Above Zero Cold Storage of Cells *ex vivo - update*: CoStorSol® with glycine: Is there a need to freeze?

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INTRODUCTION

With the advent of stem cell technology and the resulting desire to translate to cell-based therapies, the preservation and *ex-vivo* processing of various therapeutic cells is now becoming a topic of interest. Some initial applications, such as hematopoietic stem cell therapy and cord blood banking, have relied heavily on freezing of cells to be later used in the patient. While logistically beneficial, the cryopreservation method of storing valuable stem cells has some inherent pitfalls that could potentially hinder growth in the emerging cell therapy field. Issues of recovery and efficacy of frozen stem cell samples is a concern[1], as well as toxicity due to nonphysiological cyropreservatives[2], causing some interest in alternate storage methods. Such methods as vacuum desiccation or freeze dying have shown some promise but remain problematic[3]. Hypothermic storage, or storage in refrigerated conditions, is another possibility. Indeed some authors, while investigating mechanisms of cell injury models, have utilized common organ preservation solutions in order to store cells such as human renal tubular or umbilical cord endothelial cells with success[4-6]. Typically, growth of cells requires complex growth media, often containing metabolites like glucose, yet conflicting evidence exists as to the suitability of cold storage of cells in such media. To determine the effectiveness of organ transplant solutions such as CoStorSol® (i.e. UW Solution), or other common solutions as cold storage media for mammalian cells, a cell culture model was employed using monolayers of either mouse fibroblasts (L929) or human umbilical vein endothelial cells (HUVECs). Monolayer cultures were exposed to various solutions, placed in refrigerated conditions, and monitored over time to test the efficacy of such solutions as storage time passes. These data were compared to monolayers exposed to normal growth media, which would normally be used at higher temperatures in order to stimulate cell proliferation. Overall, the results look promising for storing both types of cells, including the human cell HUVECs, for up to eight days at high viability and possibly longer with minimal loss. Interestingly, early findings suggest that a simple modification to the organ preservation solution, CoStorSol®, can improve storage times even further, up to 12 days or more according to this model.

METHODS

Obtaining HUVECs/L929, seeding and subculture

Cryopreserved vials of HUVECs were purchased from Lonza (Clonetics™ Endothelial Cell Systems). Cells were of first passage when frozen (primary cells) from a pooled donor population. The typical concentration of the frozen vials was around

1.0 x 10^6 cells/mL. Mouse fibroblasts (L929) were originally purchased from Sigma-Aldrich or Diagnostic Hybrids and continually kept in culture, subculturing cells as needed. Thawed cryovials of HUVECs or subcultured aliquots of L929 fibroblasts were seeded onto 175cm^2 culture flasks containing 35-40 mL of EGM-2 (endothelial cell growth media, Lonza; for HUVECs) or MEM with 10% FBS (minimal essential media, Gibco; for L929s). Initial seeding density of flasks was about 5,700 cells/cm^2, within the range of the recommended level. Following about 5-7 days of growth, flasks were subcultured to a new passage of cells while some cells were used to seed several 24-well plates.

Cold Storage Testing

For each experiment, when cells on the 24-well plates were near full confluence (90-100%), bathing media was removed and replaced with several test solutions before storage in refrigerated conditions. Typically one plate was checked each day following initiation of the experiment, each plate containing one or more wells dedicated to each of the test solutions. As a control, a typical culture prior to refrigeration was photographed to compare cells before and after storage (note Fig. 1A). Table 1 summarizes various storage solutions that were tested and some morphological observations of the cells throughout the test period. Cells were often stained, *in situ*, following storage, utilizing the Trypan blue vital dye. Following fixation with 10% formalin, cells were counted using an inverted microscope with counting reticule. Thus, an average percent viability of cell monolayers was obtained at various time points, by using multiple plates, after initiation of cold storage.

RESULTS

Morphology and Monolayer Viability Staining

Morphological observations were made for all experiments. Overall, 22 separate, daily observations were made on HUVECs for up to 8 days. Similar observations were made for L929 cells showing similar results (data not shown). Key morphological features were estimated or noted, such as percent confluence, percent rounding of cells, intercellular gaps, mitotic figures, etc. A healthy culture, with no cold storage time would typically be 95-100% confluent with little or no cell rounding (≤ 5%). Cell rounding is a well known morphological sign of degradation or damage in cells as evident in cytotoxicity test models. As cells round, percent confluence likewise diminishes as cells pull apart from each other and lose their characteristic spindle shape. Consequently, intercellular gaps also appear. Even though many of these morphological measures are difficult to quantify, a rough visual estimate can serve the purpose of observing general trends over time.

The morphological observations for one such experiment are shown in Table 1, for HUVECs. In this particular experiment, HUVEC monolayers were exposed to the following solutions: UW Solution (CoStorSol®), Hank's Balanced Saline Solution (HBSS),

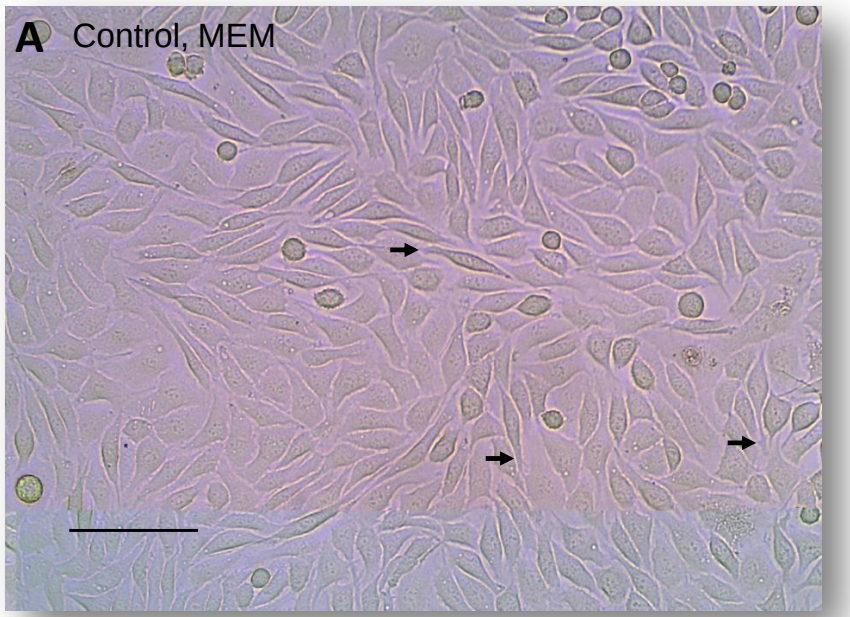
0.9% Sodium Chloride Solution (NaCl), or Endothelial Growth Media (EGM-2). HBSS, EGM-2, and NaCl showed considerable staining that progressed throughout the test period and by day 6, cell death was 65%, 100%, and 95%, respectively. HUVECs stored in the above three solutions also exhibited morphological signs of degradation, such as more cell rounding and less confluence, and prominent intercellular gaps. Evidence of cellular lysis was also present, primarily in the NaCl solution. Conversely, monolayers stored in CoStorSol®, in cold conditions, remained fairly stable. After day 6 in CoStorSol®, cells exhibited minimal staining at 5-10%, much less than observed in NaCl (95%). However, cells were beginning to show signs of pulling apart, at 50% confluence in CoStorSol® after 6 days, which was similar among all the groups. This reduction in confluence, however, did not appear to translate to an increase in rounding, as rounding was about 5% for CoStorSol® at day 6, compared to over 99% rounding of cells stored in NaCl at the same time point. Obvious rounding was present in the crystalloid solutions (NaCl/HBSS) whereas it was not so evident in CoStorSol®.

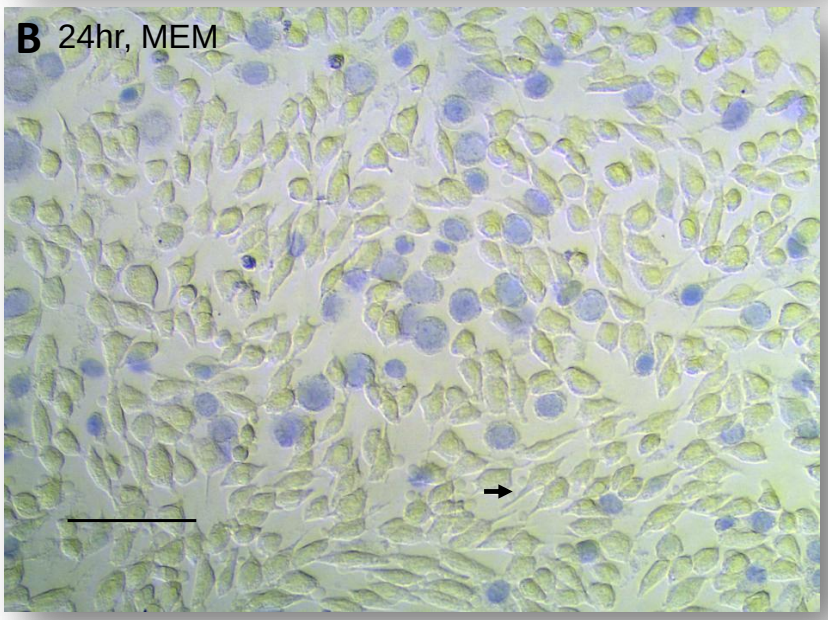
Table 1. Morphological observations and staining of HUVEC monolayers at 4-8°C

Test Solution	Day 1		Day 2		Day 3		Day 6	
CoStorSol®	95% confluent <1% rounded 1-2 mitotic bodies No intercellular gaps	pH 7.2 <5% stain	90% confluent 5% rounded No mitotic bodies Few gaps and enlarged cells	pH 7.5 <5% stain	95% confluent 1-5% rounded	pH 7.5 5% stain	50% confluent <5% rounded No mitotic bodies	pH 7.3 5-10% stain
HBSS	65% confluent 10-15% rounded No mitotic bodies Many gaps, 5% lysed	pH 7.0 15% stain	65-70% confluent 10-15% rounded No mitotic bodies Many gaps, enlarged cells**	pH 7.2 15% stain	60% confluent 95% rounded Many gaps, 5% lysed Enlarged cells	pH 7.2 40% stain	60% confluent 100% rounded Enlarged cells	pH 7.0 65% stain
NaCl (0.9%)	60% confluent 35% rounded No mitotic bodies Many gaps, 10-15% lysed	pH 5.8- 6.0 35% stain	60% confluent 45% rounded No mitotic bodies 30% lysed, enlarged cells***	pH 5.8 35% stain	60% confluent 80-85% rounded Many gaps Enlarged cells	pH 5.5 35- 40% stain	50% confluent 99% rounded Enlarged cells	pH 6.0 95% stain
EGM-2	60-65% confluent 5-10% rounded No mitotic bodies Many gaps, 5% lysed	pH 8.0 20% stain	60% confluent 50% rounded, small Enlarged cells	pH 7.8 90% stain	40% confluent 95% rounded Enlarged cells	pH 8.3 >95% stain	100% rounded Small cells Dark nuclei	pH 8.1 100% stain

**enlarged cells not stained ***some enlarged cells stained/some not

Table 1. Morphological observations and staining of HUVEC monolayers. Separate culture plates of HUVECs were obtained. Growth media was supplanted with test solutions and morphological observations were made after up to 6 days in cold storage (4-8°C). Attributes, such as percent confluence, were estimated by trained eye. Solution pH was measured with test color strips (Fisher), staining was done with Trypan blue and total percent of stained cells was estimated by aid of a reticulated eye piece over several fields of view.





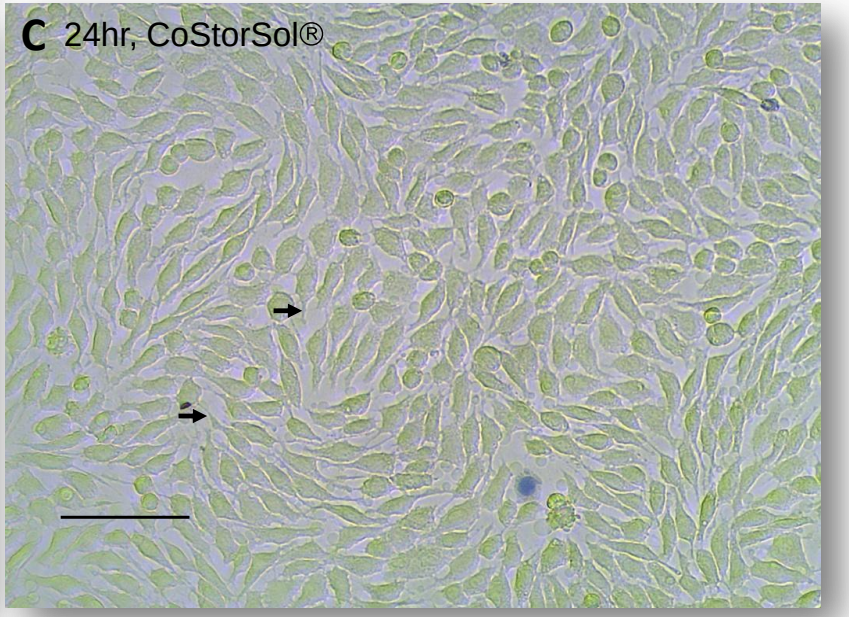


Figure 1: Culture of mouse fibroblast cells (L929) with various storage solutions at 4°C. L929 cells were grown in MEM media with 10% fetal bovine serum (FBS) at 37°C until cells reached a monolayer with approximately 90% confluence. Media was then decanted and replaced with one of several test solutions and stored in refrigerated conditions (4-8°C) for up to 14 days. Multiple culture plates were used to allow sampling throughout the storage period. Each day after storage, wells for each solution were stained with Trypan blue and fixed with 10% formalin. **A**: Cells taken directly from incubation at 37°C, stored in MEM and immediately processed (i.e. stained with Trypan blue and fixed), remained largely viable with >98% cells unstained. Cells show processes and a spindle shape (arrows). **B**: Following cold storage for one day in growth media (MEM 10% FBS, Gibco), cells began showing cell death with approximately 40% exhibiting staining with Trypan blue. Rounding in shape of the cells became more prevalent in storage with media, occurring commonly among stained dead as well as unstained live cells. **C**: Following storage for one day in CoStorSol® (also known as UW Cold Storage Solution), cells remained largely viable with >90% cells unstained. Cells retained processes and spindle shape (arrows). *Scale bar = 100µm; approximate magnification: 100X.*

The pH of the various test solutions was generally stable during the test period (see Table 1). Between the solutions, however, pH varied from acidic to basic. Bathing solutions of NaCl ranged in pH from 5.5 to 6.0. This is relatively acidic for most cells. Conversely, EGM-2 exhibited more alkaline pH levels, from 7.8 to 8.3. CoStorSol® and HBSS exhibited pH levels in a more physiological range. The pH levels ranged from 7.2 to 7.5 for CoStorSol® and from 7.0 to 7.2 for HBSS. Figure 1 shows a visual example of a control L929 monolayer, prior to storage, along with examples of L929 monolayers that have been placed in storage for 24 hours in various solutions and then stained with Trypan blue. Cells taken directly from incubation at 37°C, stored in MEM and immediately processed (i.e. stained with Trypan blue and fixed), remained largely viable with >98% cells unstained. Cells show processes and a spindle shape. Following storage for one day in CoStorSol®, cells remained largely viable with >90% cells unstained. Cells retained processes and spindle shape (arrows). Following storage for one day in MEM, cells began showing cell death with approximately 40% exhibiting staining with Trypan blue. Rounding in shape of the cells became more prevalent in storage with MEM, occurring commonly among stained, dead as well as unstained live cells. Similar but less drastic results were obtained with NaCl when compared to MEM.

Figure 2 shows similar examples for HUVECs at shorter and longer storage times. Cells stored in CoStorSol® for 16 hours remained largely viable with >98% cells unstained. Cells showed elaborate processes and spindle shape. Following storage for two days in CoStorSol®, cells began to exhibit Trypan blue staining (approx. 15% stain) yet retained processes and general spindle shape. After storage for 16 hours in Saline for Injection (0.9% NaCl, Baxter), cells exhibited no staining, although rounding in shape of the cells became prevalent. After storage for two days in saline, HUVECs were mostly stained and rounded.

Counting HUVECs/L929 for average viability

In many cases, cell viability was also measured utilizing a microscope fitted with a grid based reticule following the initial visual assessment for morphology. Figure 3 summarizes the quantitative results of average cell viability (percentage of unstained cells) throughout the test period for L929 mouse fibroblasts. Cells were placed in refrigerated conditions for up to 14 days and subsequently stained (as above) and counted. Each data point consisted of an average of nine fields of view from a single plate well at a particular time period. This was then used to obtain an average viability. The average viability values (each constituting an 'N' value) were then binned into two day periods and graphed (i.e. days 1-2, 3-4, and so on). After a period of 3 to 4 days in cold storage, L929 cells showed 97% (N = 13) viability when stored in CoStorSol®. A significant reduction in viability was seen after storage in NaCl for the same period, to about 56% (N = 7; $P < 0.01$) viability. A further reduction, especially after storage in NaCl, was observed with only 19% (N = 4) of cells being viable at 7 or 8 days. This is in contrast to average viability of 87% (N = 11) after 8 days storage in CoStorSol®. Cell viability remained high in L929 cells stored in CoStorSol® until about 8 days storage, with longer times showing some reduction. However, CoStorSol® with glycine (3mM) added exhibited significantly higher viability of 82% (N = 8; $P < 0.001$) after 9 or 10 days of storage when

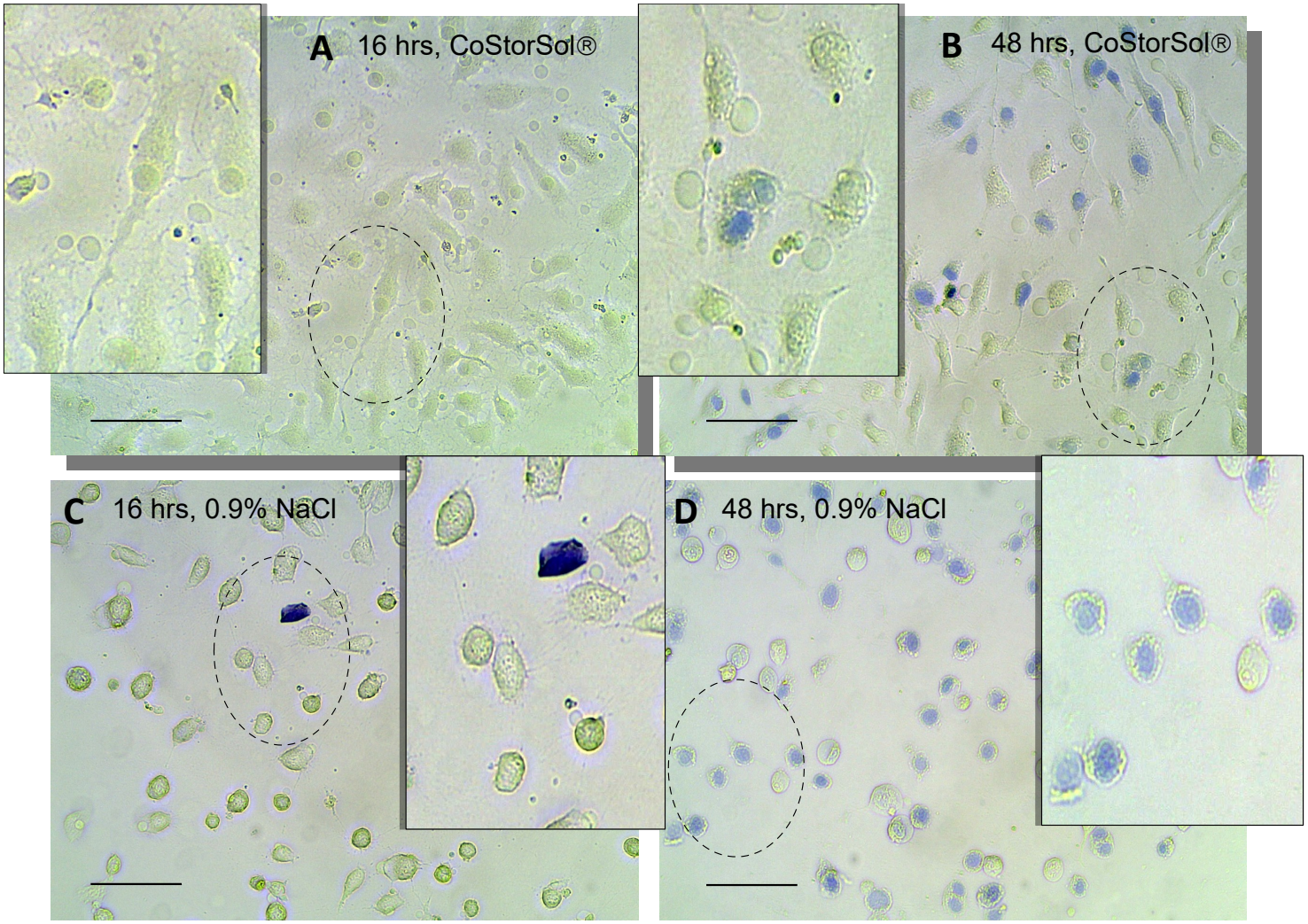


Figure 2: Storage of HUVECs at short, long storage times. HUVECs were grown in EGM media as before (see Fig. 1 for detailed method). Media was replaced with one of two test solutions and stored in refrigerated conditions (4-8°C) for up to two additional days. **A**: Cells stored in CoStorSol® for 16 hrs. remained largely viable with >98% cells unstained. Cells showed elaborate processes and spindle shape (*see inset, ~2x*). **B**: Following storage for two days in CoStorSol®, cells began to exhibit Trypan blue staining (approx. 15% stain) yet retained processes and general spindle shape. **C**: After storage for 16 hrs. in Saline for Injection (0.9% NaCl, Baxter), cells exhibited no staining, although rounding in shape of the cells became prevalent . **D:** After storage for two days in saline, HUVECs were mostly stained and rounded. *Scale bar = 100µm; approximate magnification: 100X (inset ~200x).*

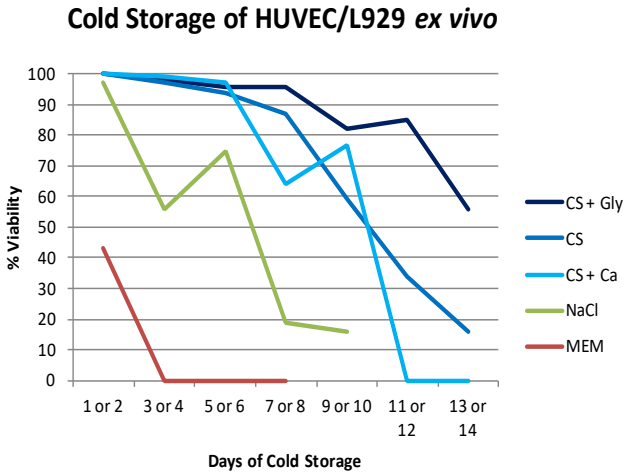
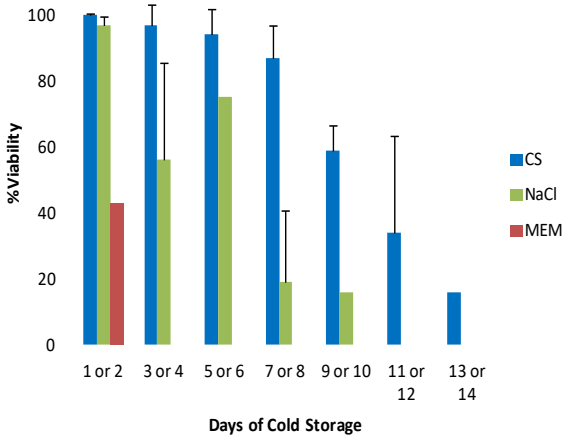
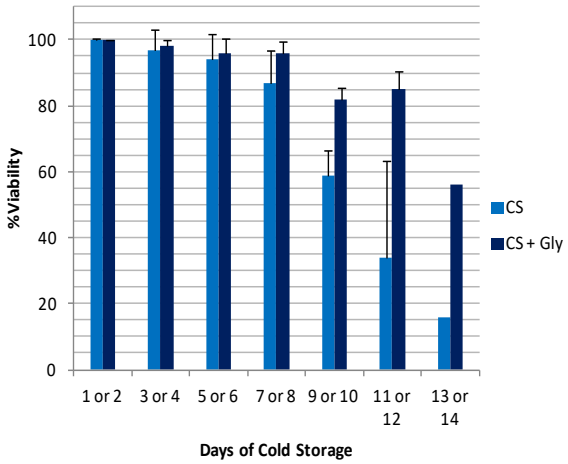


Figure 3. ***CoStorSol®-cell preservation ex vivo.*** **Mouse fibroblasts (L929) were grown to near 100% confluence on 24-well plates. Cells were exposed to either CoStorSol® (CS) or CoStorSol® with glycine (3mM-Gly) or calcium ion (3mM-Ca) added. Other solutions commonly used for storage were also tested including normal saline (%0.9-NaCl) and cell growth media (MEM). Cells were then placed in refrigerated conditions for up to 14 days. After a period of 1 to 4 days in cold storage, L929 cells showed about 100% viability (Trypan blue staining method) when stored in CS-based solution (CS-blue, CS+Gly-dark blue, CS+Ca-light blue). Conversely, cells stored in NaCl (green) or MEM (red) showed deterioration in viability within the first few days.**





compared to CoStorSol® alone (59%, N = 10). Even at storage times of 11 or 12 days, CoStorSol® plus glycine additive showed high viability (85%, N = 4). When L929 cells were stored in MEM growth media, the viability diminished quickly. Only 43% (N = 4) of cells were viable at 1 or 2 days after being stored in MEM in refrigerated conditions, proceeding to 0% afterwards.

When HUVECs were stored in the cold with the same solutions, very similar results were obtained. Figure 4 summarizes the quantitative results of average cell viability (percentage of unstained cells) throughout the test period for human umbilical cord endothelial cells. Cells were placed in refrigerated conditions for up to 10 days and subsequently stained (as above) and counted. After a period of 3 to 4 days in cold storage, HUVECs showed 87% (N = 3) viability when stored in CoStorSol®. A trend of reduction in viability was seen after storage in NaCl for the same period, to about 55% (N = 3) viability. A further reduction, especially after storage in NaCl, was observed with only 17% (N = 2) of cells being viable at 8 days. This is in contrast to average viability of 86% (N = 2) after 8 days storage in CoStorSol®. No statistical comparison was made between the two test groups, given the low N value, although a trend for reduction of viability appears robust for saline (e.g. non-overlapping error bars and agreement with visual,

morphological data). Data assessment will continue in order to perform a t-test on the data. As the dashed line shows for CoStorSol®, there appeared to be no difference between L929 cells and HUVECs in viability during cold storage. Both types of cells also appeared to react in a similar fashion when stored in the other solutions (data not shown) like EGM/MEM, NaCl, and CoStorSol® plus glycine.

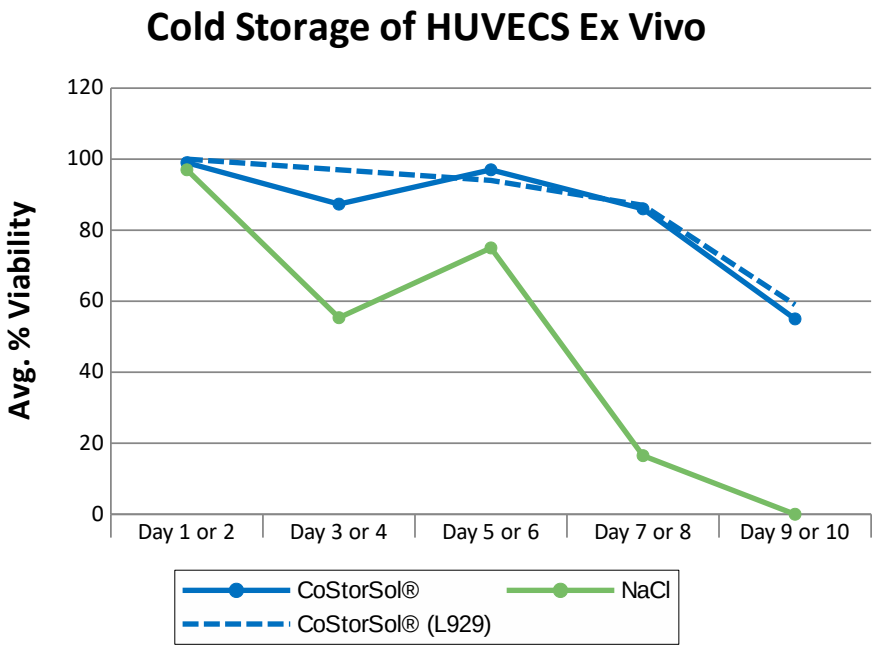


Figure 4. **Cold storage culture of Human Umbilical Vein Endothelial Cells (HUVECs) stored in CoStorSol® as compared with regular saline for injection (0.9% NaCl). HUVECs were grown to near 100% confluence on 24-well plates and subsequently exposed to either CoStorSol® (blue) or saline (NaCl)(green). Cells were then placed in refrigerated conditions for up to 10 days. After a period of 3 to 4 days in cold storage, HUVECs showed 87% viability (Trypan blue staining method) when stored in CoStorSol®. A slight trend of reduction in viability was seen after storage in NaCl for the same period, to about 55% viability. A further reduction, especially after storage in NaCl, was observed after 6 days with only 17% of cells being viable at 8 days. This is in contrast to average viability of 86% after 8 days storage in CoStorSol®. There appeared to be no difference in viability for CoStorSol® storage between HUVECs and L929 cells (dashed blue line).**

SUMMARY AND CONCLUSION

It was apparent from this study that, on a morphological and quantitative basis, CoStorSol® (i.e. UW Solution) was superior for cold storage of human umbilical vein and mouse fibroblast cultured monolayers. As mentioned above, cells stored in crystalloid solutions, such as NaCl (0.9%) or HBSS, deteriorated in terms of rounding and evident lysis as soon as day one of cold storage. This observation appears to be corroborated by the quantitative counting of cells, showing a trend for reduction in viability when cells are stored in plain saline. Growth media was by far the least effective solution in terms of cold preservation of cells in this model, perhaps due to the fact that media contains nutrients such as glucose, intended to stimulate

the growth and metabolism of cells. Several studies have mentioned this problem of storing cells in media in cold conditions[3,7]. Possibly, media stimulates energy production in cells that in turn is counterproductive to effective static cold storage, as one study suggests[8]. The pH of the solutions may also be a factor in reduction of viability, with saline showing nonphysiological pH values over time. It is well known that cells typically switch to anaerobic glycolysis under cold conditions, thereby promoting lactic acid build-up. Therefore, the protective effect of CoStorSol® may be partly due to the presence of a buffer in this solution. Additional protection could be offered by some of the other constituents of CoStorSol®, however, as suggested by the absence of effective preservation by HBSS, a buffered saline solution. It is interesting to note that with a simple additive - namely, the amino acid, glycine - to the solution, CoStorSol® becomes even better suited for the purpose of cold storage of cells. Normally, CoStorSol® is intended to statically store organs in a viable state for transplant procedures in refrigerated conditions until they can be used. Undoubtedly, cells in culture or suspension are more exposed than they would be within an organ, and perhaps this simple addition provides them a little more protection or adds some factor already present in a typical organ's reserve.

Glycine, an amino acid that is present and produced within the human body, has been shown to have certain wide-ranging biological functions. These include, among others, to act as an intermediary or component in metabolic pathways. For example, glycine is a component of glutathione, an endogenous cellular antioxidant. Glycine is also an inhibitory neurotransmitter with ionotropic receptors primarily in the spinal cord. The cytoprotective effects of glycine involve direct suppression of necrotic pathways, possibly by prevention of plasma membrane disruption[10]. In addition other, less direct pathways of glycine protection have been shown, such as an immunomodulatory role in the reduction of inflammation through more complex signaling pathways[10].

Currently, in the cell therapy clinical setting, autologous transplants and many allogeneic stem cell transplants necessitate cryopreservation protocols. However, protocols exist in certain situations utilizing fresh stems cells in allogeneic transplantation provided the transfer from donor to recipient can be established within 72 hours[9]. Encouragingly, this study shows that with very simple and practical modification, organ preservation solution can be augmented to extend cold storage of cells, without freezing, to 11 or 12 days in this model.

About the author:

***James Einum**, PhD, is currently Manager of Solutions Research at Preservation Solutions, Inc. In that role for ten years, he has developed and advanced medical projects, working in solution formulation and design of organ/tissue preservation systems. Concurrently he has worked to develop and maintain design control and risk management systems at PSI and implement these quality controls in compliance with ISO/FDA regulations. His undergraduate work was done at University of Wisconsin-Madison with a major in biology. PhD work, at Marquette University, Milwaukee WI, focused on physiology (nervous system) and, followed by post-doctoral work abroad in Bordeaux, France and Montreal, Canada, nurtured a broad biological background including physiological as well as molecular techniques and concepts. He also has other industry experience, including a year employed in a medical testing laboratory (LabCorp, Elmhurst, IL) and several semesters' worth of teaching experience. He continues striving to improve and innovate in the medical field, including new regulatory submissions.*

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Data Collected and prepared by/dates: James Einum/August 2013 to present

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