Hypothermic Preservation of Keratinocytes and an Engineered Skin Construct:

Enhancement in cell quality and storage interval through modulation of apoptotic and necrotic cell death

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Introduction and Background

The growth of the Regenerative Medicine markets and the use of engineered tissues in the In Vitro Toxicology arena have presented the challenge of developing improved methods for shipping and storing these biological products so that they can reach the global marketplace. Currently there are a variety of hypothermic solutions available for organ storage that have been tested for shipping or storing human cells in the tissue engineering markets. Many of the solutions were developed prior to the advent of the biomolecular revolution and therefore are limited in their preservation ability. We hypothesized that by studying the molecular biology of cell death that occurs during extended hypothermic storage, improved preservation solutions can be designed to extend the shipping/storage shelf life of engineered products such as stratified epidermis and dermal equivalents. Accordingly, our research team has developed a portfolio of hypothermic solutions (HypoThermosol®) that is designed to protect both the viability and function of cells and engineered tissues during transit.

Methods

Coronary Artery Smooth Muscle Cells (CASMC) were preserved for 1 day at 4°C in HTS-FRS or UW, and allowed to recover at 37°C in culture media. During the recovery period, cells were stained with Annexin-V-FITC, Propidium Iodide (PI), and Hoechst at 0, 6, 12, and 24 hours. Control cells were non-preserved samples at 37°C.

Renal Proximal Tubule Epithelial Cells (RPTEC) were preserved for 3 days at 4°C in HTS-FRS, UW, or 250µm Cisplatin (Positive Control), and allowed to recover at 37°C in culture media. During the recovery period, cells were isolated at 0, 6, 12, 24, and 72 hours. Following protein isolation, samples were assayed for caspase-3 activity by measuring fluorescent substrate cleavage (ClonTech). Control cells were non-preserved samples at 37°C.

Normal Human Epidermal Keratinocytes (NHEK) were subcultured into 96-well culture plates and tested for their abilities to withstand hypothermic preservation (between 1 and 3 days) at 4°C. The cold preservation solutions examined were HTS-BASE, HTS-FRS, HTS+Caspase Inhibitor (50µm Caspase-1 Inhibitor II, CalBiochem), culture media (KGM), or UW. Cell viability was assayed with alamarBlue™ (Trek Diagnostics, Westlake, OH), a non-toxic, fluorescent metabolic activity indicator which allows for multiple endpoint analysis. Cell death was measured using Propidium Iodide (PI) which is a nuclear stain normally impermeable to the cell membrane. Late apoptotic and necrotic cells stain positive for PI. Control cells were non-preserved samples at 37°C.

EpiDerm™ was obtained from MatTek Corporation (Ashland, MA). EpiDerm was cultured in KGM, and then preserved at 4°C in HTS-BASE, HTS-FRS, culture media (KGM), or UW. EpiDerm™ was then assessed for viability with alamarBlue™ similar to the methods for NHEK. Control samples were non-preserved EpiDerm™ at 37°C.

Results

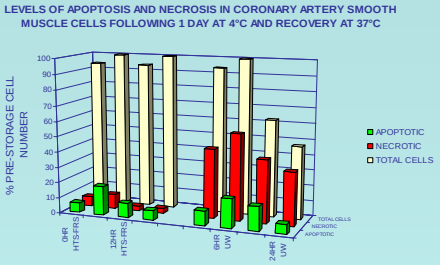

Figure 1. Coronary artery smooth muscle cells (CASMC) were preserved at 4°C in a traditional preservation solution (UW) or HTS-FRS. Following preservation, cells were stained with Hoechst (total cell number), Annexin V (early apoptotic), or Propidium Iodide (necrotic/late apoptotic). Cells stored in HTS-FRS exhibit greater cell number and decreased cell death, in comparison to cells stored in UW.

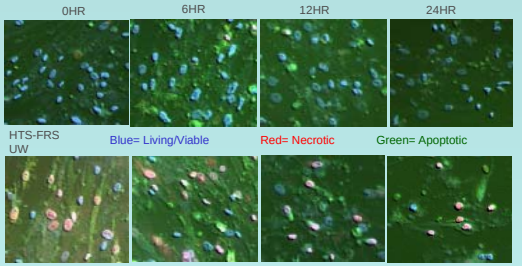

Figure 2. Fluorescent micrographs of CASMC following preservation in UW (bottom) or HTS-FRS (top). Micrographs represent the same experiment as Figure 1. Cells are stained with Hoechst (blue), Annexin (green), and Propidium Iodide (red). Cells stored in HTS-FRS demonstrate decreased Annexin/PI staining, and greater levels of viable cells (blue).

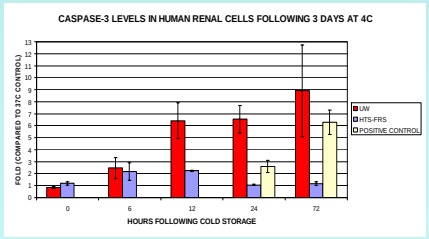

Figure 3. Renal Proximal Tubule Epithelial Cells (RPTEC) were preserved at 4°C in UW, HTS-FRS, or Cisplatin (Positive Control for apoptosis). Following preservation, cells were isolated at the indicated time points and Caspase-3 activity was measured via fluorogenic substrate cleavage. Cells stored in HTS-FRS exhibit decreased Caspase-3 activity, in comparison to cells stored in UW.

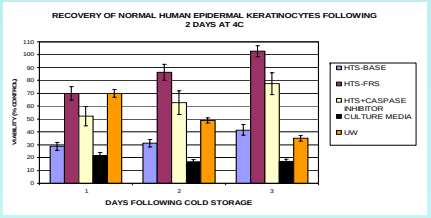

Figure 4. Normal Human Epidermal Keratinocytes (NHEK) were preserved at 4°C in HTS-BASE, HTS-FRS, HTS+Caspase Inhibitor, UW, or culture media. Following removal from cold storage, cells were allowed to recover at 37°C in culture media. During the recovery period, viability was measured with alamarBlue®. NHEK preserved in HTS-FRS demonstrate the greatest viability. In addition, NHEK preserved in HTS+Caspase Inhibitor exhibit enhanced viability, in comparison to cells preserved in HTS-BASE or UW, though not to the level of cells stored in HTS-FRS. This suggests that the targeted inhibition of apoptosis can increase the efficacy of a preservation solution, and that HTS-FRS is a more potent inhibitor of cell death in NHEK. Storage of cells in UW resulted in a gradual decline in viability during the recovery period.

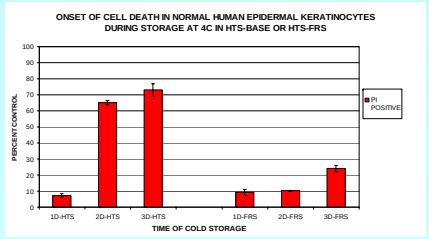

Figure 5. NHEK were preserved at 4°C in HTS-BASE or HTS-FRS for 1, 2, or 3 days. Following the indicated period of storage, cells were stained with Propidium Iodide (PI). NHEK preserved in HTS-BASE exhibit increased sensitivity to PI staining by 2 days cold storage (65%). In comparison, cells stored in HTS-FRS demonstrate lower levels of PI staining in comparison to the cells stored in HTS-BASE (9% vs. 65% at 2 days; 23% vs. 72% at 3 days).

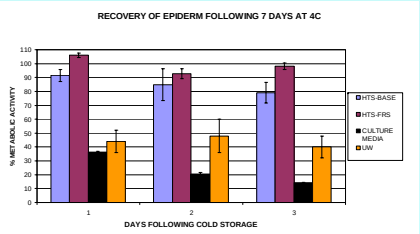

Figure 6. EpiDerm™ were preserved at 4°C in HTS-BASE, HTS-FRS, UW, or Culture media. Following removal from cold storage, EpiDerm™ were allowed to recover at 37°C in culture media. During the recovery period, viability was measured with alamarBlue®. EpiDerm™ stored in HypoThermosol® solutions (HTS-BASE, HTS-FRS) demonstrated greater metabolic viability, in comparison to those stored in culture media or UW.

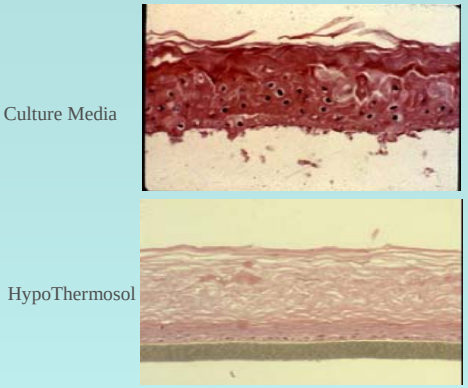

Figure 7. EpiDerm™ stored in HypoThermosol® (Right Panel) was compared histologically to EpiDerm™ stored in KGM (Left panel). Note that the EpiDerm™ preserved in HypoThermosol® appears to have a normal histology whereas this is not the case when EpiDerm™ is stored in conventional media (KGM). EpiDerm™ preserved in culture media have detached from the basement membrane. EpiDerm™ preserved in HypoThermosol® maintain tissue integrity and attachment to substrate.

Conclusions

- Hypothermic preservation is an avenue to achieve short-term (hours to days) preservation of biologics.
- Increasing the time and quality of preservation is needed for the shipping/storing of cellular and tissue products in the growing fields of regenerative medicine, tissue engineering, and pharmaceuticals.
- Extended hypothermic preservation in sub-optimal solutions leads to cell death by both apoptosis and necrosis.
- Development of solutions based on molecular biological principles has allowed for inhibition of apoptotic intermediates (caspases) and reduction in cell death.
- Custom hypothermic solutions, such as the HypoThermosol® solutions, can be developed for the improved shipping and storage of engineered human cells and tissues used in the Regenerative Medicine and In Vitro Toxicology arenas so that these important products are better able to reach a global marketplace.