxidation), UGT (7-hydroxycoumarin glucoronidation), and ST (7-hydroxycoumarin sulfation). Enzyme activities were expressed in nmole/hour/million cells.

Results

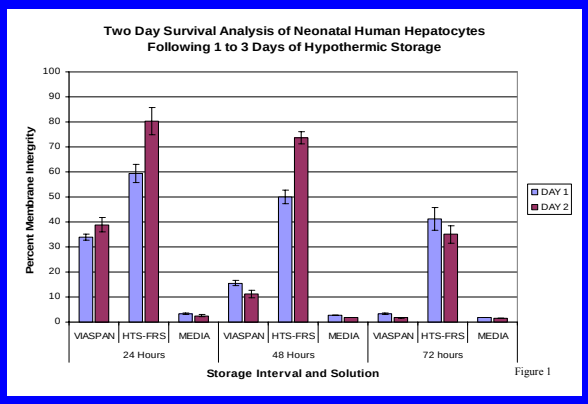

Figure 1

Figure 1: *Analysis of Hepatocyte Survival Following 1, 2, or 3 Days of Hypothermic Storage.* Hepatocytes were stored at 4°C in an adherent state for 1-3 days. Following the storage interval samples were returned to culture and assessed over a two day interval to determine true survival. The data show that hepatocytes stored in HTS-FRS maintained viability ≥50% for up to 48 hours of storage where as both culture media and Viaspan facillitated <24 hours. Further, samples stored for 72 hours in HTS-FRS yielded similar results to the 24 hour Viaspan samples.

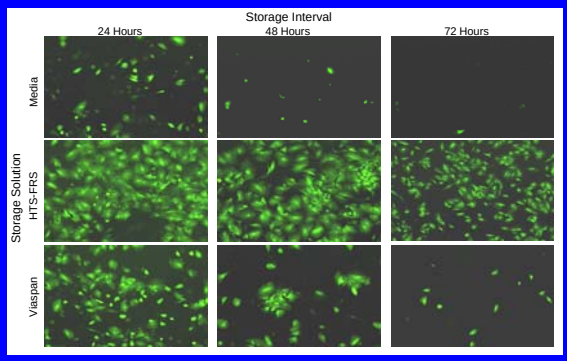

Figure 2: *Fluorescent Micrographs of Adherent Viable Hepatocytes Following Hypothermic Storage.* Hepatocytes were stored at 4°C for 1, 2, or 3 days followed by a return to culture. Following 24 hours of post-storage culture, samples were probed with Calcein-AM and fluorescent micrographs were taken to assess the degree of adherence and monolayer integrity. Micrographic analysis revealed that only the HTS-FRS samples were able to maintain a high degree of cellular adherence and monolayer integrity.

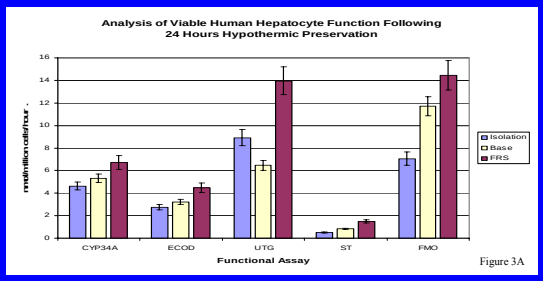

Figure 3A

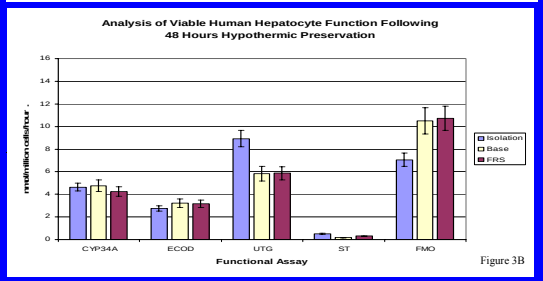

Figure 3B

Figure 3. *Analysis of Hepatocellular Function of Viable Cells following 1 and 2 Days Storage at 4°C.* Based on the viability and fluorescence microscopy data, function analysis of surviving hepatocytes from the HTS-Base (Yellow bars) and HTS-FRS (Red bars) samples were conducted and compared with non-preserved isolation controls (Blue bars). The data demonstrate that in the preserved samples, there was no significant decrease in hepatocyte function. Additionally, in the HTS-FRS samples there was a notable increase in the functional capacity of the hepatocytes compared to controls. The protective recovery property of the HTS-FRS has been attributed to the heightened levels of anti-oxidants in the solution (compared with other preservation media) thereby reducing the levels of free-radical damage experienced by cells following the isolation process.

Summary of Results

- Hepatocytes preserved at 4°C in Viaspan (the gold standard solution) for 1 day resulted in <40% survival and ≤15% after 2-3 days.
- Hepatocyte preservation in HTS-FRS resulted in a high degree of viable cells after both 1 and 2 days storage at 4°C.
- Hypothermic storage at 4°C for 3 days in HTS-FRS yielded survival equivalent to the 1 day Viaspan values.
- A high degree of monolayer integrity was observed in the HTS-FRS samples
- Analysis of hepatocellular function revealed no detrimental effect following storage at 4°C in HTS-FRS.
- Following 2 days storage at 4°C, function of hepatocytes stored in HTS-FRS remained equivalent to fresh isolation controls.

Discussion and Conclusions

In this study we evaluated the efficacy of several commercially available hypothermic storage media for the preservation of isolated primary human hepatocytes. Our data illustrates that following preservation in conventional media (culture media and Viaspan) there is a significant loss of viable hepatocytes. This loss of cells has been documented by many investigators and has been a source of concern for both cell suppliers and end users over the years. In an effort to overcome the massive cell loss experienced during the shipment process, cell suppliers have been forced to seed cultures with 2-3x the number of cells to provide end users with a hepatocyte monolayer. While a 'fix', this is a highly inefficient and costly solution.

The data presented herein illustrate the effective nature of the preservation media HTS-FRS for the cold storage of isolated human hepatocytes. Specifically, HTS-FRS provides for enhanced outcome following 24 hours storage, as well as successfully extending preservation to 3 days compared with other preservation media. While a level of cell loss remained in all conditions, the 100% survival increase in the HTS-FRS samples following 24 hour storage significantly impacted our ability to maintain a viable monolayer. Further, HTS-FRS allowed for a doubling of the storage interval while still yielding a ~50% increase in viability over the 24 hour Viaspan samples, as well as equivalent survival at 3 days to the 1 day Viaspan samples.

While increasing hepatocyte viability is critical, for true utility hepatocellular function must also be maintained. Analysis of several key enzymes demonstrated that following storage (both 24 and 48 hours) in HTS-FRS, hepatocyte function was maintained at the level of, or greater than, non-preserved isolation controls. In the case of the 48 hour preserved samples, this is significant due to the current inability to store hepatocyte samples for extended periods because of the loss of both viability and function.

The findings of this study represent a significant first-step forward increasing the usability of hepatocytes in the pharma industry. These data not only provide a basis and method for extended preservation, but may also serve as a platform for moving hepatocyte research industry from "just-in-time" to "on-demand" utilization. Ongoing investigations also are providing insight into the benefits of HTS-FRS for the preservation and transport of whole and resected liver tissue for both hepatocyte isolation as well as potential organ transplant.

References

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