Comparison of human umbilical vein endothelial cell cryopreservation in Lifor versus Cryostor

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Abstract:

Lifor cryopreservation experiments with human umbilical vein derived endothelial cells (HUVECs) at 0-10% DMSO were compared with Cryostor containing 2, 5 and 10% DMSO. Suspended cells were cryopreserved in one mL volumes in cryovials at a controlled rate of 1°C/min, with induced nucleation at -6°C, to -80°C. The cryovials were then transferred to <-135°C in vapor phase nitrogen for 3 days. After 3 days the vials were rewarmed rapidly in a 37°C water bath and placed on ice as soon as the last ice disappeared. The cells were then washed by centrifugation and resuspended. An aliquot was taken for assessment of viability by trypan blue and the remainder of the cells were plated in a single well of a 6 well plate. After overnight incubation with culture media under physiological conditions the cells were evaluated by alamarBlue, this was repeated daily for a total of 4 days. Each experiment had two duplicate cryovials and each experiment was repeated 4 times (n=8, N=4). Results were assessed using t-test and analysis of variance (ANOVA) with p-values <0.05 considered significant. The results with Lifor demonstrated excellent outcomes comparable with Cryostor5 and Cryostor10 at 3-10% DMSO concentrations. Lifor demonstrated statistically higher viability by trypan blue exclusion shortly after rewarming and similar values by alamarBlue over 4 days in cell culture post-thaw. The Lifor formulation was very effective for HUVEC cryopreservation. Future evaluations with other cell types and longer storage times are in progress.

Introduction:

There is growing demand for chemical solutions that can maintain cell, tissue and organ viability during storage and transport of biological materials. Biopreservation focuses on the development of methods for short and long-term storage of biologics with the expectation of their return to near prestorage functionality under physiological conditions. Biopreservation is expanding rapidly in areas such as cell therapy, stem cell research, personalized medicine, biopsy transport, cell banking, cancer research, tissue banking and organ preservation. These diverse areas of application drive the need for process optimization to assure post-storage and transport effectiveness (Baust, 2009).

Lifor consists of a novel chemical formulation combined with 90nm nanoparticles. The nanoparticles have a lipophilic outer layer and a hydrophilic inner core designed to transport and deliver nutrients and oxygen, while removing waste products and carbon dioxide, from tissues and organs. Previous studies have demonstrated that Lifor carries and releases oxygen effectively in kidney, heart, and liver under warm conditions (Gage, 2009; Stowe, 2007; 2008; Brockbank, unpublished results).

In this proof-of-concept feasibility study we have evaluated the potential of Lifor as a vehicle for cryoprotectants during cryopreservation. More specifically, the purpose of the experiments in this report was to determine whether the current formulation of Lifor could match industry gold standard solutions for frozen storage, Cryostor.

Materials and Methods:

HUVECs sourced from a commercial provider, the American Type Culture Collection (ATCC). Cryovials from our cell master bank containing frozen cells of each type were rewarmed in a 37°C water bath until the majority of the ice had melted. The contents of the vial was then be diluted with fresh culture medium and the cells placed in a large volume of culture medium in a tissue culture flask. Once the cells achieve confluence the cultures were passaged using trypsin with some cells being used for experiments and the remainder expanded (passaged) for the next experiment. After 3-4 expansions any further experiments performed used fresh samples from our master cell bank.

0, 1, 2, 3, 5, and 10% DMSO in Lifor were compared with commercially available Cryostor formulations (2, 5 and 10% DMSO). The cells were cryopreserved in cryovials containing 1 mL with $5x10^5$ cells suspended in cryoprotectant solution. The cells were equilibrated with experimental and control cryoprotectant solutions on ice for 20 minutes before initiating cryopreservation using a controlled rate of ~1°C/min to -80°C with nucleation at -6°C. Once the samples achieve -80°C they were transferred to vapor phase nitrogen storage below -135°C for 3 days. Samples were rewarmed by immersion in a 37°C water bath until most of the ice had melted and then subjected to viability assessment. Post rewarming assessment in HUVEC experiments was

performed by removal of a small volume of cells for trypan blue assessment and plating the remaining contents of each cryovial in a single well of a 6 well plate, two cryovials per treatment group. Cells in 6 well plates were evaluated for relative cell viability by alamarBlue after attachment to a culture substrate and overnight incubation under physiological conditions (day 0), and then again on days 1-3 to get growth/survival curves and detect the possible impact of delayed cell death. In each case the results were compared with untreated control cell samples.

HUVEC cell viability was determined by 1) the trypan blue exclusion method and 2) by measuring metabolic activity using the alamarBlue assay. The trypan blue dye exclusion test is used to determine the number of viable cells present in a cell suspension (Strober, 2001). It is based on the principle that live cells possess intact cell membranes that exclude certain dyes, such as trypan blue, whereas dead cells do not. In this test, a cell suspension is simply mixed with dye and then visually counted using a hemocytometer to determine the cell numbers that take up or exclude dye. In the protocol used here viable cells had clear cytoplasm whereas a nonviable cell had blue cytoplasm.

The rationale for using the alamarBlue assay is that we can reassess the same cell cultures or tissues over time post-treatment in cell culture (O'Brien, 2000; Brockbank, 2011). The reagents are not toxic so they can be washed off the cells and be replaced with fresh culture media. AlamarBlue is a non-toxic fluorometric indicator based on detection of metabolic activity. Fluorescence was measured at an excitation wavelength of 544 nm and an emission wavelength of 590 nm. This evaluation was performed daily for 4 days to allow characterization of re-warmed cells. After each reading the cell cultures were washed and placed back in tissue culture. Results shortly after rewarming (day 0) demonstrate cell viability. Decreases over 1-2 days of culture are indicative of cell death due to apoptosis and increases are due to cell recovery or proliferation. Data is expressed in terms of relative fluorescence units per culture well for isolated cells (mean ± 1se) in percent of untreated controls. Representative digital images of cell cultures were also taken in selected experiments on the day after plating of treated groups.

Each experiment was repeated four times (N=4) with at least 2 replicates within each experiment (n=8). Comparisons used t-tests and ANOVA (with multiple comparisons using Bonferroni adjustment of error, or Kruskal-Wallis non-parametric test and Dunns post-test as appropriate with 95% confidence intervals. The data is expressed as the mean ± 1SEM with p-values<0.05 being considered significant.

Results:

The cryopreservation experiments with human umbilical vein endothelial cells were an outstanding success, well beyond our expectations. The dye exclusion viability test using Trypan Blue demonstrated statistically significant higher outcomes for 3-10% Lifor compared with 5-10% Cryostor immediately post-thaw (**Fig. 1, Table I, Table II**). The rest of the cells were plated in cell culture and incubated under physiological conditions until the next day (Day 0). The cultures were then evaluated using alamarBlue on 4 consecutive days. The alamarBlue results demonstrate that 3-5% Lifor was essentially the same as 5-10% Cryostor. 2% Cryostor was significantly better than 2% Lifor (**Fig. 2**). Bright field microscopy demonstrated that there were more non-adherent cells (floating bright, rounded cells) in pictures of Lifor treated cell cultures taken at the end of Day 0 post-thaw (**Fig. 3**). Statistical analyses are provided in **Tables I and II**.

Discussion:

There are two types of solution in use for cell, tissue and organ storage and transport, namely intracellular-type solutions and extracellular type solutions. **Intracellular-type solutions** are typically hypertonic having a composition that is designed to restrict the passive exchange of water and ions during hypothermic exposure when cell membrane pumps are inhibited. This is achieved by raising the concentration of potassium, and reducing sodium, to mimic that of the intracellular space and thereby restricting passive fluxes of these ions. An I-type solution usually includes a non-permeating (impermeant) anion to partially replace chloride ions in the extracellular space and thereby provide osmotic support to balance the intracellular oncotic pressure generated by macromolecules and their associated counter-ions locked inside the cell (these molecules do not cross the plasma membrane even passively due to their size and charge). Energy-consuming pumps normally control the water content of cells, but during cold exposure and/or energy depletion, this control mechanism is compromised and cells imbibe water due to the oncotic pressure of the intracellular milieu. Cell swelling due to this passive hydraulic flux can be inhibited by raising the osmolality of the extracellular medium and by incorporating an impermeant anion such as lactobionate, or gluconate. Hence, these biophysical characteristics are the basis of why such solutions have been termed "Intracellular-type". Cryostor is an example of an intracellular-type cryopreservation solution

In contrast **Extracellular-type solutions** are isotonic having a plasma-like complement of ions that mimics the normal extracellular environment of cells. Examples of this type of solution can range from simple saline ("extracellular" in terms of the concentration of NaCl and osmolality) to tissue culture media that contain a more complete complement of ions, amino acids and other metabolites to mimic the extracellular composition of plasma and other body fluids. Such solutions are often, but not always, poor preservation solutions at reduced temperatures principally because they do not counteract the passive biophysical processes outlined above. Lifor is an extracellular type cryopreservation solution.

A variety of factors are known to influence cell survival during cryopreservation, these factors include:

- Freezing-Compatible pH Buffers
- Vehicle Solution Selection (may vary with cryoprotectant selection)
- Apoptosis Inhibitors (may be required to get long-term post-thaw cell survival for some cells)
- Cryoprotectant Selection (Optima may vary with vehicle solution selected)
- Cooling Rate
- Storage Temperature
- Warming Rate
- Cryoprotectant Addition/Elution Conditions (number of steps, temperature)

However, the role of vehicle solutions, the solution to which you add your cryoprotectant(s), is often overlooked. It is generally assumed that simple salt buffers or conventional culture media used to nurture cells at physiological temperatures will also provide a suitable medium for exposure at low temperatures. In a manner similar to the later discussion of optimum control of the cells' environment during hypothermic short-term storage, cryopreservation also demands consideration of the chemical composition of the buffer medium used as a vehicle for the cryoprotectants as well as the temperature to which the cells are exposed. It has been a common practice in tissue banking to use tissue culture media as the base solution for preservation media. However, for the reasons outlined below, tissue culture media, which are designed to maintain cellular function at normal physiological temperatures, are often inappropriate for optimum preservation at reduced temperatures and we have long advocated the use of intracellular-type solutions, like Cryostor, as more appropriate vehicle solutions for cryoprotectants (Biolifesolutions.com; Taylor, 2001). Maintaining the ionic and hydraulic balance within tissues during cold exposure can be better controlled in media designed to physically restrict these temperature-induced imbalances and can be applied equally to the choice of vehicle solution for adding and removing cryoprotectants in a cryopreservation protocol.

The results using HUVECs were beyond our expectations demonstrating equivalence with Cryostor at both 5 and 10% DMSO (**Figs. 1 and 2**). The trypan blue data immediately post-thaw demonstrated significantly higher cell survival in the presence of Lifor (**Fig. 1**) that was not reproduced on the following day by alamarBlue (**Fig. 2**). The explanation for the difference in outcomes using the two different viability assays is likely that there was delayed cell death in the Lifor treated groups that reduced the number of surviving cells. The observation that the Lifor treatment groups contained non-attached floating bright cells the next day after plating supports this explanation (**Fig. 3**). However, the alamarBlue data (**Fig. 2**) demonstrated that 3-10% DMSO in Lifor is equivalent to Cryostor5 and Cryostor10. It is likely that supplementation of Lifor with antioxidants (glutathione or vitamin E) or antiapoptotic agents will improve cell survival resulting in superior outcomes at all DMSO concentrations in Lifor versus Cryostor. Other potential supplement categories include the following (Brockbank, 2007):

- Impermeant anions (lactobionate and gluconate)
- Calcium channel blockers
- Protease and phospholipase inhibitors
- Iron chelators
- Metabolic substrates
- Sugars

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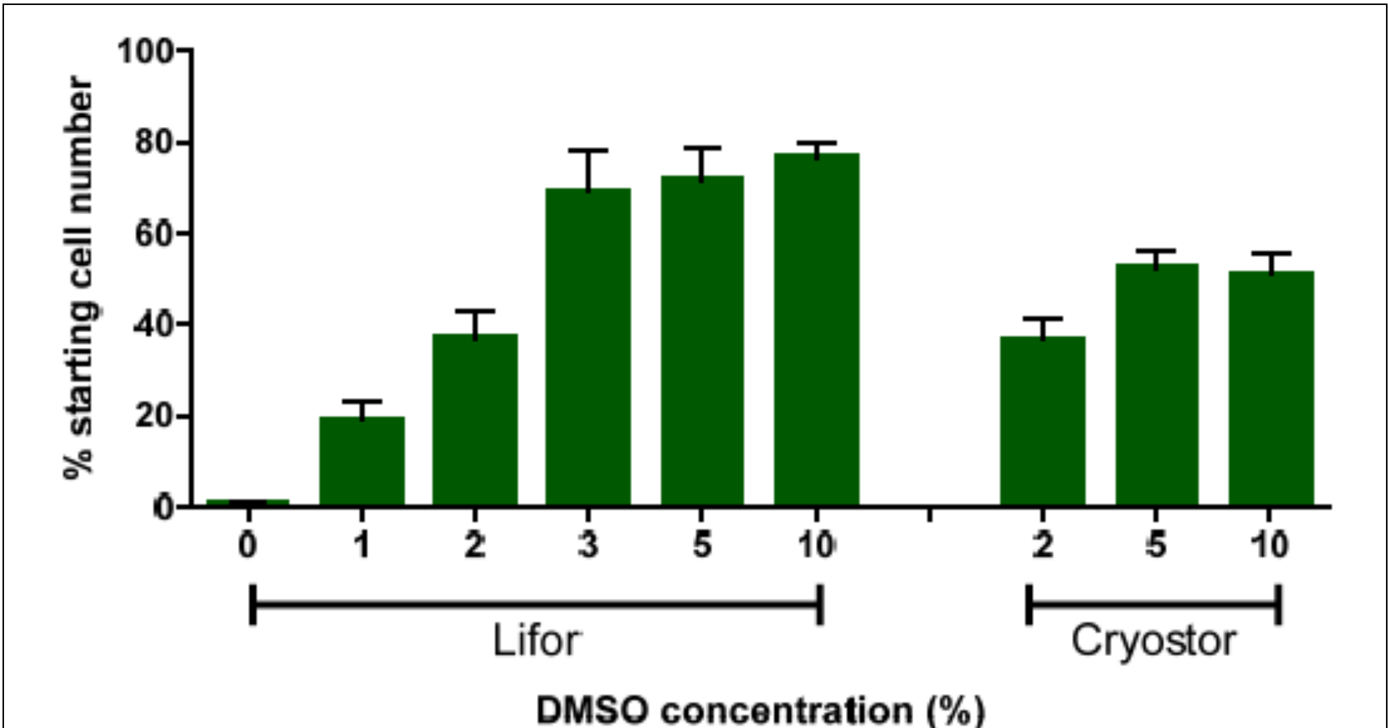

Fig. 1: HUVEC Trypan Blue exclusion viability assessment post-thaw. Cell counts were performed immediately after rewarming before plating for alamarBlue viability. Significantly higher values were observed for Lifor at 2-10% DMSO compared with Cryostor. The data is presented as the mean ± 1se (N=4, n=40). Statistics are presented in Table II.

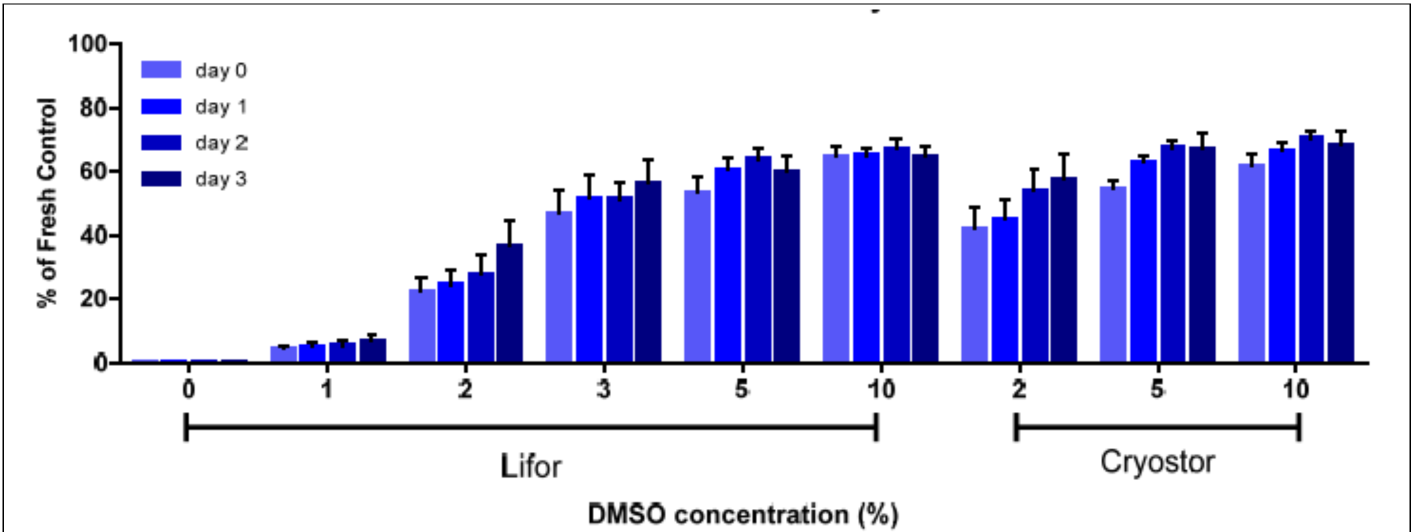

Fig. 2: HUVEC alamarBlue viability assessment post-thaw. The cells were plated after rewarming, washing, and Trypan Blue exclusion. After overnight incubation under physiological conditions the alamarBlue assay was performed (day 0) and then it was repeated on days 1-3. Excellent out comes were observed at 3-10% DMSO in Lifor similar to 2-10% DMSO in Cryostor. The data is presented as the mean ± 1se (N=4, n=40). Statistics are presented in Table III.

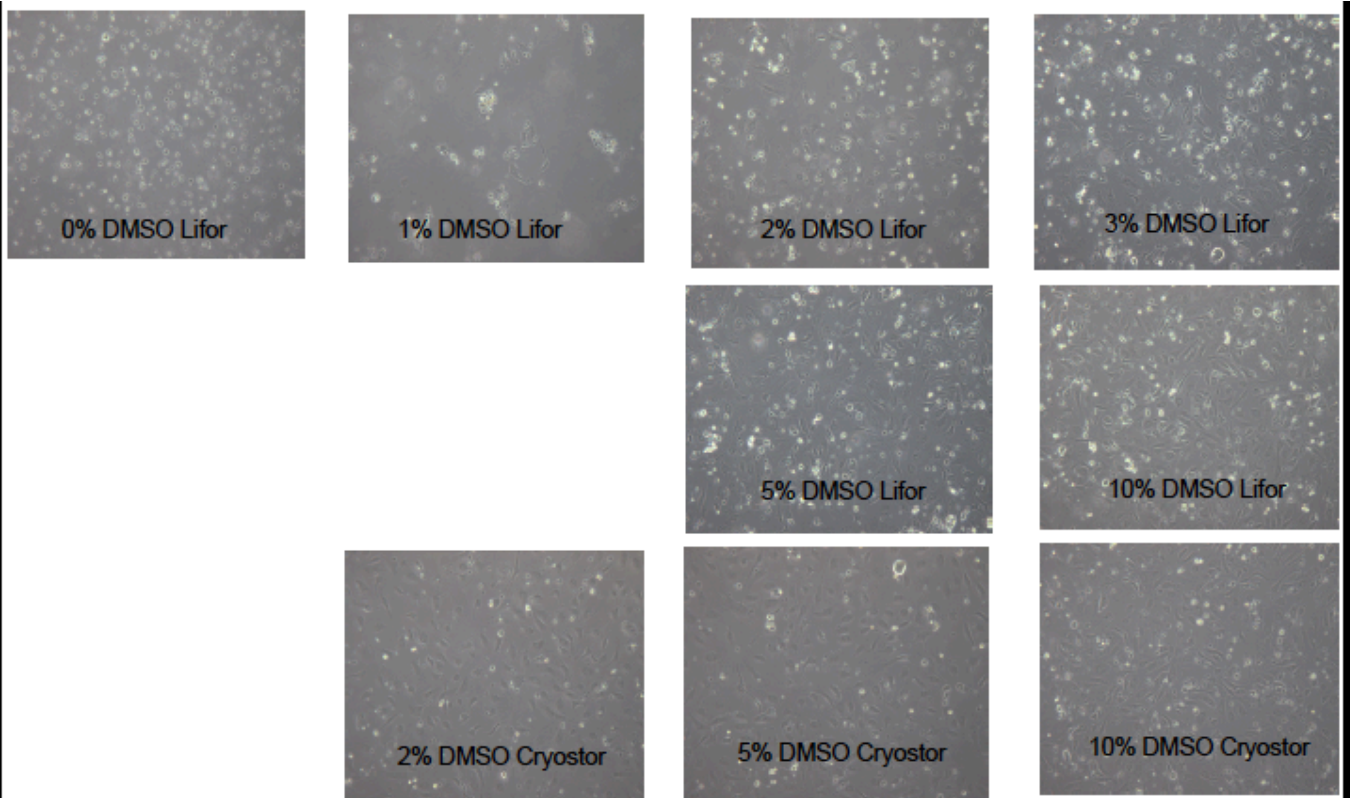

Fig. 3: Montage of micrographs taken after overnight incubation of rewarmed HUVECs under physiological conditions. Please note the increase in bright rounded cells that are floating. We believe the floaters represent cells that survived cryopreservation in Lifor but had some form of delayed cell death either due to apoptosis or damaged adhesion system. This is the probable explantation for the difference in outcomes using Trypan Blue (Fig. 1) and alamarBlue (Fig. 2). Adherent spread cells can be seen attached and spread on the cell culture substrate in all cases except the 0 and 1% DMSO groups.

Table I: HUVEC Trypan Blue data Statistical Analysis.

	ANOVA	t-test
0% lifor vs 1% lifor	no	p<0.001
0% lifor vs 2% lifor	p<0.001	p<0.0001
0% lifor vs 3% lifor	p<0.0001	p<0.0001
0% lifor vs 5% lifor	p<0.0001	p<0.0001
0% lifor vs 10% lifor	p<0.0001	p<0.0001
0% lifor vs 2% cryostor	p<0.001	p<0.0001
0% lifor vs 5% cryostor	p<0.0001	p<0.0001
0% lifor vs 10% cryostor	p<0.0001	p<0.0001
1% lifor vs 2% lifor	no	p<0.05
1% lifor vs 3% lifor	p<0.0001	p<0.001
1% lifor vs 5% lifor	p<0.0001	p<0.0001
1% lifor vs 10% lifor	p<0.0001	p<0.0001
1% lifor vs 2% cryostor	no	p<0.05
1% lifor vs 5% cryostor	p<0.01	p<0.0001
1% lifor vs 10% cryostor	p<0.01	p<0.001
2% lifor vs 3% lifor	p<0.01	p<0.05
2% lifor vs 5% lifor	p<0.01	p<0.01
2% lifor vs 10% lifor	p<0.001	p<0.001
2% lifor vs 2% cryostor	no	no
2% lifor vs 5% cryostor	no	no
2% lifor vs 10% cryostor	no	no
3% lifor vs 5% lifor	no	no
3% lifor vs 10% lifor	no	no
3% lifor vs 2% cryostor	p<0.01	p<0.01
3% lifor vs 5% cryostor	no	no
3% lifor vs 10% cryostor	no	no
5% lifor vs 10% lifor	no	no
5% lifor vs 2% cryostor	p<0.01	p<0.01
5% lifor vs 5% cryostor	no	p<0.05
5% lifor vs 10% cryostor	no	p<0.05
10% lifor vs 2% cryostor	p<0.001	p<0.0001
10% lifor vs 5% cryostor	no	p<0.001
10% lifor vs 10% cryostor	p<0.05	p<0.01
2% cryostor vs 5% cryostor	no	p<0.05
2% cryostor vs 10% cryostor	no	no
5% cryostor vs 10% cryostor	no	no

Table II: HUVEC alamarBlue data Statistical Analysis.

	day 0		day 1		day 2	
	ANOVA	t-test	ANOVA	t-test	ANOVA	t-test
0% lifor vs 1% lifor	no	p<0.05	no	p<0.01	no	p<0.01
0% lifor vs 2% lifor	no	p<0.001	no	p<0.001	p<0.05	p<0.001
0% lifor vs 3% lifor	p<0.0001	p<0.0001	p<0.0001	p<0.0001	p<0.0001	p<0.0001
0% lifor vs 5% lifor	p<0.0001	p<0.0001	p<0.0001	p<0.0001	p<0.0001	p<0.0001
0% lifor vs 10% lifor	p<0.0001	p<0.0001	p<0.0001	p<0.0001	p<0.0001	p<0.0001
0% lifor vs 2% cryostor	p<0.0001	p<0.0001	p<0.0001	p<0.0001	p<0.0001	p<0.0001
0% lifor vs 5% cryostor	p<0.0001	p<0.0001	p<0.0001	p<0.0001	p<0.0001	p<0.0001
0% lifor vs 10% cryostor	p<0.0001	p<0.0001	p<0.0001	p<0.0001	p<0.0001	p<0.0001
1% lifor vs 2% lifor	no	p<0.05	no	p<0.01	no	p<0.01
1% lifor vs 3% lifor	p<0.0001	p<0.0001	p<0.0001	p<0.0001	p<0.0001	p<0.0001
1% lifor vs 5% lifor	p<0.0001	p<0.0001	p<0.0001	p<0.0001	p<0.0001	p<0.0001
1% lifor vs 10% lifor	p<0.0001	p<0.0001	p<0.0001	p<0.0001	p<0.0001	p<0.0001
1% lifor vs 2% cryostor	p<0.0001	p<0.0001	p<0.0001	p<0.0001	p<0.0001	p<0.0001
1% lifor vs 5% cryostor	p<0.0001	p<0.0001	p<0.0001	p<0.0001	p<0.0001	p<0.0001
1% lifor vs 10% cryostor	p<0.0001	p<0.0001	p<0.0001	p<0.0001	p<0.0001	p<0.0001
2% lifor vs 3% lifor	no	p<0.05	p<0.05	p<0.01	no	p<0.05
2% lifor vs 5% lifor	p<0.01	p<0.001	p<0.0001	p<0.0001	p<0.0001	p<0.001
2% lifor vs 10% lifor	p<0.0001	p<0.0001	p<0.0001	p<0.0001	p<0.0001	p<0.0001
2% lifor vs 2% cryostor	no	p<0.05	no	p<0.05	p<0.05	p<0.05
2% lifor vs 5% cryostor	p<0.001	p<0.0001	p<0.0001	p<0.0001	p<0.0001	p<0.0001
2% lifor vs 10% cryostor	p<0.0001	p<0.0001	p<0.0001	p<0.0001	p<0.0001	p<0.0001
3% lifor vs 5% lifor	no	no	no	no	no	no
3% lifor vs 10% lifor	no	p<0.05	no	no	no	p<0.05
3% lifor vs 2% cryostor	no	no	no	no	no	no
3% lifor vs 5% cryostor	no	no	no	no	no	p<0.05
3% lifor vs 10% cryostor	no	no	no	no	no	p<0.01
5% lifor vs 10% lifor	no	no	no	no	no	no
5% lifor vs 2% cryostor	no	no	no	no	no	no
5% lifor vs 5% cryostor	no	no	no	no	no	no
5% lifor vs 10% cryostor	no	no	no	no	no	no
10% lifor vs 2% cryostor	no	p<0.05	no	p<0.05	no	no
10% lifor vs 5% cryostor	no	no	no	no	no	no
10% lifor vs 10% cryostor	no	no	no	no	no	no
2% cryostor vs 5% cryostor	no	no	no	p<0.05	no	no
2% cryostor vs 10% cryostor	no	p<0.05	no	p<0.01	no	p<0.05
5% cryostor vs 10% cryostor	no	no	no	no	no	no