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Regenerative Medicine and Tissue Engineering

Title: Low Temperature Cell Pausing: An alternative short-term preservation method for use in cell therapies

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Abstract

Encouraging advances in cell therapies have produced a requirement for an effective short-term cell preservation method, enabling time for quality assurance testing and transport to their clinical destination. Low temperature pausing of cells offers many advantages over cryopreservation, including the ability to store cells at scale, reduced cost and a simplified procedure with increased reliability. This review will focus on the importance of developing a short-term cell preservation platform as well highlighting the major successes of cell pausing and the key challenges which need addressing, to enable application of the process to therapeutically relevant cells.

Keywords: Cell pausing; Cell therapy; Cryopreservation; Hypothermic preservation; HypoThermosol-FRS.

Introduction

Encouraging advances in cell therapy research and development have produced a requirement for an effective short-term cell preservation method, enabling time for quality assurance testing and transport to their clinical destination. Due to the success of cold organ preservation before transplantation (Jamieson et al. 1988); attempts to apply hypothermic storage at the cellular level have been undertaken. Pausing cells at hypothermic temperatures (1-35°C) offers an alternative method to cryopreservation ($\leq 0^\circ\text{C}$), for the short-term storage, (minutes to weeks) and transport of cells. Unlike cryopreservation, the ‘gold standard’ for long-term cell storage (months to years), low temperature cell pausing or hypothermic preservation is a simplified process which enables storage of cells at scale with reduced cost (Figure 1). This review will focus on the importance of developing a short-term cell preservation platform as well as highlighting the current successes of cell pausing and the key challenges which need addressing, to enable application of the process to therapeutically relevant cells.

Note: Figure 1 to be placed here.

The Importance of a Short-term Cell Preservation Platform

Cryopreservation, using conventional slow freezing and storage in liquid nitrogen (the vapour phase, -150°C , or directly submerged in liquid nitrogen, -196°C) is the most commonly used method to store cells used for research (Ghera et al. 1999). Cryostorage is even utilised in medical technologies such as Dermagraft® (Advanced BioHealing, USA) (Mason & Manzotti 2010), and to preserve cells undergoing phase III clinical trials, for example CTX0E03 neural stem cells to promote recovery after stroke (ReNeuron Ltd. UK) (Stroemer et al. 2009). Cryopreservation allows for the generation of allogeneic master and working cell banks (Heidemann et al. 2010) which can deliver ‘off the shelf’ products. Whilst conventional slow freezing generally

yields over 90% viability post-thaw (Kleeberger et al. 1999; Kotobuki et al. 2005), for certain cell types such as human embryonic stem cells (hESC) grown as colonies, poor survival has been reported (Li et al. 2010; Xu et al. 2010). Vitrification is an alternative approach for storage of hESC (Li et al. 2010), yet is disadvantaged due to higher concentrations of cryoprotective agents (CPAs), smaller storage volumes (typically 1-20 μ l per cryostraw) (Unger et al. 2008; Coopman 2011; Hunt 2011) and the risk of contamination from open cryostraws (Mirabet et al. 2012). Diminished functionality of cryopreserved human hepatocytes has also been documented; with cells losing their adherence capabilities and exhibiting an altered metabolic profile post-thaw (Terry et al. 2006). Extreme temperature shifts during the process of cryopreservation cause cell damage from intra and extracellular ice formation and cell dehydration (Mazur 1984). Osmotic imbalances can injure cells and programmed cell death may be induced upon thawing (Mathew et al. 2002). Further to this, dimethyl sulfoxide (DMSO), the most frequently used CPA, is considered toxic to cells at ambient temperatures (Hunt 2011) and may induce cell differentiation (Santos et al. 2003). Patients infused with DMSO-preserved cells can also experience adverse reactions (Cox et al. 2012). Furthermore, the use of animal derived components in cryopreservation, such as fetal bovine serum as a vehicle solution, risks contamination with adventitious agents (Merten 1999). Despite some drawbacks, cryopreservation at -80°C or -20°C is commonly used to store and transfer cells between the manufacturing site and medical facility (Coopman 2011; Harel 2013). Research to improve cryopreservation protocols, using safer, naturally derived CPAs such as trehalose, and xeno-free serum alternatives, is in progress for application to therapeutically relevant cells.

Hypothermia is used as a preservative in nature and cells possess the ability to recover from exposure to low temperatures. It seems logical to apply this phenomenon to the preservation of human cells for therapeutic procedures. Clinically, hypothermia is sub-divided into four grades; mild (32-35°C), moderate (28-32°C), severe (20-28°C) and profound (20-1°C) (Marx et. al 2009). For this review, the clinical grading system for hypothermia will be applied to the low temperature pausing of cells. Preserving cells at hypothermic temperatures slows metabolic activity and cell cycle progression (Roobol et al. 2009), a process known as suspended animation. Cold-induced stress can also affect the lipid composition of cellular membranes and induce free-radical production (Fujita 1999). However, hypothermic storage effectively bypasses cell damage from ice nucleation and changes in solute concentration caused by extreme temperature shifts during freezing.

Studies have demonstrated mammalian cells can remain viable at low temperatures (6-24°C) for up to seven days (Hunt et al. 2005); supporting the potential of cell pausing as a short-term preservation protocol. Shipping

of cells within the UK or globally can take hours or days depending on distance, means of transport and delays. If cell pausing can be optimised to cell type, a preservation time frame of seven to ten days allows time for quality, safety and efficacy testing before transportation and administration to the patient. Cell pausing at ambient temperatures in particular, will reduce the need for specialist temperature controlled machinery. For example, the use of dry shippers, designed to keep cells at liquid nitrogen temperatures with no risk of spillage, can be expensive. Additionally, using ambient temperatures to preserve cells will be advantageous during a power failure, when pH, humidity and CO₂ levels will be impossible to control (Hope et al. 2011). Therefore, the successful maintenance of cells at ambient temperature and no atmospheric control for a short-period of time would be ideal for the preservation and transport of cell-based therapies.

Stem cell research is highly focused on developing scalable culture models, due to the large quantity of cells required for regenerative therapies (Mummery 2005). The exact number of cells needed is dependent on disease type and prevalence but ranges from 10⁶ to 10⁹ per patient (Mason & Dunnill 2009). Cryopreservation is limited in its ability to preserve cells at scale with most cryovials and cryobags storing between 1-20 x 10⁶ cells per ml (Coopman 2011; Heidemann et al. 2010). Being devoid of specialist storage products and apparatus, cell pausing at ambient temperatures generates opportunity for larger scale storage and transport.

Whilst low temperature pausing is focused on the manufacture and distribution of cells for allogeneic treatment, the process will also be applicable to autologous cell therapy. During surgical procedures cells will experience hypothermic temperatures and tailoring their environment in terms of media composition and atmospheric factors will optimise cell survival and quality. Furthermore, hypothermic storage will keep cells from going to waste in case of unexpected events such as patient illness on day of treatment or transport delays.

Effects of Cold-Induced Stress on Mammalian Cells

Low temperatures have wide ranging effects upon cells including the slowing of energy dependent processes such as protein synthesis, transport systems and progression through the cell cycle (Nishiyama et al. 1997). Cold-induced stress responses include changes in gene expression such as the up-regulation of cold inducible RNA-binding protein (CIRP), as a protective mechanism (Fujita 1999). Hypothermic temperatures may also affect membrane lipid bilayer integrity and cytoskeletal structure (Scott et al. 2005). When exposed to mild or moderate hypothermia, cells metabolise enough for nutrients to be depleted, waste products produced and pH lowered, leading to increased lactic acid production and an overall increase in cellular injury over time (Fujita 1999; Scott et al. 2005). Rewarming paused cells initiates resumption of cell propagation and increases energy

dependent processes to that of cells in normal culture conditions. Cell damage during hypothermia can result in further injury and cell death upon rewarming via the reactive oxygen species (ROS) produced during this process (Meng et al. 2003). This is similar to tissue ischemia and cell death upon reperfusion. Extreme temperature shifts may also induce apoptosis and necrosis (Mathew et al. 2004). Viability evaluation, either immediately after hypothermic exposure or following rewarming to normothermic conditions, may give varied results as cells may be programmed for apoptosis upon their return to 37°C or following continuous culture (Rauen et al. 2000). A full examination of these properties during and shortly after hypothermic storage has not been executed for a variety of cell types. Hence, there are opportunities for further research in this area, specifically for therapeutically relevant cells which may behave differently to hypothermic exposure.

Achievements to Date

Effective storage of red blood cells has been possible since the 1930s, using various hypothermic and cryopreservation strategies (Holovati & Acker 2011). The preservation of red blood cells led to the emergence of modern blood banks which can store chilled blood products for up to 35-42 days (Högman 1998). Hypothermia is also employed to accelerate industrial production of recombinant therapeutic molecules (Fox et al. 2005) and to enhance flexibility of cell-based assays (Wise et al. 2009). Hypothermic preservation of rat and human hepatocytes has been examined extensively, mostly due to their ease of accessibility (Meng et al. 2003). Hepatocytes are also a suitable cell to use as a test-bed for low temperature preservation, as the method used for whole liver organs can be applied at the single cell level. Single cells however, lack the 3D structure of intact liver tissue which provides a modicum of protection. Inevitably single cells are more exposed and susceptible to hypothermic damage. The protection of cells, tissues and whole organs from hypothermic damage during storage has generated the commercialisation of uniquely formulated storage media such as University of Wisconsin solution (UW) or ViaSpan®, Histidine-tryptophan-ketoglutarate (HTK), Celsior, EuroCollins, Custodial and most recently Hypothermosol-FRS (HTS-FRS) (2-8°C) and HypoThermosol-BASE (HTS-BASE) or PrepaStore (18-22°C). These cold storage media aim to support cell metabolism and inhibit post-storage necrosis and activation of apoptosis in response to cold temperatures (Hope et al. 2011). HTS-FRS has been particularly successful for the storage of cells at 4°C (Mathew et al. 2002; Mathew et al. 2004). Comprised of ionic components such as Na⁺ and Cl⁻, pH buffers, energy substrates and osmotic and oncotic stabilizers, the ionic makeup of HTS aims to balance cellular ion concentrations which are altered upon hypothermia and nutrient deprivation (Mathew et al. 2004). HEPES buffer manages pH fluctuations and provides osmotic support (Taylor 1982; Taylor & Pignat 1982). Impermeants such as lactobionate counteract cellular swelling and the

branched glucan dextran, provides support for colloid osmotic pressure (Morel et al. 1992). Low levels of glucose provide a source of energy for cell metabolism when cells are returned to optimum temperatures, without a build-up of lactic acid (Anderson et al. 1992). Notably, HTS has recently been used in conjunction with some cellular products currently under evaluation in clinical trials; for example a study by TiGenix S.A.U investigating inguinal intralymphatic administration of expanded adipose derived stem cells (TiGenix, 2013). In addition, the hypothermic storage of CTX0E03 cells in HTS-FRS for up to 7 days has been successfully achieved by ReNeuron Ltd UK (Hope et al. 2011).

The literature is limited with regards to studies focusing on cell pausing of human cells and stem cells. A summary of current research papers which focus on hypothermic storage of human cells is presented in Table 1. A current success in cell pausing under ambient conditions used human mesenchymal stromal cells (hMSC) and mouse embryonic stem cells (mESC) entrapped in a semi-permeable alginate hydrogel (Chen et al. 2013). Viability was well maintained (hMSC=80% and mESC=74%) for up to 5 days with retention of surface marker expression. Alginate entrapment of cells during hypothermic storage has previously been shown to protect against induction of apoptosis when compared to freely suspended hepatocytes (Mahler et al. 2003). Whilst gel entrapment may offer structural support to cells and protection from cold-induced injury, the cells still require retrieval and processing once delivered to the medical site. Enclosure of cells in culture chambers such as the PetakaG3, a hermetically enclosed device for cell transport between 20-30°C, can also achieve cell pausing (Celartia 2012). This closed system attains mild hypoxia which helps initiate cell cycle arrest at the G1/S interface, resulting in suspended animation. Cell pausing is clearly becoming accepted as a potential technology to preserve and transport mammalian cells. The research by Chen et al. (2013) in particular, proves that the process of cell pausing can be successfully applied to stem cells, which holds great potential for its application to future cell therapies.

Challenges

Despite some success, several challenges must be overcome before low temperature pausing can be more commonly applied to therapeutically relevant cells. Cell recovery is known to deteriorate with increased pausing time (Heng et al. 2006; Ginis et al. 2012) however; extending the shelf-life of paused cells is required for the product to become commercially viable. Lowered cell viability over time may be a consequence of a reduced nutrient supply and increase in waste and lactic production during prolonged pausing. As previously discussed, the formulation of storage media to support cells and mitigate cold induced stress responses during hypothermic

preservation is being developed. Modified media such as HTS-FRS help stabilise cells at cold temperature and can lead to an extended shelf-life compared to using standard media. The key components are considered to be potent free-radical scavengers, including Trolox, which support hypothermic storage at 2-8°C. The addition of a caspase or Rho-associated kinase inhibitor to HTS-FRS may further improve its anti-apoptotic effects and prolong duration of preservation (Watanabe et al. 2007). Conversely, the addition of drugs to the final cell product will require regulatory justification to ensure there will be no increased risk of adverse effects to the patient.

Notably, cell pausing has been achieved using standard media for recombinant CHO and HEK293 cells (Hunt et al. 2005). It will therefore be valuable to examine the benefits/costs of the individual components of hypothermic storage media such as HTS and UW. Standard culture media supplemented with a cocktail of constituents adapted for cell pausing conditions may prove just as effective as and significantly cheaper than commercially available products, however the GMP-compatibility of in-house formulations must be considered.

Contrary to organ storage, preserving mammalian cells at 4°C has proven to be less effective. Assessment of viability and quality parameters after rewarming has shown mild to moderate hypothermia to be more successful than 4°C preservation (Hunt et al. 2005). However, research to help improve cell storage at 4°C is a favorable strategy, due to current temperature controlled equipment used today. Clinics and hospitals already store chemicals and drugs in fridges (2-6°C) and many modes of transportation are built to store and transport products at cold temperatures. Whilst cell storage at ambient temperatures has many advantages in terms of enhanced viability and no reliance on temperature controlled machinery; it is important to recognise that ambient temperatures will be different across the world and in certain environments. A preservation system using 2-6°C will be more stable. Therefore, further tailoring of media composition for cell pausing at 4°C is highly valuable and easily transferable into current medical practice. However, cell products may require a rewarming step, especially if paused at 4°C. Warming cells to 37°C via incubation before infusion into the patient may be sufficient; however there is a gap in the research concerning the rewarming of cells after pausing and if the process needs to be controlled.

An additional consideration regarding pausing is uncontrolled CO₂ levels and relative humidity. Incubators for mammalian cell and tissue culture are typically set to 37°C, 5% CO₂ and a high relative humidity 95% which

creates an environment similar to *in vivo*, so that cells and tissues can thrive. Pausing cells at ambient temperatures not only inflicts cell damage from cold stress, but may additionally cause trauma via changes in other uncontrolled atmospheric factors. Low temperatures may even affect membrane permeability and gas exchange which maybe the cause of, or an additional factor for the decreased cell viability observed after cell pausing. It has been identified that more research into changes in atmospheric factors and not only temperature modifications is essential if we are to improve cell pausing protocols.

Successfully pausing suspended cells is another considerable challenge needed to simplify the preservation process. For adherent cells, this concept requires intense development as cells are known to survive better when attached to a surface. Continued cell signalling and interactions may be required for cells to survive any suboptimal conditions. More investigation into cell detachment and colony formation during pausing is needed to ascertain if cells can remain viable and functional if paused in suspension. Pausing cells in 3L bioreactors has been studied (Hunt et al. 2005) using suspension adapted CHO cells and a modified storage solution has been patented which claims to preserve progenitor and stem cells in suspension at hypothermic temperatures (2-8°C) (Hope 2011). More detailed analyses of scalable pausing protocols using non-adapted cells (including stem cells) will be required if this technique can be used for regenerative therapies.

Another factor to consider is mechanical stress encountered during transportation. Bone marrow derived stem cells show sensitivity to vibrations (Zhou et al. 2011) and a study by Nikolaev et al (2012) found a coupling effect between vibration and cold temperature storage. Mechanical stress during the transportation process requires further investigation to elucidate the effects on cell health and function and to help design appropriate packaging which is sustainable and cost effective whilst maintaining the therapeutic quality of cells (Nikolaev et al. 2012).

Methodologies for examining hypothermic preservation of cells are extremely diverse across the field and vary between research groups and investigators. These many approaches generate useful information, yet have their limitations. Viability is examined via morphology, membrane integrity and enzyme activity, as well as cell cycle analysis. Due to the extent of cell types tested in cell pausing experiments, it is of great importance that a panel of cell viability assessments is performed. In terms of membrane stability and metabolism, some cell types may be more susceptible to cold-induced exposure and injury. Furthermore, few studies focus on phenotypic and cell

quality assessments such as cell marker expression post-preservation which is crucial if the process is to be applied therapeutically. In addition, standardising methods of viability and long-term quality of stem cells used for therapy is necessary if results are to be compared amongst research groups. Cells used in regenerative medicine must not just remain viable but retain function in order to successfully treat patients; hence it is of high importance that long-term post-thaw quality assessments are carried out alongside immediate viability analysis. Heng et al. (2006) showed hESCs can be paused for 48 hours at 4°C with 95% of cells retaining markers of pluripotency. Clearly, cell pausing has potential for yielding viable, high quality cells which can function as expected. More detailed evidence is required to determine optimum length of pausing before quality starts to deteriorate.

It is of extreme importance that preservation methods are tailored to different cellular systems as each cell type has distinct metabolic, structural and physiological properties (Mathew et al. 2004). The success or failure of any cold temperature preservation protocol depends upon the rate of cooling, temperature and fluctuations, medium composition and method and rate of rewarming (Heidemann et al. 2011). All these factors may need modification for individual cell types. It would be ideal if a platform preservation method could be developed but current research indicates tailoring is needed. For example, viability in coronary artery smooth muscle cells was best maintained in HTS supplemented with Trolox for 7 days at 4°C, whereas skeletal muscle cells required the addition of an RGD peptide to maintain viability (Mathew et al. 2004). Stem cells are less robust than most fully differentiated cell types and preservation protocols need to consider maintenance of their potency characteristics. Very few cell pausing studies have focussed on stem cells (see Table 1). However, research which has applied the process to potent cells show significant potential (Heng et al. 2006; Ginis et al. 2012; Chen et al. 2013). Current advancements using devices such as the PetakaG3 and alginate gel entrapment to store cells at ambient temperatures are encouraging but limited for the storage and transport of cells at large scale.

Note: Table 1 to be placed here

As MSCs are the most extensively used stem cell in current cell therapy trials (Harel 2013), successful application of cell pausing to MSCs will provide significant benefits. It is clear, however, that knowledge of how to accommodate the metabolic and structural changes of cells during pausing needs to be developed before progressing onto more valuable and expensive cell lines. Other considerations which may influence the success of cell preservation include the way cells are harvested and passage number upon cooling, which needs to be

regulated and optimised for every cell line (Gherna 1999). Nevertheless, the promising work achieved on mammalian cells so far indicates proof of concept that the process is viable and should be applicable to therapeutically relevant cells.

The Future of Cell Pausing

Low temperature cell pausing holds great potential as a short-term storage method to transport cells between manufacturing site and medical facility. Summarising studies to date indicates that most mammalian cell types can be successfully stored for up to 48 hours at 4°C; the most favorable outcomes in terms of viability being achieved by using HTS-FRS. Together with extending the shelf-life of paused cells, more focus on ambient temperature storage is required due to its non-reliance on temperature controlled apparatus and financial benefit.

The key challenges to address before cell pausing at ambient temperatures can be utilised for the storage and transport of therapeutic cells are retention of cell viability, quality and potency post-preservation as well as resilience to ambient temperature fluctuations. Since ambient temperature will vary around the world, pausing at 4°C may be more practical. Improving storage of cells at 4°C may also ease the adaption of cell pausing into current practices. The refinement of media composition to accommodate cell metabolism and growth at low temperature is vital, especially for the expansion of this process to stem cells. Manipulating cells to survive in suspension at low temperatures will be a significant challenge. Once achieved with proven GMP-compliance (Heideman et al. 2010), pausing of cells in suspension will further ease the transportation and retrieval process of cells for clinicians. As well as allogeneic cell therapy, short-term pausing of cells will be beneficial for autologous treatment.

Regenerative medicine is a rapidly emerging field with many cellular therapies entering phase II clinical trials. Successful cell pausing will create a simpler, cost effective short-term preservation method, accelerating the progression of cellular therapies from bench to bedside.

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Table 1. A summary of current research papers focusing on hypothermic storage of human cells. HypoThermosol-Free Radical Solution (HTS-FRS), University of Wisconsin Solution (UW), Dulbecco's Modified Eagle Medium. (*) Membrane integrity (**) Membrane integrity & metabolic activity

Figure 1. Diagram to compare the process of cryopreservation to that of cell pausing, when applied to cells used for regenerative therapy. Whereas cryopreservation requires a two-step procedure involving slow-freezing cells to -80°C and transfer to liquid nitrogen, cell pausing requires just one step to expose cells to ambient temperature or 4°C . Once approved by the processing centre, batches of cells can be transported to their appropriate medical site and rewarmed ready for use. The cell pausing protocol avoids use of temperature controlled machinery for transport. Furthermore, thawing of cryopreserved vials and retrieval of cells at the clinic can be cumbersome whilst paused cells can be rewarmed to 37°C in original pausing media. In summary, cell pausing for use as a short-term preservation method, simplifies the protocol, reduces reliability of specialist machinery and eases retrieval of cells for clinicians.

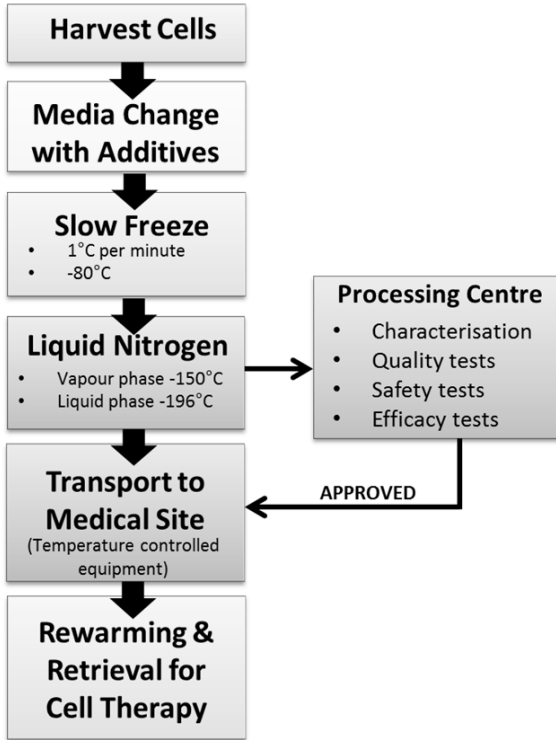
Table 1. A summary of current research papers focusing on hypothermic storage of human cells.

<i>Study</i>	<i>Cell Type</i>	<i>Storage Temperature, Exposure Time & Recovery Time</i>	<i>Storage Solution</i>	<i>% Viability (assessment method)</i>
Ginis et al. (2012)	Mesenchymal stem cells	4°C 46-96hrs 3 hrs	HTS-FRS	85 (**)
Heng et al. (2006)	Human embryonic stem cells	4°C 24-48 hrs Undisclosed 25°C 24-48 hrs Undisclosed	DMEM/F12 with serum replacement	64.4-69 (*) 71-77.3 (*)
Hunt et al. (2005)	Human embryonic kidney cells (HEK293E)	4°C & 24°C 4 days Undisclosed	Serum-free EX-cell 293 with glutamine	85 (Undisclosed)
Jannsen et al. (2003)	Human hepatocyte cells	4°C 24-48 hrs 0 & 6 hrs	UW Celsior	~42-99 (*) ~42-98 (*)

			Histidine-tryptophan-ketoglutarate	~11-92 (*)
Mathew et al. (2002)	Human renal cells	4°C 5 days 24 hrs	Renal cell culture medium HTS-BASE UW HTS-FRS	6 (*) 17 (*) 32 (*) 75 (*)
Mathew et al. (2004) (not all data shown)	Coronary artery smooth muscle cells	4°C 24 hrs 24 hrs 4°C 48 hrs Over 5days	Standard culture medium UW HTS HTS-FRS+Trolox Standard culture medium UW HTS	<10 (**) <10 (**) <10 (**) 63-65 (**) <10 (**) <10 (**) <25 (**)

			HTS+FK041	22-56 (**)
			HTS+Trolox	40-69 (**)
			HTS+Trolox/EDTA	58-81 (**)
	Coronary artery endothelial cells	4°C 48 hrs 24 hrs	Standard culture medium	0 (**)
			UW	54 (**)
			HTS	>80 (**)
			HTS+Trolox	100 (**)
			HTS+Trolox/EDTA	100 (**)
			HTS+FK041	84 (**)
	Skeletal muscle cells	4°C 7 days 24 hrs	HTS	54 (**)
			HTS-Trolox	84 (**)
			HTS-Trolox/RGD	92 (**)
	Hepatic cells (C3A)	4°C 4 days 0 hrs	All storage solutions tested	<80 (**)

Cryopreservation



Cell Pausing

