Extending Hypothermic Storage Limits of Sensitive Primary Cell Systems

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Abstract

The need for successful hypothermic storage of primary cells is growing as the field of regenerative medicine expands. For example, cardiac-directed stem cells therapies require hypothermic shipping and temporary storage prior to re-implantation. A brief period of storage would allow the identification of appropriately differentiated populations and cells unlikely to survive a clinical procedure. The conditions under which cells are maintained at 4°C are vital to the preservation outcome.

The use of an intracellular type storage solution, HypoThermosol® (HTS), was shown to provide improved recovery of cultured neonatal rat ventricular cardiac myocytes (NRVCM) following 24, 48 or 72 hr cold storage over traditional cell culture media or the "gold standard" transplantation solution, ViaSpan (UW). A triad of cellular viability assays were utilized, examining membrane integrity, metabolic activity and spontaneous contractile function. Specialized variant HTS solutions (HTS-DCC and HTS-FRS) provided survival rates 10 to 30% higher than the base solution ($p<0.05$), and over 50% versus cells stored in conventional media ($p<0.01$). At the 72hr storage interval, a significant decline in viability and function was observed in all samples, except cells stored in HTS-FRS which exhibited >75% survival ($p<0.05$). Upon return to normothermic conditions, NRVCM stored in HTS-FRS for 24 to 72 hours regained function, spontaneous cellular contraction *in vitro*, and 90-95% metabolic activity as compared to 37ºC controls. Examination of the pro:anti-apoptotic protein levels (Bcl-X_L:Bcl-X_S) in cells successfully stored at 4°C for 24 hours demonstrates a peak at the 4hr recovery timepoint, and then relatively constant levels. Cells which experienced more damage following the storage displayed downward trend in the ratio (UW solution) or a slow increase (HTS-Base) over the 24 hr observation period.

These results provide important steps towards increasing the cold storage window for obtaining fully functional cardiac cells, thus augmenting harvest and transportation protocols for sensitive biologic products.

Introduction

Cardiac transplantation exerts hypothermic, ischemic and reperfusion stress at the organ and cellular level, limiting the allowable preservation time to obtain functioning organs. In an attempt to address these issues, several differing hypothermic solutions have been developed (1, 9). One main difference between the various types of preservation solutions is the environment which they are intended to mimic, either an extra-cellular or intracellular-like solutions. Intracellular-type solutions typically have more complex compositions and do not require re-equilibrating of ion gradients during the reperfusion process. Successful hypothermic preservation requires the merging of physiological and molecular knowledge to design a solution which counteracts the adverse conditions of low temperature storage to limit damaging effects (1, 9).

Despite extensive research and solution development, successful heart preservation when coupled with hypothermic conditions to reduce physiological activity, remains limited to under 12 hours (3). The ability to store cardiac systems at hypothermic temperatures for extended periods would provide new avenues for transplantation and regenerative medicine in cardiac science. To understand the biomolecular events occurring within the heart, many studies have utilized adult and neonatal cardiac myocytes as models for hypothermic storage, ischemic conditions and reperfusion injury. (2-4, 6, 7, 10) Although adult and neonatal cardiac myocytes have been shown to have some differences, the use of neonatal rat ventricular cardiac myocytes (NRVCM) has been established as an *in vitro* model for cardiac systems (8).

Preservation of myocytes must address functionality as well as the general state of the cell. In this study, we examined the membrane integrity, metabolic activity, contractile function and expression of various proteins involved with apoptotic cascades to assess myocyte survival following cold storage in various preservation solutions. These data should provide insights into the types of solutions useful for cardiac preservation and to begin investigating the cellular and molecular damages occurring to cardiac systems following hypothermic storage.

Results

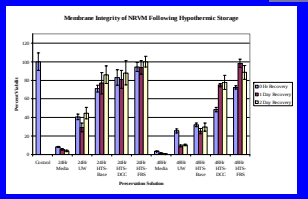


Figure 1. Membrane integrity of NRVCM following 24 or 48 hours cold storage in 24 or 48 hours in either media, UW, HTS-Base, HTS-DCC or HTS-FRS. Calcein-AM fluorescent units given as percentages of 37°C controls (±SD), representative of three experiments. Membrane integrity was maintained above 75% in the HTS based solutions following 24hr storage, whereas media and UW samples did not survive the preservation intact. Extension of the storage period to 48 hours resulted in the HTS-FRS and HTS-DCC stored NRVCM yielding system survival above 75%.

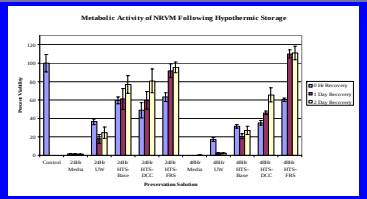


Figure 2. Metabolic activity of NRVCM monitored over 2 days following a 4°C cold storage for 24 or 48 hours in either media, UW, HTS-Base, HTS-DCC or HTS-FRS. Dashed bars indicate spontaneous contractions observed upon rewarming. Fluorescent units (alamarBlue) given as percentage of controls (±SD). Analysis of system metabolic activity following 24hrs storage revealed cell viability of 60% in HTS-Base and HTS-DCC, and at extended storage time (48hr) viability dropped below 40%. Samples preserved in HTS-FRS for 24 or 48 hours maintained viability levels of >90% in comparison to controls.

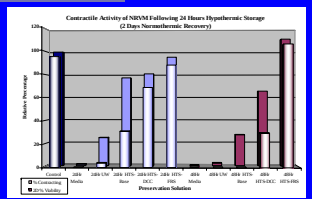


Figure 3. Metabolic and contractile activity of NRVCM 2 days following 24 or 48 hours cold storage (4C) in various preservation solutions. Shaded bars indicate percent of remaining population exhibiting contractile activity. Cell survival percentage determination was based upon fluorescent metabolic activity analysis in comparison with 37C controls.

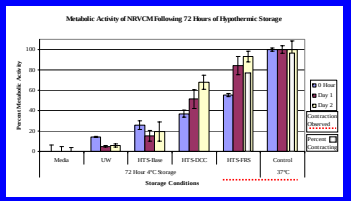


Figure 4. Metabolic activity of NRVCM monitored over 2 days following 4°C cold storage for 72 hours in either media, UW, HTS-Base, HTS-DCC or HTS-FRS. Dashed bars indicate spontaneous contractions observed upon rewarming. Fluorescent units (alamarBlue) given as percentage of controls (±SD). Samples preserved in HTS-FRS maintained viability levels and contractile activity in comparison to 37°C non-stored controls.

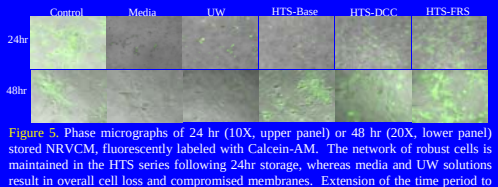
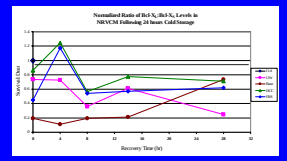


Figure 5. Phase micrographs of 24 hr (10X, upper panel) or 48 hr (20X, lower panel) stored NRVCM, fluorescently labeled with Calcein-AM. The network of robust cells is maintained in the HTS series following 24hr storage, whereas media and UW solutions result in overall cell loss and compromised membranes. Extension of the time period to 48hrs resulted in the preservation of the cardiac system in only the HTS-FRS samples.

Figure 6. Western blot of Bcl-X_L (upper bands) and Bcl-X_S (lower bands) levels in NRVCM at 0, 4, 8, 14, 28hrs recovery following 24hrs cold storage in UW, HTS-Base, HTS-DCC or HTS-FRS. Quantification of protein levels, normalized to non-stored controls (ratio=1) is represented by the ratio of Bcl-X_L to Bcl-X_S levels in the line graph.



Methods

Isolation of Neonatal Rat Ventricular Cardiac Myocytes (NRVCM)

Sprague-Dawley rat pups, postnatal day 1-3 (6 to 10g) were sacrificed by decapitation in accordance with IACUC protocols. All animals received humane care in compliance with the "Principles of Laboratory Animal Care" formulated by the National Society for Medical Research and the "Guide for the Care and Use of Laboratory Animals" published by the National Research Council (1996). NRVM were isolated according to the Worthington Biochemical Neonatal Cardiomyocyte Isolation Kit (Worthington Biochemical, Lakewood, NJ) substituting SigmaBlend H collagenase (Sigma Chemical Co., St. Louis, MO).

Isolated cardiac cells were pre-plated for 10 minutes at 37°C, 5% CO_2 to select NRVCM by differential adherence. The NRVCM enriched supernatant was plated at a density 75,000 cells/cm² onto tissue culture wear. Cells were cultured in complete DMEM for 7 days to obtain a synchronously contracting monolayer.

Hypothermic Exposure of NRVM Cultures

Twelve hours prior to hypothermic exposure, media was exchanged to reduce effects of serum deprivation. All preservation solutions {complete DMEM, UW Solution (Barr Labs), HypoThermosol® (HTS) HTS-Base, HTS-DCC, HTS-FRS (BioLife Solutions, Inc., Binghamton, NY)} were pre-cooled to 4°C, media was removed from NRVM cultures and replaced with sufficient volume of preservation solution to cover the surface. Cultures were maintained at 4°C for 24 to 72 hours.

Viability Assays

Upon removal from 4°C storage, the preservation solutions were promptly removed and replaced with 37°C complete DMEM. Assessment of the metabolic activity of the cells was determined utilizing the alamarBlue™ (Trek Diganostic) fluorescent probe multiple-endpoint assay, and membrane integrity was assessed using Calcein-AM (Molecular Probes). Briefly, media was removed from each well, 100ul of fluorescent probe was replaced and incubated for 60 minutes at 37°C, 5% CO_2. Fluorescent readings were obtained on a CytoFluor 4000 (Applied Biosystems, NJ) at excitation and emission wavelengths of 530nm and 590nm for alamarBlue and 485nm and 530nm for Calcein-AM. Spontaneous cardiomyocyte contraction was observed by phase contrast microscopy. Digital micrographs were acquired on a Zeiss Axiovert 200 using the AxioVision 3.1 software, Chroma FITC and DIC interference filters.

Protein Analysis

Cells were isolated at 0, 4, 8, 14 and 28 hours recovery following 24 hours cold storage in each of the five storage solutions and protein isolated by RIPA buffer containing protease inhibitors. Protein content was quantified using the Bradford method (Bio-Rad). Proteins were separated by 12% SDS-PAGE and transferred to PVDF membrane by semi-dry transfer. Membranes were probed (1:250) for Bcl-X_L and Bcl-X_S (Transduction Laboratories). Primary antibody binding was detected by HRP conjugated secondary antibodies, developed with Lumiglo Reagent (Cell Signaling Technology) and visualized on CL-Xposure film (Pierce). Densitometry was performed using the LabWorks software package (UVP Biolmaging Systems)

Summary of Results

- The HTS-FRS solution provided the highest viability at all hypothermic intervals examined maintaining the contracting monolayer of cells
- Metabolic activity and membrane integrity of FRS stored samples were 95% of controls at day 1 following hypothermic storage
- Micrographs demonstrate the preservation of the cardiac cell monolayer, including cardiomyocytes and other supporting mesenchymal cells in the HTS series (24 hour storage) and in HTS-DCC and HTS-FRS (24 or 48 hour storage)
- Disintegration of cell monolayer and breakdown of individual cells is evident in the media stored samples
- Cells which survive hypothermic storage (>65% CAM, AB, contractile activity) also exhibit similar trends in anti- and pro-apoptotic protein levels, with a peak in survival protein ratio at 4hr recovery
- Cells exhibiting moderate damage following storage show depressed survival protein ratios as compared to controls

Discussion and Conclusions

These data demonstrate differential survival of NRVCM when cold stored for 24 to 72 hours in various preservation solutions and the importance of assessing preservation success by multiple methods. Examining membrane status, metabolic activity, functional capability and protein expression provide supporting data to more accurately quantify the overall state of the cells following hypothermic preservation.

Hypothermic preservation solutions which are formulated to counteract free radical damage or chelate divalent cations (HTS-FRS and HTS-DCC) provided protection from stress incurred as a result of hypothermic storage. Cells stored in these solutions maintained adherence to culture dishes and exhibited positive viability data. Maintainence of mitochondrial function and ion stores are likely to be essential for regaining contractile function following hypothermic storage.

The survival ratio (Bcl-X_L to Bcl-X_S protein levels) drops below controls in all conditions of 24 hour/4°C stored cells, but those conditions which do not afford protection demonstrate a more pronounced drop to below 50% of control samples. This may implicate the initiation of apoptotic cascades resulting from increased cell stress during the storage and recovery periods.

Regenerative medicine/cell therapies and transplantation science face a bottleneck of transport and storage of sensitive biologic products. The ability to preserve myocytes (*survival and function*) and supporting cellular matrix may be useful in advancing these fields over preservation hurdles.

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