Low-temperature pausing: an alternative short-term preservation method for use in cell therapies

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Low Temperature Pausing: An Alternative Short-Term Preservation Method for Use in Cell Therapies

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by

Nathalie Jayne Robinson

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Centre for Biological Engineering

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ABSTRACT

LOW TEMPERATURE PAUSING: AN ALTERNATIVE SHORT-TERM PRESERVATION METHOD FOR USE IN CELL THERAPIES

By Nathalie Jayne Robinson

With encouraging advancements in cell therapies, there is a requirement for an effective short-term cell preservation method, enabling time for quality assurance testing and transport to their clinical destination. This project aims to 'pause' cells at ambient temperatures, whilst maintaining viability and function post-preservation. Ambient cell preservation bypasses ice crystal exposure and toxic solute concentrations experienced with cryogenic storage. Storage in ambient conditions also avoids use of toxic cryoprotectants and aims to greatly reduce costs and reliability on specialist machinery.

Early work used HOS TE85 cells (derived from an osteosarcoma) as a model. When atmospheric factors were controlled, HOS TE85 cells demonstrated effective recovery in terms of morphology, membrane integrity (viability >90%) and fold growth expansion when paused at ambient temperature for up to 144 hours. Without atmospheric control, addition of the buffering agent HEPES (25mM) to cell medium was required to keep viability above 70%, as well as to maintain yield and continual passage following 144 hours pausing. The pausing potential of therapeutically relevant human mesenchymal stem cells (hMSCs) from three individual donors (M2, M3 and M4) was tested by keeping cells in suspension for up to 72 hours. Using standard medium with the addition of 25mM HEPES, average membrane integrity was maintained above 70%. Following pausing for between 24–72 hours, hMSC

tri-lineage differentiation capacity (osteogenesis, adipogenesis and chondrogenesis) remained similar to non-paused cells. Apart from a short lag phase on the first passage, hMSC fold growth expansion level was consistent with the control for all three donors over 3 x 6 day passages. The colony forming unit (CFU) efficiency of paused cells was significantly reduced when compared with non-paused M2 and M4 lines, whilst M3 retained a similar CFU efficiency to its non-paused counterpart. On return to normal culture conditions, hMSCs had comparable metabolic activity rates with non-paused cells for up to 9 hours.

Stable pH is vital during pausing and additional antioxidants or apoptotic inhibiters may be required to keep average viability well-above the 70% threshold, set by the US Food and Drug Administration. Collectively, results have been encouraging and show potential for the movement towards using ambient temperature preservation as an option for the short-term storage and transport of cells for therapy.

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EQUATIONS

ABBREVIATIONS

4-MU 4-methylumbelliferone

4-MUP 4-methylumbelliferyl phosphate

ATP Adenosine triphosphate

ALP Alkaline phosphatase

ACA Anti-clumping agent (Agent A)

AO Acridine orange

ANOVA Analysis of variance

BSC Biological safety cabinet

CD Cluster of differentiation

CFU Colony forming unit

CHO Chinese hamster ovary cells

CIRP Cold inducible RNA-binding proteins

CSP Cold shock proteins

CPA Cryoprotective agent

CRF Controlled rate freezer

cGMP current Good Manufacturing Practice

DAPI 4',6-diamidino-2-phenylindole

dH$_2$O distilled H$_2$O

DMEM Dulbecco's Modified Eagle's Medium

DMSO Dimethyl sulphoxide

EDTA Ethylenediaminetetraacetic acid

EIF Extracellular ice formation

EMA European Medicines Agency

ECACC European Collection of Cell Culture

FBS Foetal bovine serum

FDA Food and Drug Administration

GCP Good Clinical Practice

GMP Good Manufacturing Practice

GvHD Graft versus host disease

HCL Hydrochloric acid

HSP Heat shock proteins

HEK Human embryonic kidney cell

hESC human embryonic stem cell

HFEA Human Fertilisation and Embryology Authority

hiPSC human induced pluripotent stem cell

HTK Histidine-tryptophan-ketoglutarate

hMSC human mesenchymal stem cell

hPL human platelet lysate

HSC Haematopoietic stem cell

hSA human serum albumin

HTS-FRS® Hypothermosol-free radical solution

IHC The International Council for Harmonization

IIF Intraceullar ice formation

IPA Isopropyl alcohol

ISCT International Society for Cellular Therapy

IVF In vitro fertilisation

MCB Master cell bank

MEM Minimum Essential Medium

MEM/EBSS MEM with Earle's Balanced

mESCs mouse embryonic stem cells

mRNA messenger ribonucleic acid

MSR Modular signal recorder

MTT (3-(4,5-Dimethylthiazol-2-yl)-2,5-Diphenyltetrazolium Bromide)

NEAA Non-essential amino acids

NC-100 NucleoCounter-100

NC-3000 NucleoCounter-3000

PBS Phosphate buffered saline

PI Propidium iodide

P68 Pluronic F68

PEG Polyethylene glycol

PES polyethersulfone

PPG Polypropylene glycol

PLA Pseudo Lyte A

RNA Ribonucleic acid

ROS Reactive oxygen species

RT Room temperature

QC Quality control

TNS Tumour necrosis factor

ULA Ultra-low attachment

UV Ultra-violet

UW University of Wisconsin solution

WCB Working cell bank

CHAPTER 1: INTRODUCTION

The global marketplace of regenerative medicine has expanded impressively over the past decade, with cell therapies such as Prochymal[TM] (Osiris Therapeutics) gaining regulatory approval in Canada and New Zealand and more entering phase II/III clinical trials (Culme-Seymour, *et al.* 2012). These therapies not only offer a treatment for incurable diseases, but also have the potential to repair, regenerate or replace the injured or damaged tissue and restore function. Regenerative medicine encompasses small and large molecules, devices and cells, with cell therapies being just one facet. There are between 300–700 companies including small University spinouts and multinational companies such as GlaxoSmithKline, Johnson and Johnson, ReNeuron, Pfizer and Genzyme all actively pursuing cell based therapies (Mason, *et al.* 2013). The global regenerative medicine market is set to have an annual compound growth rate of 23.2% during 2014–2020 (Lewes, 2014) and the cell therapy industry alone is now known as a billion dollar business (Mason *et al.* 2011). Additionally, the use of stem cells is prevalent amongst scientific research industries and in clinical and toxicology studies (Mathew *et al.* 2004).

Though marked by some failures, there have been major breakthroughs in regenerative medicine including the first successful transplantation of a synthetic tissue engineered windpipe in 2008 (Macchiarini, *et al.* 2008) and the generation of commercial products for skin ulcers and sports injuries. The central focus of regenerative medicine is human stem cells, which can be somatic, embryo-derived or reprogrammed from adult cells to a stem-like state (Mason and Dunhill, 2008).

Research so far has centred on the expansion and manufacture of cells for therapy as the number of cells required, whilst dependent on dose and type of disease, can be in the order of trillions (Kirouac and Zandstra, 2008; Heathman, *et al.* 2015). Preservation, storage and delivery are often considered insignificant during early development of a cell therapy product. With cell therapies moving closer to commercialisation, attention has now turned to the safe distribution and administration of cellular products to patients. This movement has necessitated a requirement for a short-term preservation method, which enables the transfer of cells from manufacturing site to medical facility, whilst providing adequate time for batch release testing and delays to transport or surgery. The short-term storage of cells without the need to use cryopreservation will be important for both allogeneic (off the shelf) therapies and autologous treatments (personalised).

This thesis highlights the need for an alternative cell preservation technique to cryopreservation, the current standard procedure for cells in the research or clinical setting. Cryopreservation is commonly used for long-term storage (months-years) of cells in master cell banks, as cellular function is often lost during continual culture (Hourd, *et al.* 2014). However, as will be discussed in section 2.4.1, cryopreservation is limited for the storage of cells for hours–days due to the damage that can arise from the freeze-thaw procedure (Mazur, 1970). Hypothermic preservation, whilst not a new concept in terms of the food industry and organ preservation, will be introduced as an alternative approach for the storage and transport of cells for therapy. With a strong history in hibernation, torpor, brain and heart surgery and the biotechnology industry, it seems worthy to exploit this natural ability of mammalian cells to recover from cold

temperatures. The term cell 'pausing' will be utilised throughout this thesis, which refers to the slowing metabolic and growth rate of cells experienced during cold temperatures compared with freezing cells, which halts metabolism and proliferation altogether. Whilst the phenomenon of hypothermic cell preservation has been touched upon in the literature, there are significant gaps in the research concerning optimal preservation conditions; the effects of low temperature on the cell; how different cell types respond to hypothermia (specifically therapeutically relevant cells); and the potential for ambient cell preservation. This review will outline the current options available for biopreservation and introduce hypothermia as a short-term stem cell preservation method, with a focus on chilled and ambient storage. The effectiveness of pausing as a useful alternative to existing strategies including cryopreservation and cell culture will be evaluated, as its requirement in the laboratory and for clinical use is becoming more recognised.

1.1 PROJECT AIMS

To design a lab-scale process to determine the effects of low temperature pausing on cells and to identify the key factors important for successful cell pausing.

1. **Define/refine the pausing process**

    a. To assess whether pausing a human osteoblast-like cell line for 48 hours at ambient temperatures is feasible

    b. To examine the effect of ambient temperature storage on cell viability and quality

    c. To determine important factors involved for successful cell pausing.

2. **<u>Optimise medium composition for pausing</u>**

   a. To investigate the importance of medium composition to support cell pausing for short-term time periods.

3. **<u>Pausing cells in suspension</u>**

   a. To determine the potential for transporting adherent cells as a single cell suspension, at ambient temperatures, during short-term preservation.

4. **<u>Application to a therapeutically relevant cell type</u>**

   a. To examine if cell types currently utilised in cell therapy e.g. human mesenchymal stem cells (hMSC), can withstand the stress of pausing

   b. To elucidate whether pausing can be applied to cell therapy products

CHAPTER 2: CRITICAL LITERATURE REVIEW

2.1 INTRODUCTION

Cell preservation is defined as the storage of cells, tissues or whole organs overtime, without losing biological function and is applied to support advances in the study of disease progression, treatment and toxicology. A vital component behind the medical developments seen today, cell preservation incorporates biobanks, which hold a diverse range of samples and specimens for research or industrial purposes. Successful biopreservation effectively conserves original cell and tissue morphology and functionality upon return to physiological conditions. Recent progress in cell therapy, stem cell research, personalised medicine, cell banking and cancer research has necessitated improved protocol designs for cell storage; this is due to the deterioration in cell health and function by the initiation of cellular stress pathways, during and post-preservation.

This literature review will introduce the phenomenon of cell 'pausing' by hypothermic storage at low to moderate temperatures as an option for preserving cells used in the biotechnology or cell therapy setting. Pausing, by way of reducing the surrounding temperature of the cell, slows its internal metabolic and proliferative capacity; this aids maintenance of cellular health for short-time periods (hours to days), as an alternative option to the harsh cryopreservation process. As confidence in the cell therapy industry is growing, shown by the activity of clinical trials in the past decade (Culme-Seymour, *et al.* 2012), it is clear that more options for cell preservation and transport must be explored for various cell types and therapies. Being able to transfer

cellular products safety from their manufacturing site to their clinical destination will support the commercialisation of many future regenerative therapies.

Firstly, stem cells will be introduced (section 2.2); the main attraction in regenerative medicine. The options available for biopreservation of these cells will be described (sections 2.4 and 2.8), together with achievements using hypothermic preservation (section 2.12) and the challenges yet to be addressed (section 2.10). A detailed insight into the effects of low temperature on the cell itself (section 2.11), the composition of tailored media to offer a form of protection against cold stress (section 2.10.1) and the application of pausing to therapeutically relevant cells will be also covered (section 2.12).

2.2 INTRODUCING STEM CELLS

The definition of a 'stem cell' is one that is undifferentiated and has the ability to differentiate into a specialised cell type or to produce more stem cells. The use of stem cells to treat disease is by no means a new concept (Hunt, 2011), with the first transplantation of bone marrow in the 1950s (Mathe, *et al.* 1959) and the identification of the MSC, a separate non-haematopoietic stem cell population with multi-lineage potential, in 1976 (Friedenstein, *et al.* 1976; Hunt, 2011).

Once the true potential of stem cells had been uncovered to the public in the 1990s (Caplan, 1991), the hype and interest in these cells grew together with a marked increase in publications and an acceleration in cell-based therapy research. The industry suffered in the early 2000s due to the high failure rate of clinical trials, resulting in a loss of funding for the industry to progress (Lysaght and Hazelhurst, 2004). Since then, the cell therapy arena has regained widespread interest,

demonstrated by the number of clinical trials currently in practice (Culme-Seymour, *et al.* 2012). The allogeneic MSC-based therapy Prochymal[TM] is now approved for use in Canada and New Zealand to treat graft-versus-host disease (GvHD) and in 2010, the autologous prostate cancer vaccine Provenge (Dendreon) gained approval in the USA.

The therapeutic action of cells can either be from the cells themselves or the soluble factors that they produce. Engraftment and homing of implanted cells to the site of injury or disease can replace damaged tissue and promote healthy cellular function (Shah, *et al.* 2010). Additionally, cytokines, chemokines and growth factors that are secreted by stem cells can elicit therapeutic effects indirectly via paracrine signalling to help promote the induction of cellular processes such as angiogenesis and anti-inflammation (Ratajczak, *et al.* 2011).

The process of cell differentiation is complex and takes the cell through many different stages (figure 1); at each stage the cell becomes more specialised (National Institutes of Health, 2015). The mechanisms behind stem cell differentiation are not fully defined; however, internal genomic influences and extrinsic cues such as chemicals or molecules secreted by neighbouring cells as well as physical contact are thought to instruct the cell to differentiate into a specific lineage (Caplan, 1991; National Institutes of Health, 2015).

2.2.1 ADULT STEM CELLS

Most tissues in the body harbour a population of adult stem cells, which remain dormant for years, becoming active when tissue is damaged or diseased. Adult stem cells can be isolated from various tissues in the body including bone marrow (National Institutes of Health, 2015); skeletal muscle (Sambasivan and Tajbakhsh, 2015); blood

(National Institutes of Health, 2015); skin (Blanpain and Fuchs, 2008); brain (Bjornson *et al.* 1999); dental pulp (Huang, *et al.* 2009); heart (Leri, *et al.* 2011); gut (Barker, 2014); and even menstrual blood (Mou, *et al.* 2013). Isolation of stem cells is dependent on tissue type; for example, haematopoietic stem cells (HSC) can be collected from bone marrow, peripheral blood and the umbilical cord.
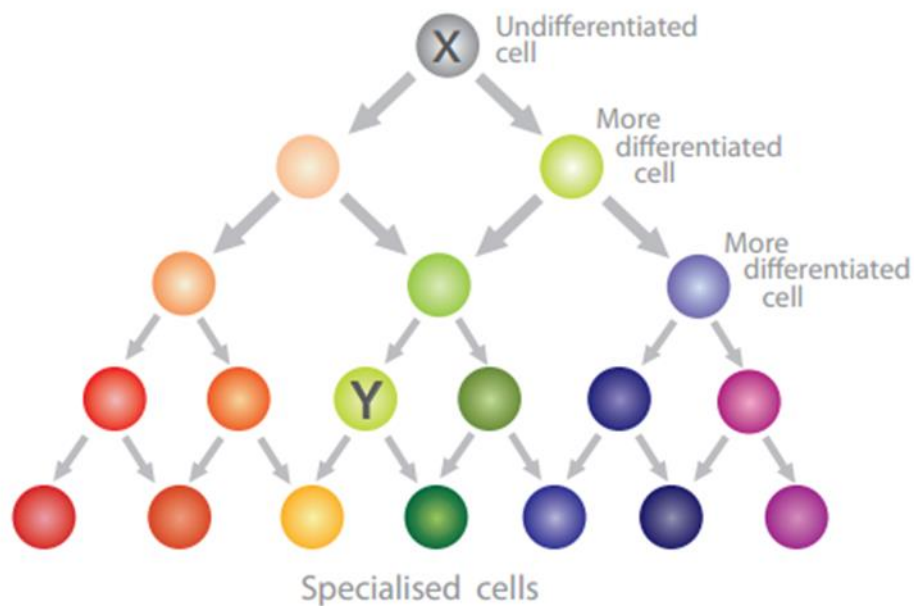
Figure 1: Cell differentiation. When a cell undergoes differentiation, it progresses through many stages, each time becoming more specialised. This limits the amount of cell types the cell can become, for example, X has the potential to become many more cell types than Y. Taken from Barfoot, *et al.* 2016.

Other sources of stem cells include the amniotic fluid (Antonucci *et al.* 2012) and developing organs such as the foetus and the placenta (Mathews *et al.* 2015). These cells are multipotent, meaning they can differentiate into many but not all cell types.

All types of blood cell including myeloid and lymphoid cells differentiate from a pool of haematopoietic stem cells (HSC). HSCs are important for the treatment of blood cancers like leukaemia and multiple myeloma (American Cancer Society, 2013).

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The liver is known for its ability to grow rapidly after resection of more than 50% of its mass (Fausto and Campbell, 2003); this shows promise for patients with liver disease. However, progression in this area has been limited due to the loss in function in hepatocytes, the main cell of the liver, following cryopreservation procedures. Although the reasoning behind loss of hepatocyte function is not clearly understood, evidence implicates mitochondrial sensitivity and impairment following thawing and exposure to hyperosmotic solutions (Stephenne, *et al.* 2009). Improved cryopreservation procedures and alternative storage options for these cell types such as hypothermic temperatures are required.

2.2.2 EMBRYONIC STEM CELLS (ESCS)

Embryonic stem cells (ESCs) are derived from the inner cell mass of a pre-implantation blastocyst, most commonly from an artificially fertilised ovum donated for research with informed consent. ESCs are pluripotent and are not limited to a fixed number of mitotic divisions (Caplan, 1991); in terms of scale this makes them an ideal candidate for regenerative medicine.

There is much potential for the use of ESCs to help cure major diseases such as multiple sclerosis, diabetes and cancer; however, the ethical issues restrict their use in certain parts of the world making their progression for use in cell therapies difficult. One disadvantage of using ESCs is their ability to form teratomas, a non-cancerous type of tumour, which is a safety concern (Hentze, *et al.* 2009). Opponents to the use of ESCs have highlighted the potential of adult stem cells and stressed that more money and time should be focussed on MSC research (Barfoot, *et al.* 2016). The Human Fertilisation Act in 1990 allowed the UK to be the first country in the world to

regulate artificial reproduction and embryo research, and was revised in 2000 to allow human embryonic stem cell research (EuroStemCell, 2012). Within these guidelines, there is a rule that no embryo can be kept or used for research after 14 days of development. The isolation, storage and use of ESCs is carefully regulated under the 1990 act, which means the use of these cells has to be approved by the Human Fertilisation and Embryology Authority (HFEA).

Views on the use of stem cells vary considerably throughout Europe. In Germany, the creation of embryos is illegal; however, they are allowed to use imported embryos (EuroStemCell, 2012). On the other hand, Lithuania does not allow any form of embryonic research (EuroStemCell, 2012). Financial drawbacks have also minimized the use of ESCs in America, with no Federal government funding permitted for ESC research during 2001–2009. Rules have since changed with each state at liberty to decide on which research can be permitted (EuroStemCell, 2007).

With all the controversy over the use of ESCs for research and global differences in opinions, the common approach is to use adult stem cells for research, namely MSCs.

2.2.3 INDUCED PLURIPOTENT STEM CELLS (IPSS)

Induced pluripotent stem cells (iPSs) are a somatic cell which has been reprogramed back to an undifferentiated state to behave like a stem cell. This method of 'inducing pluripotency' uses a viral vector carrying genes encoding *c-Myc*, *Klf4*, *Oct4* and *Sox*, which can reprogramme the cell to lose its specialist function and return to a stem like state (figure 2), bypassing the need for ESCs (Takahashi, *et al.* 2007).

iPSs offer many advantages over adult and ESCs as any cell type from the body can be reprogrammed, which makes them easily obtainable with fewer ethical concerns. The issue of immune rejection, prominent when using allogeneic therapies, could be eradicated with the possibility of generating patient-specific cells for transplantation. The main concern with iPS cells is the use of viral vectors to gain pluripotency, which increases the risk of producing cancerous cells (Barfoot, *et al.* 2016). Research to produce iPSs without the need for viruses is ongoing (Zhou, *et al.* 2009).
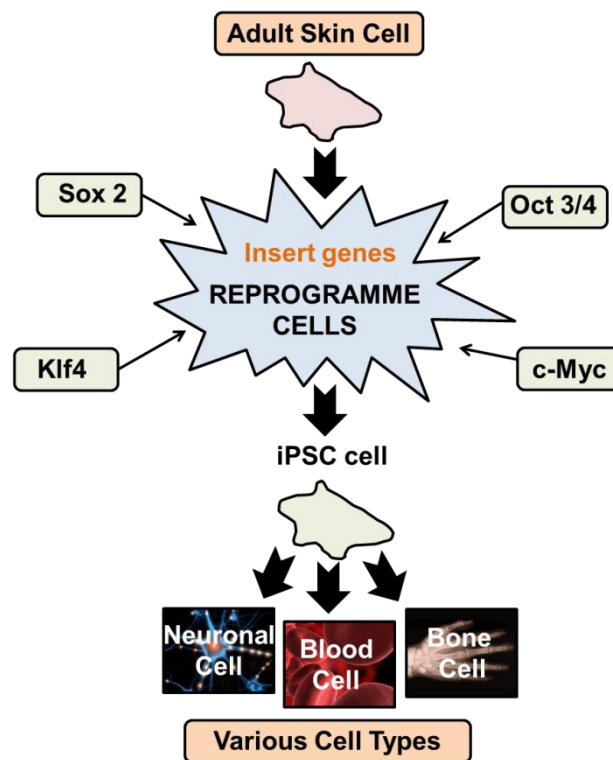
Figure 2: Induced pluripotent stem cells. Schematic demonstrating how an adult cell can be reprogrammed to become pluripotent by the insertion and expression of genes typically associated with embryonic stem cells.

2.2.4 MESENCHYMAL STEM CELLS (MSCS)

MSCs are the most characterised and utilised amongst regenerative research today, mainly due to fewer ethical restrictions and ease of access compared to other types of stem cells. Being able to differentiate into many lineages (figure 3) such as bone,

cartilage, muscle, ligament, tendon, adipose and stromal cells, means they are a valuable candidate for curing a wide range of diseases (Pittenger, *et al* 1999).

Whilst MSCs are used in the forefront of cell therapy, there is still much to learn regarding what defines an MSC. Variations in nomenclature, with some using mesenchymal stem cell (Caplan, 1999; Pittenger, *et al*. 1999) and others referring to mesenchymal stromal cell or skeletal stem cell (Bianco and Robey, 2015) already pose issues for standardising the definition of an MSC. There are also concerns with where these cells are located in the body. Residing mainly in the bone marrow, various extraction methods are used and it is uncertain whether enough cells are isolated and if the cells retrieved are all stem cells.
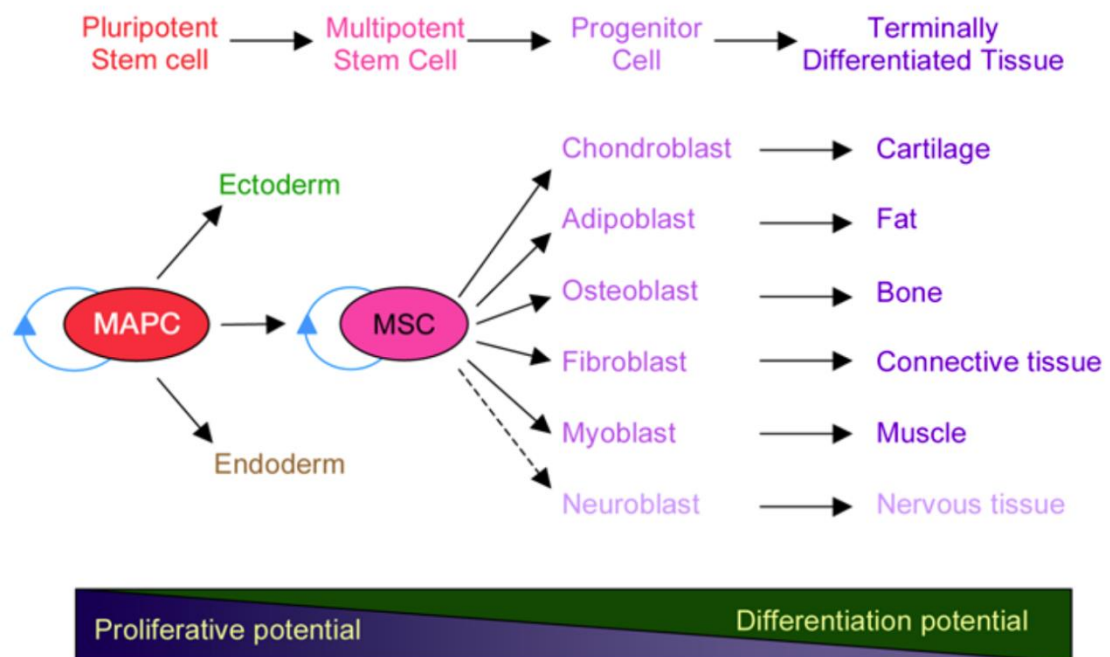
Figure 3: Mesenchymal stem cell (MSC) differentiation. Schematic showing the differentiation capacity of MSCs. Taken from Oreffo *et al.* 2005.

The difficulty in defining a multipotent MSC lead to the development of the International Society for Cellular Therapy (ISCT) criteria in 2006 (Dominici, *et al.* 2006). For a cell to be considered an MSC, the guidelines state that a cell must be able to attach to tissue culture plastic, have positive and negative expression of certain surface markers (CD105+, CD73+, CD90+, CD45-, CD34-, CD14- or CD11b-, CD79alpha- or CD19- and HLA-DR-) and be able to differentiate into osteocytes, chondrocytes and adipocytes. Although the criteria have provided guidance and improved the ability to compare studies between research groups, they are limited due to the large amount of antigens possibly expressed on MSCs. Furthermore, none of the markers are unique to MSCs and differences may arise between differentiating cells *in vitro* compared with the *in vivo* microenvironment. Since the industry is moving forward and the amount of MSC research has expanded, these criteria may require an update and further guidance via collaboration. Due to the uncertainty of MSC identity, the industry is starting to move towards assaying cells based on function (e.g. production of immunomodulatory cytokines (Kyurkchiev, *et al.* 2014)).

A recent search on the ClinicalTrials.gov database (Heathman, *et al.* 2015) demonstrates how much progress there has been with this cell type by the number of actively recruiting phase I and II trials. The main indications for hMSC therapy were neurology and cardiology. Other ongoing trials include safety and efficacy assessments of transplanting allogeneic MSCs to heal tibial closed diaphyseal fractures (Royan Institute, 2016) and using MSCs to treat acute severe respiratory failure (Asan Medical Center, 2016).

As MSCs are the most commonly used stem cell used in clinical trials today, this project will focus on preservation methods for the storage of MSCs.

2.3 CELL PRESERVATION

The successful preservation of stem cells is fundamental for their utilisation either for research or as a therapy. Stem cell research is highly focused on how to develop scalable culture methods, due to the large quantity of cells required for regenerative therapies (Mummery, 2005). The exact number of cells needed is dependent on the type of disease or injury as well as its severity and can range from 10^6-10^9 per patient (Mason and Dunnill, 2009). However, what is equally important is how to preserve these cells at scale (Coopman, 2011). In order for stem cells to be used as therapeutic products, they must undergo screening and quality control tests. These tests ensure product safety and efficacy before their distribution and final delivery to the patient, necessitating the need for cell storage (Holovati and Acker, 2011). The two methods currently used to preserve cells during laboratory research are slow-freeze cryopreservation and maintaining cells in culture with repeated passaging (Mazur, 1970). Both methods have limitations for transient cell storage such as cellular injury during the freeze-thaw process (Fowler and Toner, 2005) and increased risk of genetic modifications and infection from continual passaging (Diaferia, *et al.* 2011). Consequently, a short-term storage technique such as the use of hypothermia, keeping cells biologically stable, maintaining cellular integrity and functionality after warming, with fewer requirements for maintenance is highly advantageous. The process of hibernation is naturally occurring evidence that mammalian cells have the potential to withstand temperatures below 37°C. Additionally, viability results for the

low temperature pausing of mammalian cells have been encouraging (Meng, 2003; Mahler, *et al.* 2003; Hunt, *et al.* 2005; Ostrowska, *et al.* 2009), however its application in stem cell preservation has not been thoroughly investigated.

2.4 CRYOPRESERVATION

The most widely used approach to preserve cells is cryopreservation, which is already utilised in medical technologies such as Dermagraft (Organogenesis Inc. USA) (Mason and Manzotti, 2010) and in phase III clinical trials (ReNeuron Ltd. UK) (Stroemer, *et al.* 2009). Cryogenic temperatures convert all water inside a cell to ice and subsequently halt all chemical and metabolic processes. Successful cryopreservation generally yields over 90% cell viability (measured by membrane integrity) following thawing (Kleeberger, *et al.* 1999; Kotobuki, *et al.* 2005) although it is strongly dependent on cell type. ESCs are particularly sensitive to cryopreservation processing with poor survival post-thaw being reported (Li, *et al.* 2010; Xu, *et al.* 2010). Inevitably, viable cells will be lost following cryopreservation and any subsequent wash steps may lead to further cell losses. Work to mitigate the loss in viability upon thawing and to maintain cell function has generated highly controlled processes (Jesson *et al.* 2016).

Since the first successful cryopreservation of spermatozoa with glycerol in 1949 (Polge, *et al.* 1949), our understanding of cryobiology and methods to improve cell health and viability upon thawing have developed. The current practice of cryopreservation is carefully controlled, firstly by slowly freezing the cells to -80°C and then placing the cells in liquid nitrogen (-196°C) or liquid nitrogen vapour phase (-150°C) (Jesson *et al.* 2016).

A proficient cooling rate is slow enough to allow sufficient efflux of water to avoid ice crystals forming inside the cells and fast enough to prevent toxic solute concentration and cell shrinkage (figure 4). To counteract these events, a cooling rate of $1°C.min^{-1}$ is generally used during the intermediate zone of temperature (-15 to -60°C) to protect cells against the formation of ice crystals and steep osmotic gradients (Mazur, 1984: Jesson, *et al.* 2016). Both dehydration and high solute concentrations can have adverse effects on cell metabolism and the lipid-protein complexes of cell membranes. A freezing rate of $1°C.min^{-1}$ can be achieved using a controlled rate freezer (CRF) or freezing containers such as a Mr Frosty™ or CoolCell® (Biocision, 2015), which all require validation. To mediate the detrimental effects during freezing, a cryoprotectant agent (CPA) is also used to regulate the rate of water transport and crystal growth. The most commonly used CPA is the solvent dimethyl sulphoxide (DMSO) (Van Buskirk, *et al.* 2004). Glucose, trehalose, hydroxyethyl starch as well as ethylene glycol, can also work as cryoprotectants; however, none have proved more effective than DMSO in providing viable cells following preservation (Scheinkonig, *et al.* 2004; Rodrigues, *et al.* 2008). Commercially available freezing solutions such as CryoStor® are also pre-formulated containing 2–10% DMSO (Jesson *et al.* 2016).

Vitrification, a rapid freezing method, has been proven for embryo and human ESC preservation (Zhou, *et al.* 2004). This form of cryopreservation uses high CPA concentrations and cooling rates to enable a higher glass transition temperature and avoids both intra- and extracellular ice crystal formation. Since vitrification usually uses open cryostraws, the process is vulnerable to microbial contamination. Furthermore, each cryostraw is restricted to small volumes, typically 1–20 µL, which in terms of scale is severely limited for cell therapy (Jesson *et al.* 2016).

Slow Cooling = Solute Cryoinjury

- Cells lose water and dehydrate
- Volume shrinkage
- High solute solution
- No IIF

Rapid Cooling = Lethal IIF

- Intracellular water is not lost
- Cells super cooled
- IIF and EIF to obtain equilibrium

Ideal Cooling Rate

- Slow enough to avoid IIF
- Fast enough to prevent toxic solute concentrations and cell shrinkage
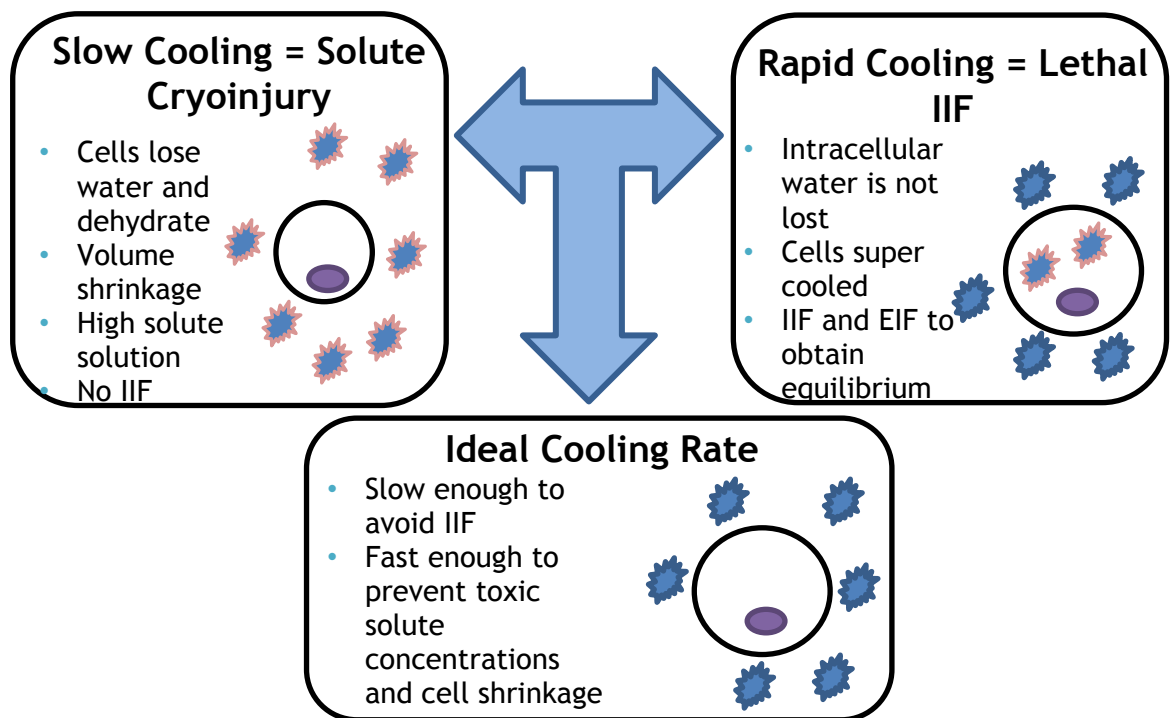
Figure 4: Effects of cooling rate on cellular health: An effective cooling rate is required to manage the adverse effects during the cell freezing process (EIF-Extracellular ice formation, IIF-Intracellular ice formation) Taken from Jesson, *et al.* 2016.

2.4.1 CHALLENGES OF CRYOPRESERVATION

One of the main challenges for cryopreservation is the use of DMSO as a CPA. Despite its success as the most effective CPA to date, it is as yet unclear whether its benefits outweigh the risks for cell therapy applications. DMSO works as an intracellular CPA and is thought to be cytotoxic to cells at room temperature (Scheinkonig, *et al.* 2004). Graded as a class 3 solvent (ICH, 2012), patients should receive no more than 1g.kg^{-1}.day^{-1} DMSO (Pomper, 2011; Jesson *et al.* 2016). Upon administration of DMSO-containing therapies, adverse patient reactions have been observed (Brobyn, 1975). It is unclear as to whether these symptoms arise from the DMSO, or the action of cellular infusion itself (Pomper, 2011; Jesson *et al.* 2016). DMSO can also dose-dependently initiate cell differentiation (Pal, *et al.* 2012). Given these risks, alternative

CPAs for freezing cells, have been investigated. Dissacharides such as trehalose and sucrose can be a replacement for DMSO, however their protective efficiency is hindered; since being large molecules, they are unable to penetrate the cell. Work to deliver trehalose inside cells including electroporation (Zhou, *et al.* 2010) and cell permeabilisation (Eroglu, *et al.* 2000; Sharp, *et al.* 2013) have been explored. The recent trend is to reduce DMSO concentration from the standard 10% to 5%, or to use a cocktail of DMSO and a non-toxic cryoprotectant to try and achieve the same level of effectiveness (Rodrigues, *et al.* 2008). With a lack of evidence and guidelines, it is unclear what percentage concentration of DMSO is safe to use in cell therapy, whilst maintaining the same level of cell protection; therefore alternatives are desirable (Jesson *et al.* 2016).

The thawing process of cryopreservation is of equal importance to the freezing procedure. During thawing, cells will be exposed for a second time to potentially damaging ice crystals when reaching the intermediate zone of temperature. To combat this, it is considered best practice to thaw cells 'quickly' (2–4 minutes) in a water bath at 37°C (Morris, 2007). More controlled methods of thawing are currently being explored such as the VIA Thaw device (Asymptote Ltd, 2015), a form of electrical thawing and the Genesis Plasmatherm (Genesis BPS™, 2015), a sealed class II device for warming and thawing blood and plasma products. Whilst the thawing step is of fundamental importance for retrieving live healthy cells post cryopreservation, it has become clear that a fully regulated and standardised thawing process has been neglected when compared to freezing protocols. This indicates a greater issue from a cell therapy perspective, particularly if a centralised manufacturing site is implemented. When cells are needed for a treatment, they are generally shipped

frozen, which means the vial will need to be thawed, reformulated and processed at the clinical site (Jesson *et al.* 2016). The thawing procedure is therefore performed by multiple clinical staff which can ultimately lead to variations in execution and a highly uncontrolled process. Furthermore, different thawing methodologies may hinder or vary the therapeutic action of the cells. A controlled approach may increase cell viability post preservation and reduce variability between samples (Morris and Acton, 2013). Performing the thawing process at the manufacturing site and shipping the cells hypothermically may be a beneficial alternative to controlled thawing at the clinic. The use of cold storage and shipping is of course dependent on whether a centralised or distributed manufacturing strategy is undertaken. Having one principal facility for the manufacture and scale-up/out of cells, together with hypothermic preservation and delivery, makes the process easier to validate; however the distribution of the hypothermic therapy will also need validating. Centralised manufacture also ensures quality consistency and no requirement for knowledge and technique transfer to additional sites (Coopman and Medcalf, 2014). Alternatively, a decentralised manufacturing site is particularly advantageous for autologous therapies due to being closer to the final point of administration. However, this 'point-of-care' approach using distributed facilities faces challenges such as process consistency (Coopman and Medcalf, 2014; Jesson *et al.* 2016).

2.5 BIOTECHNOLOGY INDUSTRY

Animal cells and tissues have been used in biotechnology for many years, including the use of human diploid fibroblast cells for the manufacture of vaccines and Chinese hamster ovary (CHO) cells for the production of therapeutic proteins (Fox, *et al.* 2005).

Cryopreservation, using liquid nitrogen, has been employed in the biotechnology field for the generation of homogeneous aliquots of cells, which aim to minimise genetic variation and allow for the testing of contamination before use in vaccine production (Stacey, 2004). Cryopreservation is the most successful method used today for mammalian cells and gametes. It is a costly process with a high risk of cell damage, particularly during and post-thaw. Other options such as hypothermic preservation and dry storage, in the form of lyophilzation, offer an alternative approach (Loi, *et al.* 2013). Mild hypothermia (30–33°C) is already successfully employed for large scale commercial production of therapeutic proteins from recombinant CHO cells (Sunley, *et al.* 2008; Kim, *et al.* 2012). Subculturing cells at these low temperatures leads to an adapted cell population, which achieve a growth rate two folds greater than non-adapted cells and leads to an elevated production in proteins (Sunley, *et al.* 2008). The exchange of sperm samples between research facilities has been successfully achieved at cold temperatures for up 48 hours (Takeo, *et al.* 2012). Work to extend this time frame and to attain successful fertilisation has included the use of the preservation medium Lifor®; a non-animal protein solution, which is designed to support cells and tissues at low temperatures, including ambient (Cell Preservation Solution LLC, 2015). Fertility in turkey sperm is known to be lower, when the sperm is stored hypothermically for more than 6 hours (Donoghue and Donaghue, 1997). Improved preservation protocols in agriculture maintain sperm fertility for longer, exploiting anti-oxidants (such as butylated hydroxytoluene and vitamin E), to limit to peroxidation and the production of reactive oxygen species (ROS) during cold temperatures (Aitken and Clarkson, 1988; Maxwell and Stojanov, 1996).

The process of freeze drying cells has been utilised in the preparation of pharmaceuticals and vaccines (Zhang, *et al.* 2010) and comes with the advantage of ambient temperature storage, subsequently easing transport regimens (Xiau, *et al.* 2004; Wolkers, *et al.* 2002). In the past, this method was limited to prokaryotes due to injuries implicated from desiccation (Zhang, *et al.* 2010). Recent studies have shown that cells, including human erythrocytes and platelets, have been successfully freeze dried whilst retaining physiological viability upon resuscitation with an aqueous solution (Wolkers, *et al.* 2002; Török, *et al.* 2005; Arav, 2013). Studies are ongoing to apply freeze drying to complex cells such as MSCs (Yang, *et al.* 2005; Zhang, *et al.* 2010). The rehydration process for dried cells is particularly severe, with colloidal osmotic and crystalloid pressure affecting cell recovery (Han, *et al.* 2004). Research to improve cell outcome following lyophilzation has used lyoprotectants like trehalose and polyvinylpyrrolidone (Yang, *et al.* 2005). Whilst there is activity in developing freeze drying as a cell preservation method, the procedure is currently difficult to control and too risky for cell therapy. Freeze drying does however have its uses in vaccines and in food and agriculture based industries.

It is clear that alternative methods of preservation such as cell pausing not only have a place in the regenerative medicine arena but can also be established for vaccine development, sperm and oocyte transport and recombinant protein production.

2.6 AN INTRODUCTION TO HYPOTHERMIA

Hypothermia is described as a condition in which core body temperature is reduced below the required temperature of 35°C for normal metabolic function to take place (Polderman, 2004). Body temperature is safely kept between a range of 36.5-37.5°C

through a process of homeostasis and thermoregulation (Benzinger, 1969). When exposed to cold temperatures, the body reacts by sacrificing the provision of heat to the extremities and maintaining core temperature at 35°C or above (Rodahl, 2005). Exposure to severely cold temperatures can sometimes result in a failure of internal metabolic mechanisms to replenish heat loss, which inevitably leads to a reduction in core temperature. Any core body temperature below 35°C is diagnosed clinically as hypothermia (McCullough and Arora, 2004); however, there are different degrees of the condition (Jolly and Ghezzi, 1992) as indicated in table 1. The same terminology and ranges of temperatures are used when applied to cells with some variation in the literature regarding what temperatures represent the levels of hypothermia.

Table 1: Various degrees of hypothermia

Mild	32-35°C
Moderate	28-32°C
Severe	20-28°C
Profound	<20°C

2.6.1 HYPOTHERMIA: A NATURAL PROCESS

Hypothermia is employed in nature as a method to survive cold temperatures during the winter by means of lowering internal core body temperature and conserving energy. Mammals who undergo torpor (temporary hibernation) can overcome cold induced stress due to innate molecular response mechanisms, which reduce physiological and metabolic activities (Hunt *et al.* 2005). This means enzymes and metabolites are preserved and biologic stability is maintained for a short period of time until temperature rises enough to initiate metabolism.

Hibernation in the Ground Squirrel, *Citellus tridecemlineatus* (figure 4), has also been found to provide tolerance to hypoxia, aglycemia and hypothermia compared to its active counterparts and non-hibernating species (Frerichs and Hallenbeck 1998). The Arctic Ground squirrel endures some of the harshest winters, surviving temperatures ranging from -8°C to -26°C (Barnes and Buck, 2000).

The potential to recover from hypothermia may depend on cell type. For example, tissues such as skin and testis are maintained below core body temperature and have adapted to these conditions (Hunt *et al.* 2005). An understanding of the mechanisms that help certain cells manage cold induced stress is required in order to facilitate and tailor environmental conditions during hypothermic preservation.

Figure 5: Arctic ground squirrel in Alaska (Wikipedia, 2016)

2.6.2 CLINICAL APPLICATIONS OF HYPOTHERMIA

Hypothermia has been used clinically since ancient times, when Hippocrates used snow and ice to reduce haemorrhage in wounded patients (Hippocrates 460-375 BC). In modern medicine, hypothermia has been used therapeutically since the 1940s when it was found to have neuroprotective properties in traumatic brain injury (Fay, 1945)

by reducing overall metabolism and blood flow to the tissues. Thereafter, hypothermia became used intraoperatively, particularly for cerebral protection during cardiac surgery (Bigelow, *et al.* 1950). Progress was made in 1980s when it was realised that mild hypothermia can be as beneficial as moderate or severe hypothermia with fewer side effects (Polderman, 2004). At present, hypothermia is induced deliberately in cardiac surgery to lessen the risk of myocardial ischemia (Mauney and Kron, 1995) and to minimise severity to traumatic brain injury (Marion *et al*. 1997).

With the emergence of organ transplantation in the 1960s, came the preservation of whole organs using low temperatures and modified media such as University of Wisconsin (UW) solution for short-term kidney and liver preservation (24–48 hours) (Belzer, *et al.* 1967; Jamieson, *et al.* 1988). Additionally, driven by direct clinical need (Scott, *et al.* 2005), the effective storage of red blood cells was accomplished using various hypothermic and cryopreservation strategies (Holovati and Acker, 2011). The biopreservation of red blood cells lead to the emergence of modern blood banks, which can successfully store blood in hypothermic conditions between 35–42 days (Högman, 1998).

As previously mentioned, hypothermia is also used in CHO cell bioprocessing to enhance the production of therapeutic molecules such as recombinant human glycoproteins for biochemical and preclinical studies (Sisken, *et al.* 1965; Fox, *et al.* 2005). Secretion of therapeutic proteins is one mechanism of action for MSCs; controlled hypothermic exposure, as well as preserving MSCs, could actively enhance their functionality.

As hypothermia already has many clinical applications and cold chain logistics already exist in the food industry and for vaccine supply, its progression into the laboratory as a cell preservation technique seems practical.

2.7 THE IMPORTANCE OF A SHORT-TERM CELL PRESERVATION PLATFORM

Stem cell preservation is employed continually throughout the development of a cell therapy. The scale up, transport and delivery of donor cells with the transplantation process and ensuring product quality can be challenging, hence a preservation method introduces more flexibility into the system (Frey, *et al.* 2006). Short-term storage options are valuable throughout development of cell therapies to allow enough time for safety and quality control testing (Jesson et al. 2016). During cell manufacture and also in the research setting, cells may be exposed to suboptimal conditions for hours at a time, referred to as 'in process' pausing. This exposure mainly arises from experimental delays, quality tests and equipment failures (Jesson *et al.* 2016). Cryopreservation is the preferred method of cell storage amongst cellular research institutes and industries. Although under researched, hypothermic preservation is becoming much more recognised as an option for the short-term storage of cells as it avoids the use of sub-zero temperature exposure. The literature describing the phenomenon of low temperature cell storage and the biological mechanisms behind it is developing; however there is certainly a gap in current research to highlight its potential for cell therapy applications.

2.8 LOW TEMPERATURE BIOPRESERVATION METHODS

2.8.1 CHILLED STORAGE

An alternative approach to cryopreservation suitable for short-term cell preservation is chilling cells to between 2–8°C. Hypothermic temperatures are already used clinically to store organs before transplantation (Collins, *et al.* 1969). Low temperature preservation (i.e. refrigeration) is employed for the storage of red blood cells for transfusion purposes (Hess, 2012). Refrigeration, alongside specialist formulations has enabled a shelf-life of up to 42 days (Hess, 2012). With regards to cells and tissues, the US industry guidance for current Good Manufacturing Practices (cGMP) sets 4°C as the target point for hold and transport (Mathew, 2013). Although stem cells are more complex than red blood cells, attempts to chill stem cells have been promising to date. For example, hMSCs have been successfully stored, in terms of membrane integrity and metabolic activity (85% viability), for up 96 hours at 4°C in the commercial hypothermic storage medium Hypothermosol®-Free Radical Solution (HTS-FRS, BioLife Solutions, Inc.) (Ginis, *et al.* 2012). Additionally, human ESCs preserved for 48 hours at 4°C in standard medium retained 64% membrane integrity (Heng, *et al.* 2006; Jesson *et al.* 2016)

When considering acceptable survival time for chilling, it depends upon whether the treatment is autologous or allogeneic. For example, HSC for transplantation can be processed and administered within a few hours when using freshly isolated cells (Giralt and Bishop, 2009). Cells may need to be expanded at the manufacturing site and transported to the clinic, which may require a storage length of days to weeks. Most research reports give examples of successfully chilling mammalian cells for 24–48 hours with some stating successful recovery after a week (Mathew, *et al.* 2004; Heng,

et al. 2006; Ginis, *et al.* 2012). Transporting a product from the UK to anywhere in Europe would typically take less than 24 hours, however extra hold time needs to be considered for potential transport delays, custom checks and setbacks to surgery (Jesson *et al.* 2016). The main challenge is to ensure that stem cells are viable and functional after chilling for this time frame. Given the cold-chain supply chain which already exists in the food and pharmaceutical industries, this method could be easily incorporated into cell therapy storage and transport procedures (Jesson *et al.* 2016).
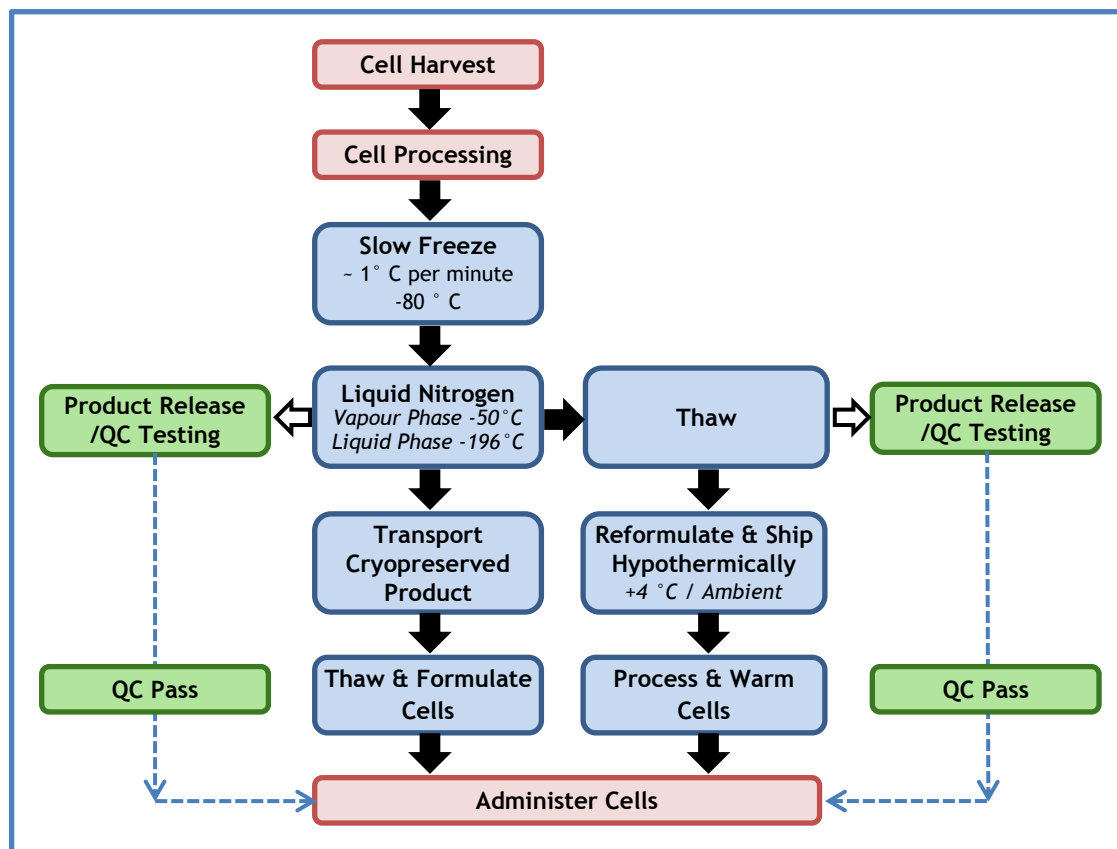
Figure 6: Preservation and Transport of Cellular Therapies - Flow diagram illustrating the main steps of cryopreservation and cell pausing processes for cell therapy. Taken from Jesson, *et al.* 2016.

Unlike cryopreservation, cell metabolism and progression through the cell cycle does not halt during hypothermic exposure. Metabolic reactions and energy dependent

systems such as protein synthesis and membrane transport slow down as temperature decreases, hence the term cell 'pausing'. For every 10°C decrease in temperature, metabolic reactions will slow by 50% as described by the Q_{10} temperature coefficient, (Reyes, *et al.* 2008). Over time, energy substrates in the pausing medium will become depleted, meaning insufficient adenosine triphosphate (ATP) can be generated to sustain cell integrity. Energy derived from the lactic acid cycle can temporarily compensate, but will not maintain cell energy over relevant storage periods once glucose is depleted (Mathew, 2013; Jesson *et al.* 2016).

Cells do not experience ice crystal formation or toxic solute concentrations during chilling, but hypothermic stress can still damage cells. Whilst hypothermia provides a protective mechanism by reducing energy dependent processes, over time ATP-driven ion pumps, which maintain osmotic gradients, membrane stability and stop free radical accumulation will similarly decrease, leading to cold-induced cell damage (Mathew, 2013). Cellular swelling (Plesnila, *et al.* 2000), up-regulation of cold shock proteins (Fujita, 1999), protein uncoupling (Tseng, *et al.* 2011) and the unfolded protein response (Corwin, *et al.* 2011) can be consequences of hypothermic storage. The more stress that accumulates with increased pausing time, the higher the risk of initiating apoptotic and necrotic pathways leading to cell death. During the rewarming of chilled cells to normothermic temperatures, metabolic processes are reactivated, which may lead to increased free radical production, efflux of waste products and cell death signalling pathways. It can take many hours or even days for cell death to manifest following thawing or rewarming and this is commonly referred to as 'delayed onset cell death' (Baust, 2006). This delay highlights the importance of viability assessment timing and the need to perform functional assays during the period from

24 hours to a week after thawing/warming. Without a suitable potency assay, shelf life cannot be accurately measured (Jesson *et al.* 2016).

2.8.2 AMBIENT TEMPERATURE PAUSING

Ambient temperature storage provides another option for the preservation and transport of cells for therapy. A hypothermic approach simplifies the preservation process and greatly reduces cost as well as reliance on specialist equipment such as the dry shippers, fridges or liquid nitrogen vessels used in cryopreservation. The literature for ambient temperature preservation is limited compared to cryogenic and chilled techniques (Robinson, *et al.* 2014), particularly for therapeutically relevant cells such as hMSCs. The main achievements have included alginate gel entrapment (Chen, *et al*. 2013) and enclosure in hermetic culture chambers such as the PetakaG3 (Celartia, 2015). A closed system is thought to protect cells from cold-induced stress and injury by sustaining a mild hypoxia, helping to arrest the cell cycle at the G_1/S interface. The drawback to using gel entrapment is the addition of a cell retrieval step, likely to be performed at the clinical site and introducing more time and variability into the process. Following ambient temperature storage of hair follicles adequate hair graft recovery has been reported (Hwang, *et al.* 2002) and mammalian cells have been successfully paused (4–24°C) in a variety of culture vessels including micro-centrifuge tubes, spinner flasks and 3L bioreactors (Hunt, *et al.* 2005; Jesson *et al.* 2016).

Ambient temperature pausing might be a promising strategy for use during 'inter-process pausing'. It is inevitable that during the manufacture of cell products (figure 6), cell pooling will be required and delays may ensue due to further processing, failed machinery or operator issues. A clear understanding of the length of time cells can be

paused for whilst still remaining viable and functional after warming would be highly valuable during cell manufacture and similarly in the biotechnology industry (Jesson *et al.* 2016). Due to the inability to define a temperature for ambient storage it is difficult to pin point which studies in the literature are most relevant. Referring again to the Q_{10} temperature coefficient, metabolic activity and energy dependent systems are supressed by 50% for every 10°C decrease in temperature. Therefore, the rate of reactions and cell cycle progression is slowed but not as significantly as when cells are chilled. Cells preserved in mild to moderate hypothermia metabolise enough for nutrients to be depleted and generate waste products and lactic acid which lowers pH and contributes to cellular injury over time (Fujita, 1999; Scott, *et al.* 2005). As with chilled cells, storage at ambient temperature will result in osmotic induced swelling and free radical production as well as affect membrane lipid bilayer integrity and cytoskeletal structure (Scott, *et al.,* 2005). The up-regulation of p53, WAF1 and cold inducible RNA-binding proteins (CIRP) between 25–33°C, offer a protective mechanism and help cell survival from recurrent stress (Nishiyama, *et al.* 1997; Matijasevic, *et al.* 1998; Ohnishi, *et al.* 1998; Sonna, *et al*. 2002). For example, a decrease in temperature from 37°C to 28°C prolongs the cell cycle of cultured mouse leukemic cells seven-fold (Watanabe and Okada, 1967; Jesson *et al.* 2016).

2.9 SELECTION OF BIOPRESERVATION METHOD

One of the most important decisions in cell manufacture and distribution is determining which preservation procedure(s) (freezing, chilling or ambient storage) is the most appropriate for the given therapeutic application. The preservation method must be tailored to the cell therapy product/process to enable the best outcome in

terms of therapeutically effective cells. For cell banking, which requires long-term storage (months to years), cryopreservation is the most suitable option. When shipping cells, on the other hand, hypothermia may be the most feasible method to keep cells 'paused' for short-time frames (days). Choosing a preservation and shipment method, therefore, also depends on the length of the journey from manufacturing to clinical site e.g. national or transcontinental; and the ability of the storage system to support live, functional cells for this duration. A 'one size fits all' approach is unlikely for hypothermic preservation media, as cell types differ in their response to supplements (Van Buskirk, *et al*. 2004). Whilst promising results have been achieved when pausing mammalian cells in ambient conditions, a comprehensive investigation is yet to be undertaken on stem cells. Research to develop media and additives that support metabolism and cell survival at a range of temperatures (e.g. 15–25°C) is currently ongoing. Simpler hold and transport protocols are desirable for cell products in the future. Ultimately, the chosen method to process and preserve cells should be tailored to each cell therapy product to provide the desired potency, therapeutic effect and cGMP compliance (Jesson *et al.* 2016).

2.10 CHALLENGES OF LOW TEMPERATURE BIOPRESERVATION

2.10.1 CHILLED STORAGE

One of the main concerns with hypothermic preservation is cell deterioration with time. As cellular systems do not stop under hypothermic conditions, a gradual decrease in cell viability over time could be due to reduced nutrient supply and a build-up of waste and metabolites. To combat these toxic accumulations, specialised intracellular-like media that are based on the ion concentrations the cells experience

during hypothermia have been developed to help stabilise cells at cold temperatures (Mathew, 2013). Numerous solutions were initially developed for tissue preservation, prior to the development of our understanding of the mechanisms behind hypothermic stress and apoptotic pathways (Van Buskirk, *et al.* 2004). Currently, HTS-FRS, a medium formulation designed for cold storage (2–8°C), is the leading commercial product for the hypothermic preservation of cells. BioLife Solutions Inc. has submitted a drug master file to the FDA for the company's HTS product and it is currently being used in clinical trials for heart disease, stroke, cancer and neuronal disorders (BioLife Solutions, Inc. 2015). Irvine Scientific®, a global supplier of cell therapy media, have demonstrated effective storage of hMSCs for 5 days at 4°C in their defined hypothermic preservation medium, PRIME-XV™ (Irvine Scientific, 2016). Cell viability was maintained (>80%) 24 hours post recovery. These specialised media aim to modulate the stress response cells undergo during temperature changes, particularly during the re-warming phase. Hypothermic storage media generally contain ionic components such as Na^+ and Cl^-, pH stabilisers, glucose and oncotic and osmotic stabilisers. These constituents aim to balance ion concentrations, manage pH fluctuations, aid ATP production upon warming and provide osmotic support during hypothermic stress (Jesson *et al.* 2016).

Whilst commercial media are being designed and established, another approach for the effective storage of live cells at low temperatures is the development of stabilisers or supplements to standard cell culture medium. Rather than replacing existing medium with a complete preservation medium before storage, simply adding a supplement to the culture medium can protect cells against hypothermic damage. Rokepie® is a non-toxic medium additive, which works to pause cells (2–8°C), based on

the chemistry of hibernation (Rokepie®, 2015). As the supplement is not damaging to cells, this avoids the requirement of a removal step before processing. Drawbacks to using media additives and supplements are GMP compatibility and the build-up of waste products before and during cell preservation (Jesson *et al.* 2016).

The importance of examining post pause cell viability and function is a theme within the literature regarding preservation. Whilst initial viability results may look promising, upon thawing/rewarming a decline in viability is commonly observed. This may be due to the phenomenon of delayed onset cell death, where the activation of caspase cascades can occur up to several days following warming to normothermic conditions (Van Buskirk, *et al.* 2004). When the cells are paused during low temperature exposure, a lag period can be observed when placed back into normal culture conditions before exponential growth is resumed. From a cell therapy perspective, this might be problematic if the cellular product once administered takes 24 hours or more to take effect either by engraftment or another mechanism of action (Jesson *et al.* 2016).

It is also uncertain whether cells will need to equilibrate to room temperature or be warmed to body temperature before administration to the patient. The current process for chilled blood products prior to transfusion employs the use of a blood warming device which heats to near body temperature to avoid hypothermia and other complications (Greenblatt, 1987). For cell therapies, clarification by product release testing will provide information as to whether the cells survive the stresses of rewarming before administration. It is unclear whether a wash step or dilution will be required before cells are delivered to the patient and will be determined by the ingredients of the pausing medium and if they are FDA approved. If a wash step is

required, effective removal of the medium or additives needs to be demonstrated with no risk of cell diffusion or uptake. The more complex the final processing steps, the more need for appropriate facilities and sufficiently trained staff (Jesson *et al.* 2016).

2.10.2 AMBIENT TEMPERATURE PAUSING

The main drawback with using ambient temperatures to store and transport cells is the definition and control of ambient temperature. Atmospheric temperatures can significantly vary globally, seasonally and on a smaller scale between countries, cities and rooms; therefore, an ambient temperature range, between which cells are able to successfully recover and function therapeutically, is difficult to define. The range would be dependent on the specific journey and estimated temperatures the cells may be exposed to; it should also be broad enough that the transit can occur but narrow enough so that the product is not compromised. If the product breaches these temperature boundaries, the cells would have to be discarded (Jesson *et al.* 2016).

With the exception of a few cell types (e.g. HSC), most cells used for therapeutic application are anchorage-dependent. However, to reduce transportation volumes and avoid the need for clinical staff to harvest cells from culture vessels, cells should be transported in suspension, in sealed vials or bags. Manipulating adherent cells to survive in suspension at ambient temperature is particularly difficult due to the increased level in cell-cell attachment and aggregation. During cell death, nucleic acids are released from cells; these nucleic acids promote cell adhesion and can lead to cell aggregate formation. Research to maintain a single cell suspension during pausing has produced chemically defined formulations which can be incorporated into cell culture

media such as Gibco® Anti-Clumping Agent and Benzonase® Endonuclease (Smith, *et al*. 2001). These tools effectively remove DNA and RNA to reduce cell clumping in suspension culture. It is unclear whether these cell aggregates can be safely delivered to patients without systemic reactions or risk of vascular occlusion and will be strongly dependent on aggregate size. Additionally, if cells were to aggregate due to lowered temperature during inter-process pausing, this may cause complications for downstream processing (Jesson *et al.* 2016).

Another consideration with ambient storage is uncontrolled atmospheric factors including CO_2 concentrations and relative humidity, which in typical cell conditions are set to 5% and 95% respectively to simulate an *in-vivo* environment (Robinson, *et al.* 2014). Standard cell culture medium usually relies on sodium bicarbonate as a buffer system, which depends on CO_2 diffusion, in order to maintain a physiological pH of 7.4. Additional buffer systems will be required in order to regulate pH and minimise damage from deviations (Jesson *et al.* 2016).

2.11 EFFECTS OF PAUSING ON THE CELL

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 CIRP, as a protective mechanism (Fujita, 1999) to help the cell survive and recover from current and subsequent stresses. Hypothermic temperatures may also affect membrane lipid bilayer integrity and cytoskeletal structure (Scott *et al.* 2005). During physiological temperatures, the cell membrane is in a fluid state. Upon a reduction of temperature,

lipids transition into a gel state, which can make various membrane proteins and lipids segregate and become leaky (Rubinsky, 2003). Together with ionic leakage and a reduced efficiency of energy-dependent ion pumps, this can lead to osmotic imbalances and protein denaturation. Furthermore, low temperatures can weaken hydrogen bonds and ionic clustering in the lipid bilayers of the cell membrane making them vulnerable to rupture. 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 and consequently cell death (Fujita, 1999; Scott, *et al.* 2005).

2.11.1 MODES OF CELL DEATH

Extreme temperature shifts which occur during cryopreservation and hypothermic storage are associated with various modes of cell death including physical ice related cell disruption and the induction of molecular pathways (figure 7) including apoptosis and necrosis (Mathew, *et al.* 2004). Necrosis can be described as an energy-independent, pathological form of cell death. It is characterized by cellular swelling, impaired membrane integrity, rupture of lysomes and DNA degradation leading to cell lysis (Baust, 2007) and an inflammatory response *in vivo*. On the other hand, apoptosis or programmed cell death is an energy-dependent process and is extremely important for the homeostatic maintenance of cell number and tissue development in complex organisms. For example, apoptosis is employed for the deletion of excess cells in the developing humans webbed fingers and toes and assists in the removal of damaged cells from the body (Baust, 2007). If energy in the form of ATP is not available, cells will switch to a necrotic form of cell death, known as secondary apoptosis. The association

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of apoptosis as a consequence of cryopreservation was identified in 1998 (Baust *et al.* 2009). Stresses including cytotoxic agents, temperature deviations, anoxia and nutrient deprivation have been demonstrated to elicit the effects of apoptosis. Apoptosis occurs through a cascade of events with the commencement of caspase activation and mitochondrial release of cytochrome c, through to the terminal stages of DNA cleavage, phospholipid inversion, cellular shrinkage and membrane blebbing, also known as apoptotic bodies.

One of the ways these cellular pathways are initiated is through the binding of cell membrane protein receptors including several from the tumour necrosis factor (TNF) superfamily. The mitochondrion and the nucleus are also primary sites for apoptosis activation. A cells decision to induce apoptosis is further based on the present ratio of anti and pro apoptotic proteins. The manifestation of these molecular pathways does not take place immediately post-preservation (Baust *et al.* 2001), with a delayed peak in necrotic and apoptotic activity post-thaw at 6 and 12 hours respectively. This phenomenon, known as delayed onset cell death, has led to researchers performing viability and functionality tests not only post-thaw but at certain time points (hours to days) following resuscitation to ensure all effects from freezing/chilling are accounted for. 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 during hypothermic exposure compared to cryopreservation.

During the rewarming period, energy-dependent systems such as cell propagation are resumed to that of normal culture conditions. As well as a recommencement of cellular metabolic and proliferative activities, other energy-dependent mechanisms such as apoptosis and the production of ROS will be initiated (Meng, 2003). This is similar to tissue ischemia and cell death upon reperfusion.

2.11.2 EFFECTS OF PAUSING ON GENE REGULATION AND PROTEIN EXPRESSION

Transcription and translation of new proteins takes place continuously within mammalian cells at 37°C, yet when temperature is reduced, these processes slow down or are halted altogether (Fujita, 1999). During hypothermia, the expression of some cellular proteins including p53, WAF1 and CIRP are elevated at temperatures ranging between 25–33°C (Nishiyama, *et al.* 1997; Matijasevic, *et al.* 1998; Ohnishi, *et al.* 1998; Sonna, *et al.* 2002).

p53 is a tumour suppressor protein, encoded by the gene *TP53*, which regulates the cell cycle and is associated with initiating apoptosis (Liu, *et al.* 2003). Human fibroblasts cultured at 28°C undergo a reversible growth arrest proposed to be mediated by p53 (Matijasevic, *et al.* 1998). WAF1 is a protein directly regulated by p53 (Ohnishi, *et al.* 1998) and has similar tumour suppressive properties. The increased expression of these proteins suggests a protective mechanism, by arresting cell growth when environmental conditions are below optimum standards for proliferation. It has been demonstrated that upon thawing and rewarming mammalian cells to 37°C, the expression and activation of heat shock proteins (HSP) is also induced (Holland, *et al.*

1993; Liu, *et al.* 1994). HSP are a form of stress protein with a fundamental protective

role in physiological homeostasis when environmental factors are sub-optimal
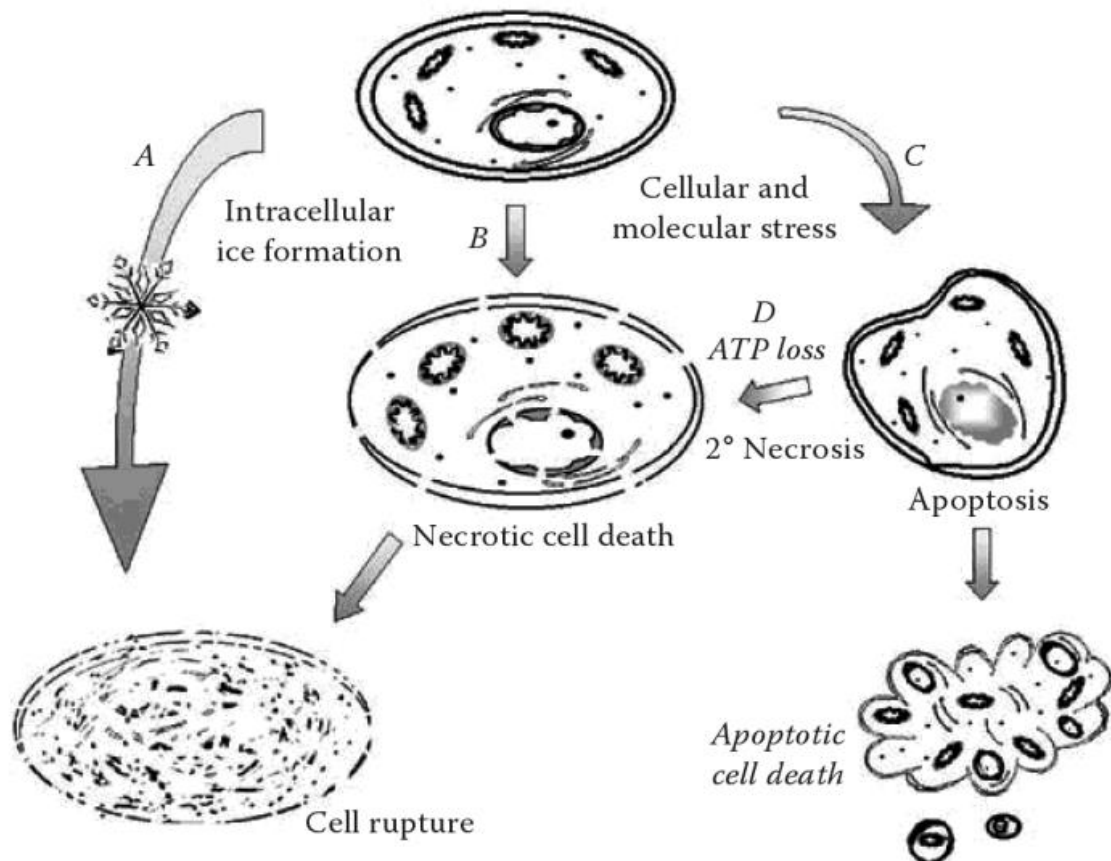
Figure 7: Modes of cell death associated with cryopreservation: (A) Physical ice rupture **(B)** Necrotic cell death **(C)** Apoptotic cell death **(D)** Secondary necrosis. The molecular mechanisms of cell death (A,B) can also be initiated by hypothermic preservation. Taken from Baust, 2007.

The elevated expression of HSP upon exposure to increased temperatures has been

well studied and these proteins are also present as molecular chaperones at

normothermic temperatures, having roles in the folding/unfolding and transport of

proteins (Fujita, 1999). It seems that the ability of a cell to adapt to increases in

environmental temperature is fundamental to survival in both prokaryotes and

eukaryotes as the synthesis of these highly conserved HSPs has been observed in nearly all multicellular organisms (Lindquist and Craig, 1988).

Compared to HSPs, less is known about cold shock proteins (CSP); however the same principal occurs where upon exposure to decreased temperature (e.g. mild to moderate hypothermia 25–33°C) CSP gene expression is rapidly elevated as a protective mechanism (Sonna, *et al.* 2002). It seems most cellular responses to cold shock are similar to those that occur during heat shock; see further details in table 2.

The best characterised CSP is CIRP, made up of 172 amino acids with an RNA-binding domain at its N-terminus and a glycine-rich carboxyl terminal (Nishiyama, *et al.* 1997). Using cultured BALB/3T3 mouse fibroblasts, it has been found that a decrease in temperature from 37°C to just 32°C induces CIRP expression and cold-induced suppression of proliferation (Nishiyama, *et al.* 1997). As well as its role in suppressing cell proliferation, CIRP is important for the stabilisation and increased translation of target mRNA as it enhances its resistance to degradation by RNases (Sonna, *et al.* 2002). The CIRP gene is highly conserved and constitutively expressed at low levels in rodent species as well as in humans (Xue, *et al.* 1999).

Interestingly, Lindquist and Craig (1988) explain that HSPs become the predominant proteins synthesized during hypothermic exposure and recovery. Comparable to HSPs, CIRP expression can be induced not only by decreases in temperature but also exposure to ultra-violet (UV) radiation and hypoxia (Fujita, 1999). Upon UV radiation, CIRP is induced and translocated from the nucleus to the cytoplasm to increase stabilisation of specific transcripts to help with cell survival against genotoxic stress (Yang and Carrier, 2001). Moreover, cells transfected with an antisense CIRP vector

had an impaired ability to survive UV irradiation (Yang and Carrier, 2001). CIRP may have additional roles in cold-induced cell cycle arrest. In 1997, Nishiyama and colleagues (1997) found that the overexpression of CIRP reduced growth rates at 37°C with an extended G_1 phase.

RNA-binding motif 3 (RBM3) is another member of the glycine-rich RNA-binding protein (GRP) family that has been studied in some detail and shares a similar structure to CIRP, however, it does not appear to be implicated in cold-induced growth suppression (Danno, *et al.* 2000).

2.11.3 EFFECTS OF COLD INDUCED STRESS ON THE CELL CYCLE

As explained previously, cold shock is associated with prolonging or pausing the cell cycle altogether (Rieder, *et al*. 2002). For example, Watanabe and Okada in 1967 found that lowering the temperature of cultured mouse leukemic (L5178Y) cells from 37°C to 28°C prolongs their cell cycle time seven-fold.

The phases of the cell cycle that are most susceptible to hypothermic temperatures are the beginning growth (G_1) phase, synthetic (S) phase and particularly mitosis, illustrated in figure 8 (Rao and Engelberg, 1965). To demonstrate this, spindle formation in rat kangaroo kidney (Ptk1) cells was found to take 8–10 hours in hypothermic conditions, whereas at optimum temperature the process usually takes ~50 minutes (Rieder, 1981).

It is understood that if upon cellular stress a cell has already committed to mitosis, the cell progresses through the cellular division process (Rieder, 1981). Alternatively,

before commitment the cell decondenses and proceeds to the terminal stage of G_2 also known as antephase (Bullough and Johnson, 1951; Pines and Rieder, 2001).

Table 2: Cellular responses to non-lethal heat and cold shock (Table information from McGarry & Lindquist, 1985).

Effects	Cellular Response to Heat Shock	Cellular Response to Cold Shock
Proteins	Increased denaturation and misaggregation	Increased denaturation and misaggregation
Cell Cycle Progression	Reduced/Inhibited	Reduced
Transcription/translation	Reduced/Inhibited Translation of normal cellular mRNA is reduced and translation of mRNA encoding HSPs such as HSP70 is enhanced	Reduced Translation of normal cellular mRNA is reduced and translation of mRNA encoding CSPs such as CIRP is enhanced
DNA Synthesis	Reduced/Inhibited	Reduced/Inhibited
Membrane Permeability	Reduced/Inhibited	Reduced/Inhibited
Enzyme Activity	Increased, especially the proteasomal and lysosomal pathways/Inhibited	Decreased
Transport systems	Reduced/Inhibited	Reduced/Inhibited
Metabolism	Inhibited	Reduced
Lipid Bilayer Composition	Damaged	Damaged
Gene Expression	Overall decreased expression/ Increased expression of HSP	Overall decreased expression/ Increased expression of CSP

These cells re-enter mitosis upon warming to 37°C and complete mitotic cell division. Rieder and colleagues (2002) explains that there may be a regulatory mechanism in the G_2 phase which is temperature dependent and controls entry into mitosis.

The degree of hypothermia can determine the rate of progression through the cell cycle, for example, mild hypothermia results in a slower movement through the cell cycle and moderate to severe hypothermia can block the cell cycle in the G_2 phase or

at the G_1/S boundary (Rieder, *et al.* 2002). Most research undertaken in the field of cold tolerance has focused on microorganisms and plants. Hence, further research is required on mammalian cell lines as well as stem cells as hypothermic preservation procedures gain more popularity in the clinic.
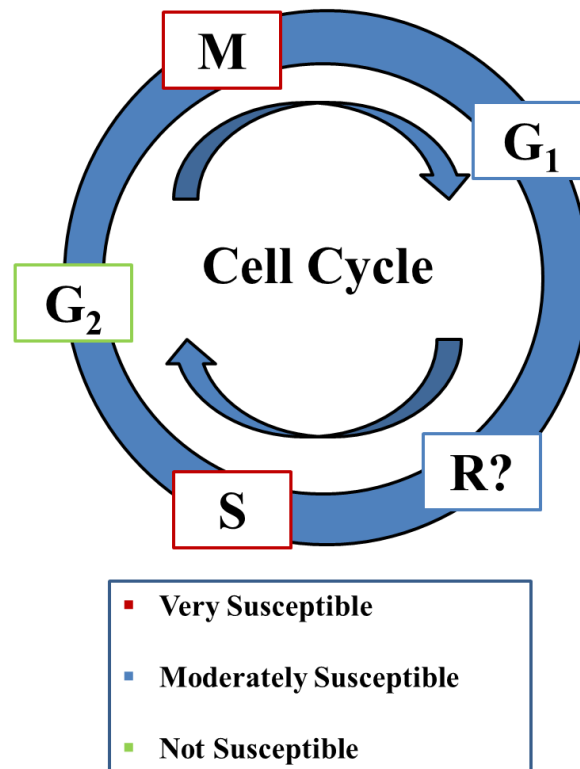
Figure 8: Effects of hypothermia on cell cycle progression - The diagram above shows which parts of the cell cycle are most affected by hypothermic temperatures. Cell growth and replication is a highly regulated cycle consisting of a number of checkpoints which decide whether a cell is able to progress through the cell cycle successfully or enter apoptosis. The cycle begins with cell growth (G_1) where the cell increases in size and its internal composition is analysed as well as DNA damage to determine whether the cell enters the synthetic (S) stage or is programmed for apoptosis (R). DNA replication and preparation occurs during the S phase and the cell prepares to divide through G_2. Cell division occurs at the mitotic phase, producing two daughter cells in which the cell cycle begins again.

2.12 ACHIEVEMENTS IN CELL PAUSING TO DATE

Effective storage of red blood cells has been possible since the 1930s, using various hypothermic and cryopreservation strategies (Holovati and 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, 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 commercialization of uniquely formulated storage media such as University of Wisconsin solution (UW) or ViaSpan®, Histidine-tryptophan-ketoglutarate (HTK), Celsior, EuroCollins, Custodial and most recently HTS-FRS and HypoThermosol-BASE (HTS-BASE) or PrepaStore (2–8°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, see figure 9 (Mathew, *et al.* 2002; Mathew, *et al.* 2004).

HEPES buffer manages pH fluctuations and provides osmotic support (Taylor, 1982; Taylor and Pignat, 1982). Impermeants like 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). HTS has 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).
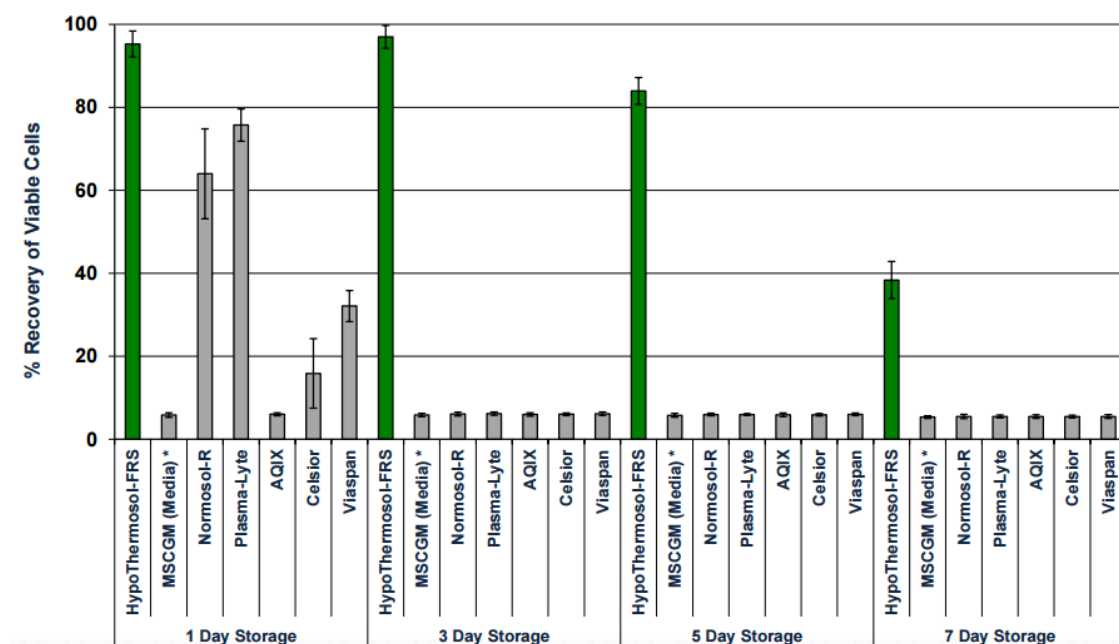
Figure 9: The hypothermic storage (2–8°C) of human mesenchymal stem cells in various media including Hypothermosol® - This graph shows data from BioLife Solutions, Inc. and indicates the percentage of viable cells, 24 hours post storage via alamar blue staining for metabolic activity (*contains serum). Taken from BioLife Solutions, Inc. 2015.

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 3. One particular success in cell pausing under ambient conditions used hMSCs and mouse ESCs (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. Most recently, human adipose derived stem cells encapsulated in alginate gel and stored for 72 hours at 15°C were capable of attachment and displayed normal growth kinetics in comparison with control cells (Swioklo, *et al.* 2016). Alginate entrapment of cells during hypothermic storage has previously been shown to protect against induction of apoptosis when compared with 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, 2015). This closed system attains mild hypoxia which helps initiate cell cycle arrest at the G_1/S interface, resulting in suspended animation. Cell pausing is clearly becoming more accepted as a potential strategy 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.

2.13 REGULATORY HURDLES

Whilst cell therapies offer a novel approach to the treatment of a vast amount of diseases and will no doubt be touted as a salvage therapy for some patients, they face stringent regulatory challenges (Feigal, *et al.* 2014). The US FDA and the European Medicines Agency (EMA) are global regulatory bodies that provide guidance on the safe and efficacious development of cell therapies (Jesson, *et al.* 2016). Since 1997, more than 300,000 patients have been treated with a regulatory approved cell-based product (Mason and Manzotti, 2010). Whilst this may encourage patient expectations, strict regulatory boundaries ensure patient safety and the manufacture of these products under GMP (Heathman *et al.* 2015; Thomas *et al.* 2008). In the case of cell therapies, the cell is the product; thereby stringent potency assays can provide a form of validation test which measures consistency, therapeutic quality and risk of tumourigenicity. Whilst these tests provide information on the safety and efficacy of the cell in an *in vitro* setting, there are still concerns over donor acceptance and whether the products therapeutic action will be delivered once *in vivo*. Moreover, the use of animal-derived products in the culture of some cell-based therapy products calls for additional regulatory constraints.

One major regulatory hurdle for allogeneic cell therapies is the variation encountered in terms of input material and processing. Global differences in regulations have caused uncertainty regarding cell sourcing and donor eligibility which necessitates convergence of guidelines amongst worldwide regulatory bodies and the need for additional guidance.

Table 3: A summary of current research papers focusing on hypothermic storage of human cells. Taken from Robinson, *et al.* **2014** HypoThermosol-Free Radical Solution (HTS-FRS), University of Wisconsin Solution (UW), Dulbecco's Modified Eagle Medium. (*) Membrane integrity (**) Membrane integrity and metabolic activity

Study	*Cell Type*	*Storage Temperature, Exposure Time & Recovery Time*	*Storage Solution*	*Viability (%)*
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 Histidine-tryptophan-ketoglutarate	~42-99 (*) ~42-98 (*) ~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 (*)

Study	Cell Type	Storage Temperature, Exposure Time & Recovery Time	Storage Solution	Viability (%)
Mathew *et al.* (2004) (All data not shown)	Coronary artery smooth muscle cells	4°C 24 hrs 24 hrs	Standard culture medium	<10 (**)
			UW	<10 (**)
			HTS	<10 (**)
			HTS-FRS+Trolox	63-65 (**)
		4°C 48 hrs Over 5days	Standard culture medium	<10 (**)
			UW	<10 (**)
			HTS	<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 (**)

The International Council for Harmonization (IHC) has since published a guidance

document aiming to harmonize world-wide regulatory differences for the

development cell therapy (ICH Guidelines, 2015). Regardless of these efforts, clearer guidance is required for specific cell therapies and should be considered from the start of its development (Jesson *et al.* 2016).

The delivery of cell-based products leads to another regulatory challenge. Maintaining cell viability and quality whilst in transit for a short-time frame (hours to days) is of vital importance for cell therapies to be administered to patients. The most recent example of the delivery of a non-cryopreserved personalised therapy is Provenge, which uses peripheral blood mononuclear cells to treat advanced stage prostate cancer. The cells are suspended in Lactated Ringer' Injection solution and can be stored at 2–8°C for 18 hours only whilst lot release testing is performed simultaneously to product shipping (Provenge: EPAR-product information).

2.13.1 PACKAGING

For the biopreservation of cell and tissue products, the container must be stable, easily accessible and hermetically sealed to ensure sample integrity (Woods and Thirumala, 2011). Ideally, the chosen medium and packaging must maintain cell viability and function throughout the holding and transportation processes at levels measured prior to shipment (Snyder, *et al.* 2004). The most commonly used vial type in research is the screw cap which is limited for clinical use due to low integrity and high risk of contamination. Cell freezer bags are more ideal for use in clinical practice as they can be hermetically sealed and hold larger volumes of cellular product. Vials and bags that are currently available are typically made with ethylene vinyl acetate and polyvinyl chloride. These particular materials carry risks for storage at cryogenic temperatures, as they become brittle and susceptible to breakage. Furthermore, the

plasticizers used to make PVC flexible are prone to leaching into the surrounding media. More thought into the appropriate materials for cell and tissue storage containers has generated commercial products such as CellSeal® and Crystal® vials; closed system cryovials made with USP class VI materials, which are resistant and durable at cryogenic temperatures (Jesson *et al.* 2016).

Traceability of cellular products, whether allogeneic or autologous, is essential for their safe delivery to patients. Cell therapy packages or vials must be labelled appropriately detailing batch number, expiration date and processing details upon receipt (Jesson *et al.* 2016).

2.13.2 THE XENO ISSUE

Fetal bovine serum (FBS) is a common component added to cell culture medium to aid cell attachment and growth and is also used as a vehicle solution for cryopreservation. Whilst the mechanism by which serum assists cell growth and attachment is not fully understood, it has been used widely used in cell culture for over a decade. However, for research supporting cells for use in cell therapies, there are safety concerns from using animal-derived reagents (Patrikoski, *et al.* 2013). Not only are the extraction methods for obtaining FBS from cattle considered unethical but the sheer quantity of serum required for cell therapies causes issues regarding batch variation; a bovine fetus near term yields only ~550mL serum (Jochems, *et al*. 2002) and the production rates of cGMP grade serum may only be sufficient for one blockbuster cell therapy (Brindley, *et al*. 2012). hMSC research using FBS has been criticised for the potential introduction of zoonotic contamination to the cell product such as prion disease and its potential effects on the immunomodulatory properties of the cells (Shahdadfar, *et*

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al. 2005). To date, most clinical trials have used hMSCs previously expanded in FBS (Horwitz, *et al.* 1999; Horwitz, *et al.* 2002; Koç, *et al.* 2000; Koç, *et al.* 2002); however, anaphylactic reactions have been observed in patients who received repeated administration of dendritic cells cultured in FBS (Mackensen, *et al.* 2000). With cells previously cultured or stored in FBS, there is a risk of xeno-originating components being transferred to patients, even if numerous wash steps are incorporated into the process. Due to the regulatory concerns of the FDA, the restricted availability of FBS for cell therapy production and the impact this will have on commercialisation, the development of serum-free and chemically defined options is now a major area of research including the generation of CryoStor (BioLife Solutions, Inc.) and Stemulate^TM (Cook Regentec), which is a pooled human platelet lysate.

2.14 THE FUTURE OF CELL PAUSING

Low temperature cell pausing demonstrates great potential as a short-term storage method for allogeneic and autologous cell therapies. Its non-reliance on temperature controlled apparatus means ambient temperature storage is particularly advantageous and signifies its reliability as well financial benefit. Alternatively, improving storage of cells at 4°C may ease the adaption of cell pausing into current practices.

Whereas cryopreservation requires a multi-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. Furthermore, thawing of cryopreserved vials and retrieval of cells at the

clinic can be cumbersome whilst paused cells could potentially be rewarmed to 37°C in original pausing media.

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. The refinement of media composition to accommodate cell metabolism and growth at low temperature is vital, especially for the translation of this process to stem cells. Manipulating cells to survive in suspension at low temperatures will be a significant challenge. Once achieved with proven cGMP-compliance (Heidemann, *et al.* 2010), pausing of cells in suspension will further ease the transportation and retrieval process of cells for clinicians.

Whilst still in its infancy, low temperature pausing has huge potential for improving transport protocols for use in cell therapy as well in biotechnology. In summary, cell pausing (chilled or ambient) simplifies the protocol, reduces reliability of specialist machinery and eases retrieval of cells for clinicians. With the amount of research and finance being invested into cell therapy, it is crucial that the cell storage and transport methods are developed alongside the therapy itself so that the best preservation option can be appointed and fewer regulatory hurdles encountered when approaching approval and commercialisation. This will in turn limit the pitfalls of drug development and lead towards more regenerative therapies on the market.

CHAPTER 3: MATERIALS AND METHODS

3.1 CHEMICALS, REAGENTS, CONSUMABLES AND EQUIPMENT

All chemicals and reagents, unless stated were purchased from Sigma-Aldrich, UK and consumable equipment was obtained from Scientific Laboratory Supplies (SLS), UK. A list of equipment and suppliers is provided in section 3.12.

3.2 CELLS

3.2.1 HUMAN OSTEOSARCOMA CELL LINE (HOS TE85)

An adherent human osteosarcoma cell line (HOS TE85) obtained from the European Collection of Cell Culture (ECACC) was used for initial investigations. These adherent cells were derived from an osteosarcoma of a 13 year old female Caucasian (ECACC, 2015). HOS TE85 cells were expanded to produce a master cell bank (MCB) and were cryopreserved (1×10^6 cells/mL) in FBS (Fisher Scientific, UK) with 10% (v/v) DMSO. HOS TE85 cells were thawed for these experiments from passage 54 and after reaching 80–90% confluence, were passaged at least twice before all hypothermic investigations. Briefly, following culture for 24–48 hours in a humidified incubator set at 37°C, 5% CO_2, cells were washed with $0.1mL/cm^2$ phosphate buffered saline (PBS) without calcium chloride and magnesium chloride (Life Technologies, UK). Cells were detached from tissue culture plastic by incubating with $0.04mL/cm^2$ of 0.25% trypsin-EDTA for 5–10 minutes. Any cells still attached after this time were dislodged by gentle tapping of the flask. Trypsin-EDTA was quenched with complete minimum essential medium with Earle's Balanced Salts (MEM/EBSS) (Fisher Scientific, UK) supplemented with 10% (v/v) FBS, 2% (v/v) L-glutamine (Life Technologies, UK) and 2% (v/v) non-essential amino

acids (NEAA) (Life Technologies, UK) using a 1:3 dilution (for medium formulations see section 3.5). A cell sample, if needed for viability or total count, was taken here. Please refer to section 3.9.2 for detailed cell count methodologies. The cell suspension was centrifuged at 1200g for 5 minutes at room temperature to produce a cell pellet. Following this, the supernatant was aspirated away and the pellet was resuspended in fresh medium. Cells were then seeded into new vented T-flasks or well plates at 1.2×10^4 cells/cm^2; table 4 describes the growth areas and volumes of reagents used for various sized culture vessels.

Table 4: **Summary of the various cell culture vessels and volumes of reagents used throughout general culture.**

Culture Vessel	Growth Area (cm^2)	Medium Volume (mL)	Trypsin-EDTA Volume (mL)
24-well plate	1.9	1.5	0.1
12-well plate	3.8	2	0.2
6-well plate	9.5	3	0.4
T25	25	5	1
T75	75	15	3
T175	175	35	7

3.2.2 HUMAN MESENCHYMAL STEM CELLS (HMSC)

hMSCs were initially extracted from bone marrow by Lonza, USA and shipped as P0 mononuclear cells. Cells were derived from three healthy donors, described in-house as M2, M3 and M4, who each provided informed consent. Cells were cryopreserved at passage 0 and 1 (1-2x10^6 cells) in a 2mL cryovial (see section 3.3). Further information on donor characteristics is provided in table 5.

For all hMSC hypothermic investigations, a vial of cells banked at passage 1 from all three donors (M2, M3 and M4) was thawed and passaged at least once. Briefly, cells were cultured for 6 days in a humidified incubator set to 37°C and 5% CO_2, with a full

medium change on day 3. On day 6, cells were enzymatically detached from the tissue culture plastic as described in section 3.2.1. The cell suspension was centrifuged at 300g for 5 minutes at room temperature to produce a cell pellet. The pellet was resuspended in fresh medium and cell sample was taken at this point if required. Cells were seeded into a new vented T-flask at a density of 5×10^4 cells/cm^2.

Table 5: Donor human mesenchymal stem cell (hMSC) characteristics, based on information provided from Lonza.

hMSC Donor Nomenclature	Donor Age (years)	Donor Gender	Donor Ethnicity
M2	19	Female	Black
M3	24	Male	Caucasian
M4	25	Female	Hispanic

3.3 CRYOPRESERVATION

Following passage, 1×10^6 cells were suspended in 1mL of cryomedium, which consisted of FBS as a vehicle solution with 10% (v/v) DMSO as a cryoprotectant. The cells in cryomedium were loaded into 2mL cryovials. The vials were transferred into a Nalgene® Mr. Frosty™ freezing container (Fisher Scientific, UK) and then into a -80°C freezer for slow cooling. The Mr. Frosty contained 100% isopropyl alcohol which helped to achieve a freezing rate close to 1°C/minute. A CoolCell® freezing container (BioCision, USA) was also used for slow freezing which uses thermos-conductive alloys and highly insulating outer materials to maintain a freezing rate of 1°C.min-1. After a minimum of 24 hours at -80°C, the vials were transferred to the vapour phase of liquid nitrogen (-150°C) in cryostores.

3.4 THAWING AND CELL RESUSCITATION

A vial of cells was retrieved from the liquid nitrogen cryobank and immediately placed in a 37°C water bath to thaw until only a sliver of ice remained. Meanwhile, a 15mL centrifuge tube was prepared with 9mLs of warmed standard culture medium. Once the vial had thawed, it was transferred into a biological safety cabinet (BSC). The cells were added to the medium and mixed thoroughly by drawing the medium up and down the side of centrifuge tube with a stripette. A 200μL sample was taken at this point if required for cell counting (details described in section 3.9.2). Cells were seeded at the required density in the appropriate sized tissue culture flask or plate with standard culture medium, as detailed in table 4.

3.5 MEDIA FORMULATION

All water used for medium preparation was obtained from a MilliQ ultrafiltration unit and all sterile filtering was performed using either a 0.22μm filter (Millipore, UK) or a Nalgene^{TM} Rapid-Flow^{TM} Sterile Disposable Filter Unit with a polyethersulfone (PES) membrane (Fisher Scientific, UK). Medium was stored between 2–8°C and was used within 2 weeks of preparation.

3.5.1 HOS TE85

Cells were cultured using MEM/EBSS supplemented with 10% (v/v) FBS, 2% (v/v) L-glutamine and 2% (v/v) NEAA at 37°C, 5% CO_2 and 95% humidity. For initial pausing investigations, cells were paused in standard MEM with supplements as described. For medium formulation experiments, the addition of 25mM HEPES buffer and 1μM Trolox to standard MEM with supplements was used. The commercial solution HTS-FRS was obtained from BioLife Solutions, USA and Pseudo Lyte A (PLA) was made in-house (see

appendix A) with 5% (w/v) human albumin, to mimic Plasma Lyte A. Medium for single

cell suspension experiments consisted of standard MEM with supplements with the

addition of various concentrations of anti-clumping agents. These included, Pluronic

F68 (PF68) and anti-clumping agent (Agent A) obtained from Fisher Scientific, UK and

polypropylene glycol (PPG), polyethylene glycol (PEG), EDTA and Tween® 80 (see

concentrations in tables 6 and 7).

Table 6: Various concentrations of anti-clumping agents used for toxicology testing

Pluronic F68 (%)	EDTA (mM)	Tween 80 (%)	Agent A (%)	PPG (%)	PEG (%)
1	0.5	0.1	0.01	0.5	0.5
2	0.75	0.2	0.2	1	1
5	1	1	1	2	2

Table 7: Concentrations of anti-clumping agents used for single cell suspension testing in ultra-low attachment plates. (All solutions are v/v)

Agent	Low Concentration	High Concentration
Propylene glycol	0.05%	2%
Pluronic F68	0.1%	1%
Polyethylene glycol	0.05%	2%
Agent A	0.1%	1%
EDTA	0.1mM	1mM

3.5.2 HMSCS

hMSCs were cultured in Dulbecco's Modified Eagles Medium (DMEM, 1g/L glucose;

Lonza UK) supplemented with 10% (v/v) FBS (Lonza, USA) and 2mM ultra glutamine

(Lonza, UK). For pausing experiments, a 1 x solution was made from 10 x DMEM (SLS,

UK) using sterile filtered water and the addition of 7.5% (v/v) sodium bicarbonate

solution. The pausing medium was further supplemented with 25mM HEPES.

3.6 PAUSING TEMPERATURE

Cells were paused at ambient temperature in the laboratory, which was measured using a modular signal recorder (MSR) data logger for the duration of each experiment. The data logger software (Version 5.12.01) was set up to take a temperature reading twice every hour; this was averaged and ranged between 18–22°C throughout all experiments (figure 10). Cells were also paused at 4°C which was achieved by storing the flasks or plates in the cold room which was kept between 2–8°C.
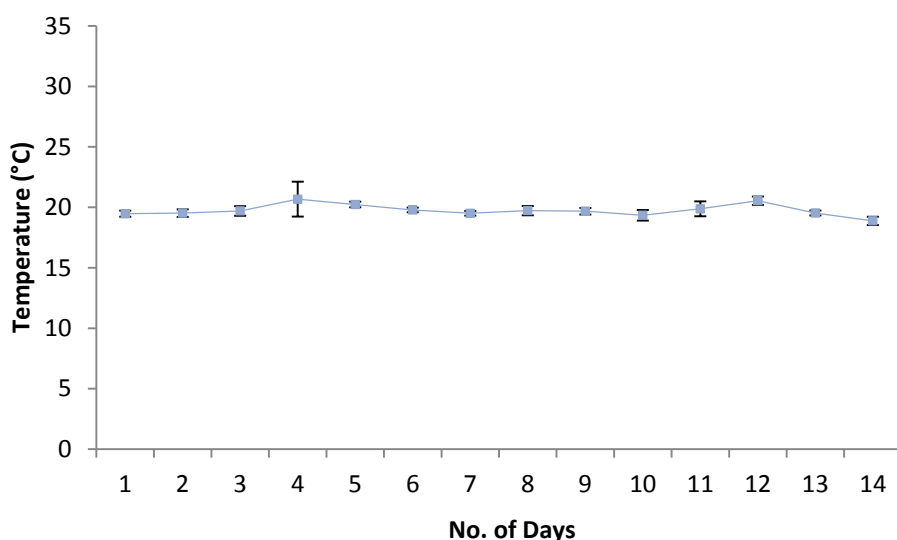
Figure 10: Average ambient temperature (°C) in the laboratory over a 14 day period. Temperature was monitored throughout all pausing experiments using a modular signal recorder data logger, which took 2 readings per hour. Shown here is the average temperature over two weeks. Error bars show the standard deviation between readings.

3.7 PAUSING CELLS AT AMBIENT TEMPERATURE

3.7.1 HOS TE85

HOS TE85 cells were seeded (6.25×10^4 cells/cm^2) into Nunc^{TM} cell culture treated 6-well plates (ThermoScientific, UK), with 3mL of MEM/EBSS medium supplemented with 10% (v/v) FBS, 2% (v/v) L-glutamine and 2% (v/v) NEAA. The cells were then incubated for 24 hours in normal culture conditions (37°C, 5% CO_2 and 95% humidity)

to allow attachment and 90% confluence. Before pausing, medium was aspirated and replaced. Cells were paused at ambient temperatures with and without atmospheric control for up to 144 hours overall. For pausing with atmospheric control, cells were stored in a Heraeus HERAcell 150i CO_2 incubator, set to ~20°C, 5% CO_2 and 95% humidity. Cells paused with no atmospheric control were kept in a sealed plastic container and placed in a cupboard at room temperature (figure 11). Similarly, HOS TE85 cells were kept at 37°C with and without atmospheric control. Cells with atmospheric control were stored in an incubator at normal culture conditions and cells without atmospheric control were also stored in an incubator set at 37°C, however CO_2 was set to 0%.

Figure 11: Ambient temperature cell pausing experiments. Cells were kept in a sealed plastic container in the dark in the cupboard indicated by the yellow star.

3.7.2 HMSCS

hMSCs were seeded at 3×10^4 cells/cm^2 into Corning Costar® 6 and 24-well ultra-low attachment (ULA) plates. These culture vessels were placed into a sealed plastic container, in the cupboard at room temperature (no atmospheric control).

3.8 PH MONITORING DURING PAUSING

Throughout ambient cell pausing, 200µL samples of medium were taken and placed in 1.5mL Eppendorf tubes. These tubes were capped and immediately taken to an EasyFerm pH probe to measure pH. Occasionally, samples were stored at -20°C and pH was measured within 2 weeks of sampling.

3.9 ANALYTICAL TECHNIQUES FOR HOS TE85 CELLS

3.9.1 CELL MORPHOLOGY

Cell morphology was examined using optical microscopy. Images were captured randomly in three areas of the flask or well, with a Nikon ECLIPSE T5100 microscope (Nikon Instruments, UK) immediately before and after cell pausing and following the recovery period and serial passages.

3.9.2 CELL MEMBRANE INTEGRITY

Viability determined by membrane integrity was assessed by trypan blue exclusion, propidium iodide and 4',6-diamidino-2-phenylindole (DAPI)/acridine orange (AO) staining. Trypan blue was added to a 100µL cell sample (1:1 dilution) and a haemocytometer was used to count the number of live/dead cells. The NucleoCounter-100 was used to perform live/dead cell counts for initial data (48 hours pausing) based on the staining of non-viable mammalian cell DNA with the fluorescent

dye propidium iodide embedded within a cassette (Sartorius, UK). The 200 μL cell sample was drawn into the cassette via a piston and was stained as it passed through the system. For all other viability results (144 hours pausing) the NucleoCounter-3000 (NC-3000) was used. The NC-3000 also used dyes embedded within a cassette; the cell permeating nucleic acid binding dye AO was used to stain all cells and DAPI was used to stain the nucleic acid of cells with broken or damaged membranes, thereby producing a live/dead cell count and percentage.

3.9.3 CELL DIAMETER

During the cell count and viability protocol, the NC-3000 also produced an average cell diameter based on image acquisition. This number was used to look at changes in cell size before and after pausing cells at ambient temperatures.

3.9.4 CELL METABOLIC ACTIVITY

PrestoBlue cell viability reagent (Fisher Scientific, UK) was used as an indicator of metabolic activity during pausing and recovery. A reduction reaction, which occurs by cell activity and oxygen consumption through metabolism, changes the non-fluorescent blue resazurin to the pink fluorescent dye resorufin. Fluorescence intensity is equivalent to cellular metabolic activity. To measure fluorescence, 10% (v/v) PrestoBlue cell viability reagent was added to the medium of each well and plates were incubated at 37°C, 5% CO_2 and 95% humidity for 40 minutes. After incubation, 100μL media from each well was transferred to a black Nunclon 96-well microplate (Fisher Scientific, UK), in triplicate. Fluorescence (excitation 544, emission 590) was measured using a FLUOstar Omega plate reader. Negative controls consisted of culture medium with no cells supplemented with 10% PrestoBlue cell viability reagent. The

positive control was made up of medium with no cells, supplemented with 10% PrestoBlue cell viability reagent which was reduced 100% by autoclaving at 121°C for 15 minutes.

$$\% \; reduction \; of \; PrestoBlue = \frac{S^x - S^{control}}{S^{100\% \; reduced} - S^{control}}$$

The percentage reduction of PrestoBlue was calculated using equation 1 (s = fluorescence signal of sample, x = treated sample) and the number of metabolically active cells was determined using a calibration curve (figure 12) ranging from 2.6×10^3-4.2×10^4 cells/cm^2.
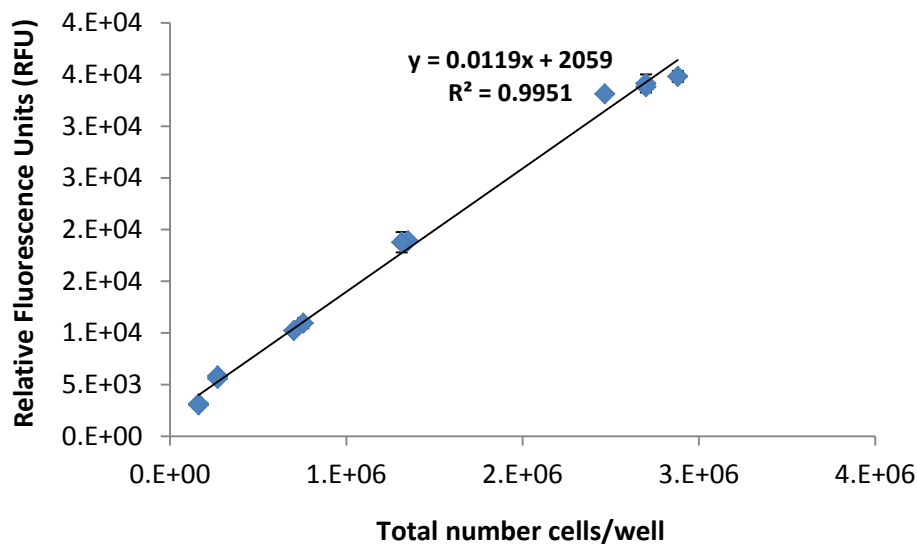
Figure 12: Presto Blue calibration curve for HOS TE85 cells. Cells were seeded at various densities and incubated in normal culture conditions for 40 minutes. Fluorescent intensity values represent the means of triplicate measurements and error bars show the standard deviation (n=3).

3.9.5 BONE SPECIFIC ALKALINE PHOSPHATASE ACTIVITY

Alkaline phosphatase (ALP) expression is used here as a hallmark of osteoblast phenotype (Lian and Stein, 2010). Bone-specific ALP is produced by osteoblasts and is believed to have roles in bone matrix calcification (Roudsari and Mahjoub, 2012). Other ALP isoenzymes exist in the liver, intestine, placenta, mammary glands and kidney (Roudsari and Mahjoub, 2012). The enzyme cleaves the phosphate group of the non-fluorescent 4-methylumbelliferyl phosphate (4-MUP) substrate, resulting in the fluorescent product 4-methylumbelliferone (4-MU). Briefly, HOS TE85 cells ($1x10^6$) were washed in 3mL PBS and centrifuged for 5 minutes at 1200g, immediately after pausing and recovery. The supernatant was aspirated away and cells were resuspended in 100µL dH_2O. Cells were kept in the fridge at 2–8°C for 24 hours and then transferred to a -20°C freezer until analysis. Two freeze thaw cycles were performed in order to lyse the cells and achieve a cell extract. A standard curve of 4-MU was prepared on a black Nunc® 96 well plate as follows. A stock solution of 12.5mg/mL 4-MU was prepared by dissolving 25 mg in 2mLs of methanol, with gentle heating at 40°C on a hot plate. A standard curve using the stock solution was achieved by performing a serial dilution (final concentrations in table 8). 10µL of cell lysate was transferred to the black 96-well plate in triplicate along with 50µL of 4-MUP. The cells were incubated for 30 minutes in the dark at room temperature. Following incubation, 10µL of 0.5M EDTA was added to each well to stop the reaction.

Table 8: Concentrations of 4-methylumbelliferone (µg/mL) used in standard curves for alkaline phosphatase activity analyses.

Standard Number	4-MU µg/mL
4	20
5	4
6	0.8
7	0.16
8	0.032
9	0.0064
10	0.00128
Blank	0

Fluorescence was measured using the FLUOstar Omega plate reader (excitation 360nm/emission 440nm). Negative controls were wells minus cells. Positive controls included the human ESC line NCL5, known to highly express ALP (O'Connor, *et al.* 2008) and hMSCs (M2), provided by a colleague in the lab.

3.9.6 BRADFORD PROTEIN ASSAY

This assay was used alongside the ALP assay to determine the concentration of protein in each sample. The Quick Start™ Bradford Assay Kit (Bio-Rad, UK) is an absorbance assay involving the binding of Coomassie Brilliant Blue G-250 dye to proteins. 5µL of cell extract was transferred to a clear 96-well microplate (Fisher Scientific, UK) in triplicate for each condition. 250µL of 1 x dye reagent was added to and mixed with each standard and sample. The samples were incubated for 5 minutes at room temperature. Following this, absorbance was measured using the FLUOstar Omega plate reader at 595nm. The standard curve using bovine serum albumin (BSA) was ran on every plate of samples measured (figure 14). Details of final protein content used for the standard curve are provided in table 9.
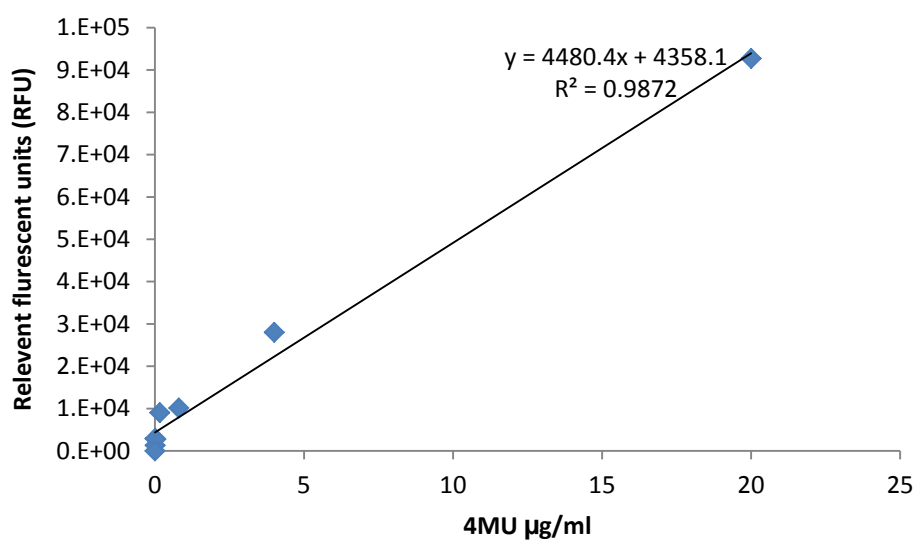
Figure 13: Alkaline phosphatase assay standard curve example. A standard curve using concentrations ranging between 0–20µg/mL of 4MU was measured in duplicate on every 96-well plate alongside cell samples.

Table 9: Bovine serum albumin (BSA) content (µg/mL) used in standard curves for the Bradford protein assay.

Standard Number	Final protein (µg/mL)
1	2000
2	1500
3	1000
4	750
5	500
6	250
7	125
Blank	0
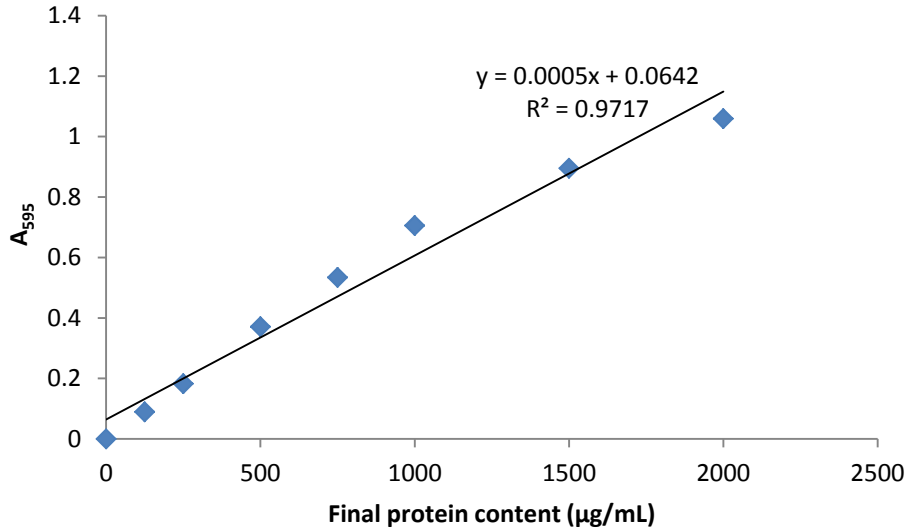
Figure 14: Bradford protein assay standard curve example. A standard curve using concentrations ranging between 0–2000µg/mL of bovine serum albumin was measured in duplicate on every 96-well plate alongside cell samples.

3.9.7 FOLD GROWTH EXPANSION

Following pausing at ambient temperature, HOS TE85 cells were seeded (1.2×10^4 cells/cm^2) in T25 Nunclon flasks (Fisher Scientific, UK). Cells were cultured for 48 hours at 37°C, 5% CO_2 and 95% humidity and passaged enzymatically using 0.25% (w/v) trypsin-EDTA. A 200µL sample was taken for cell viability analyses using either the NC-100 (48 hour data) or the NC-3000 (144 hour data) as previously described (section 3.9.2). The fold change in growth expansion was calculated by the division of the viable cell count after 48 hours and original seeding density (see equation 2). This was performed over five passages to examine changes in cell proliferation over time.

(eq.2)

$$Fold\ change\ in\ growth\ expansion\ \frac{Viable\ cell\ count}{Original\ seeding\ density}$$

67

3.9.8 CELL METABOLITE CONSUMPTION AND PRODUCTION

In order to assess the effect of pausing on HOS TE85 cell metabolite utilization and production, the amount of glucose, lactate and ammonium was measured in the medium before and after pausing determined by the NOVA BioProfile Flex Analyser. 1mL samples of medium were collected in 1.5mL Eppendorf tubes before and immediately after pausing for 24 and 48 hours and upon recovery at normal culture conditions for 24 hours. These samples were frozen at -20°C until thawed and analysed. Data are expressed as mmol/L.

3.10 ANALYTICAL TECHNIQUES FOR HMSCS

3.10.1 CELL PREPARATION FOR HMSC DIFFERENTIATION ASSAYS

Following pausing, cells were counted and centrifuged at 300g for 5 minutes to generate a cell pellet. The supernatant was aspirated away and fresh medium was added to result in a final concentration of $1x10^7$ cells/mL. 5μL droplets were placed in each well of a 12-well tissue culture plate in order to form micro masses for chondrogenesis. The plate was gently put inside the incubator at 37°C for 2 hours to allow the micro masses to attach to the tissue culture plastic. Once the micro masses had attached, 1mL/well of Gibco Chondrogenesis differentiation medium (Life Technologies, UK) was added and replaced every 72 hours.

For adipogenesis and osteogenesis, cells were seeded at ($7.9x10^3$ cells/cm^2) in 2mL of pre-warmed standard growth medium. Cells were incubated at 37°C for 24 hours to allow cell attachment. Following this, medium was replaced with either Gibco Adipogenesis or Osteogenesis differentiation medium also purchased from Life Technologies. Medium was refreshed every 72 hours. On day 21 of culture, all cells

were fixed in 1mL/well of 4% (v/v) para-formaldehyde at room temperature for 20–3 0 minutes.

3.10.2 CHONDROGENESIS

A solution of 1% (w/v) Alcian Blue in 0.1N hydrochloric acid (HCL) was prepared. After the Alcian Blue had completely dissolved, the solution was filtered using a 0.2µm filter. Following fixation, cells were washed twice with PBS (1mL/well) and stained with the filtered Alcian Blue solution (1mL/well). Cells were incubated in the solution for 60 minutes. Following incubation, the Alcian Blue solution was removed and cells were rinsed three times with 0.1N HCL (1mL/well) and twice with deionised water to reduce the acidity. Cells were visualised under a light microscope; a minimum of 3 images per well were captured.

3.10.3 OSTEOGENESIS

A 4% (v/v) solution of Napthol AS-MX phosphate alkaline solution was prepared in Fast Violet B salts solution to produce Reagent X. After cell fixation, three washes were performed with dH_2O (1mL/well). 1mL of 2.5% silver nitrate solution was added to each well and cells were incubated at room temperature under UV light for 30 minutes. Cells were washed three times with dH_2O. 1mL of Reagent X was added to each well and incubated in the dark for 45 minutes. The Reagent X was removed and cells were washed in dH_2O three times. Cells were visualised under a light microscope; a minimum of 3 images per well were captured.

3.10.4 ADIPOGENESIS

An Oil Red O stock solution was prepared by adding 150mg of Oil Red O powder to 50mL of 99% isopropanol. The solution was mixed until completely dissolved and filtered through a 0.2μm filter until clear. An Oil Red O working solution was prepared from the stock solution by mixing 3 parts Oil Red O stock solution with 2 parts dH$_2$O and incubating at room temperature for 10 minutes. The solution was again filtered using a 0.2μm filter. After fixing, cells were washed three times with PBS. Cells were then incubated with 1mL/well of Oil Red O for 5 minutes at room temperature. The Oil Red O was removed and cells were rinsed with dH$_2$O until clear. Cells were visualised under a light microscope; a minimum of 3 images per well were captured.

3.10.5 MULTIPARAMETER FLOW CYTOMETRY

To examine the immunophenotypic properties of hMSCs following pausing, a multiparameter flow cytometry technique was used (Chan, *et al.* 2014). This method effectively examines the expression of several bone marrow derived hMSC markers, simultaneously, at the single cell level. Briefly, a cell concentration of between 0.5– 1x10^6 cells/mL was obtained. 200μL of this cell sample from each time point and each donor was transferred into five wells of a V bottomed 96-well plate. The plate was centrifuged for 5 minutes at 300g. The supernatant was aspirated and 200μL of stain buffer (R&D Systems, UK) was used to resuspend the cells. The plate was centrifuged for 5 minutes at 300g. The supernatant was aspirated and the relevant antibody or isotype (0.5μL) was added to the cell pellet. The antibodies chosen were based on the recommendations of the ISCT (Dominici *et al.* 2006) and included CD34 PE-Cy5, CD73 PE-Cy7, CD90 APC, CD105 PE and HLA-DR FITC (BD Biosciences, UK). For each time

point, one well of cells was left unstained as a blank and was used for gating; one well was stained with the five relevant isotypes and three wells were stained with the five antibodies. 50μL of stain buffer was added and mixed to each well and the cells were incubated at room temperature in the dark for 60 minutes. The plate was centrifuged at 300g for 5 minutes, the supernatant aspirated away and the cells resuspended in 200μL of stain buffer. This was repeated three times. Samples were ran on the Guava easyCyte^{TM} Flow Cytometer for analysis, which recorded a minimum of 10,000 events for each sample. Data was analysed using the FlowJo computer software v7.6.5 (Treestar Inc, USA).

3.10.6 FOLD GROWTH EXPANSION

Following pausing, cells were seeded into T25 tissue culture flasks, at a density of $5x10^4$ cells/cm^2. Cells were cultured for 6 days under normal culture conditions (37°C, 5% CO_2 and 95% humidity). At the end of the 6 days, cells were enzymatically passaged using 0.25% (w/v) trypsin-EDTA and a cell count was performed on the NC-3000. Cells were reseeded into a T25 and the process was repeated 3 times to assess fold growth expansion over time following pausing. (Refer to section 3.9.7 for calculation details).

3.10.7 COLONY FORMING UNIT ASSAY

Immediately after pausing, ~250 cells were seeded into a T25 with 5mL of standard medium. The cells were cultured at normal conditions for 14 days with a medium exchange every 3–4 days. On day 14, the medium was aspirated and the monolayer was washed twice with PBS. Cells were fixed for 30 minutes in 4% (v/v) paraformaldehyde. The monolayer was washed twice with dH$_2$O. Cells were stained with 1% (v/v) crystal violet (Sigma-Aldrich, UK) for 30 minutes. The monolayer was

again washed twice with dH$_2$0. The total number of colonies on the surface of the flask were visualised using a light microscope and were manually counted.

eq.3

$$Colonal\ Efficiency = \left(\frac{Number\ of\ colonies}{250}\right) x\ 100$$

3.10.8 24 HOUR ATTACHMENT EFFICIENCY ASSAY

Following pausing, cells together with medium were taken from the 24-well ULA plate and seeded directly into a 24-well tissue culture plate. The plate was placed inside an incubator set to normal culture conditions to allow cell attachment. After 24 hours, the medium was removed and cells were enzymatically passaged and counted on the NC-3000. Results were calculated and expressed as shown in equation 4.

eq.4

$$24\ hour\ attachment\ efficiency - \frac{No.\,cells\ attached}{No.\,cells\ originally\ seeded}$$

3.11 STATISTICAL ANALYSES

All statistical analyses were performed using IBM SPSS Statistics 22.0. When comparing two experimental means, a simple t-test was used. For comparing three or more means, a one-way or two-way ANOVA was used to determine significance between paused and non-paused cell results. Tukey's range test was chosen as the multiparameter test in conjunction with the ANOVA, to find which means were significantly different from each other. In any circumstances where there were uneven sample sizes across factors, the Bonferroni post-hoc was used instead to correct for the difference. Error bars either show the interquartile range, standard error or the

standard deviation between independent experimental repeats (* indicates p≤0.05; ** indicates p≤0.01; ***indicates p≤0.0001).

3.12 EQUIPMENT AND SUPPLIERS

- BMG Labtech Ltd, UK

  o FLUOstar Omega plate reader

- Chemometec, Denmark

  o NucleoCounter NC-100 automated mammalian cell counter

  o NucleoCounter NC-3000 advanced automated cell analyser

- Eppendorf, UK

  o Eppendorf 5804 Centrifuge

- Grant Instruments, UK

  o Sub Aqua Pro 2 2L Heated Waterbath

- Hamilton, Germany

  o EasyFerm pH probe

- Merck Millipore, UK

  o MilliQ ultrafiltration unit

  o Guava easyCtye^{TM} Flow Cytometer

- MSR Electronics GmbH, Switzerland

  o Modular Signal Recorder Data Logger

- Nikon Instruments, UK

  o Nikon ECLIPSE T5100 microscope

- Nova Biomedical, USA

  o BioProfile FLEX Bioanalyser

- ThermoScientific, UK

    o Herasafe KS Class II BioSafety Cabinet

    o Heraeus Herasafe HERAcell 150 CO_2 incubator

CHAPTER 4: DESIGN AND OPTIMISATION OF THE CELL PAUSING PROCESS

4.1 INTRODUCTION

Information on the hypothermic preservation of human cells is severely limited and storing cells at ambient temperatures is a particularly niche area. As detailed in the literature review, interest in the low temperature storage of cells has stemmed from its success with organ preservation and the strong potential of implementing pausing at the cellular level. Low temperature preservation is already well established in the food industry and storing cells at 33°C already has its uses in biotechnology; to exploit CHO cell protein productivity. With more cell therapies gaining regulatory approval including Prochymal (Osiris Therapeutics Inc) and ChondroSelect (TiGenix), the focus has turned to how these therapies will be manufactured and transported to the medical site. The idea that pausing can act as a platform technology with use in cell therapies is the focus of this project. Whilst research centering on low temperature cell preservation is rare, the studies that have been undertaken on mammalian cell types have demonstrated survivability, yet is heavily dependent on cell type, length of storage, temperature and atmospheric factors. The most significant achievement so far for human cell pausing has been with blood components, which can be successfully stored within an additive solution for over a month at 4°C (Hess, 2012). Similarly, freshly isolated adult HSC have been successfully stored at 4°C for up to 48 hours prior to infusion (Andritsos, *et al.* 2007). What is clearly lacking in the current literature is data on the pausing of other complex human cells and therapeutically relevant cell types for use in stem cell and regenerative technologies, particularly at temperatures

other than 4°C. Previous research has shown cell preservation at 4°C to yield poor viability with the suggestion that higher temperatures for pausing may be more effective in maintaining cellular health and functionality (Hunt, *et al.* 2005; Ginis, *et al.* 2012). Hence, ambient temperature, which ranged between 18–22°C throughout all experiments (except temperature deviation experiments), was chosen for this research (figure 10). Whilst ambient temperature may vary between different countries, pausing cells at room temperature is convenient and advantageous, as no expensive temperature controlled equipment is required. This method is also insensitive to powered equipment failures. For clinical application, it is still best practice to store and ship cells using cryopreservation, as demonstrated with Prochymal. However, hypothermic preservation offers a more simplistic approach, without the need for toxic cryoprotectants and may be a more suitable route for the short-term storage of cells for autologous treatments and the shipment of allogeneic therapies.

This chapter explores the feasibility of human cell pausing in ambient conditions, for short-time frames (24–144 hours), in order to acquire knowledge and add to the current literature. The cell line chosen for initial studies was derived from a human osteosarcoma (HOS TE85). Originating from a bone cancer, these cells are highly robust in nature due to their proliferative phenotype, making them a suitable test-bed for a new process. Furthermore, being immortalised, the HOS TE85 cell phenotype is consistent, which means using non-paused cells as a control is an acceptable comparative measure against paused cells. As a sarcoma-derived cell line, HOS TE85 cells are useful for the early examination of pausing, however due to their cancerous

characteristics and differences to non-sarcoma cells, therapeutically relevant cells (hMSCs) are examined in chapter 5.

4.2 TESTING THE PAUSING PROCESS

To examine the potential of pausing human cells at ambient temperature with no controlled atmospheric environment, it was important to test that the HOS TE85 cells could survive for at least 24–48 hours. This pausing time frame was considered suitable, as the shipping of a cell product from one country to another would take no more than 24 hours and the extra hold time covers delays to transport, custom checks and setbacks to surgery. The 48 hour storage time would also be acceptable for preparation of autologous treatment regimens. Performing a panel of viability and phenotypic analysis on the cells immediately after pausing and following warming to normothermic temperatures offered an indication of whether pausing cells in ambient conditions was practicable and highlighted any potential limitations.

4.2.1 OVERVIEW OF EXPERIMENTAL PROCEDURE

A vial of HOS TE85 cells ($1x10^6$ cells/mL) was obtained from the MCB and thawed, as described in section 3.4, before each experiment commenced. Cells were retrieved from the bank at passage 54 and passaged at least twice before pausing, which meant all experiments were carried out between passages 56–60. Due to the immortality of the HOS TE85 line, it is unlikely that cell age would be a contributing factor to cell survival after pausing. Nevertheless, ensuring experiments were performed with cells between a small range of passages and population doublings, lessened the risk of cell age impacting survival.

Firstly, pausing for up to 48 hours only was performed, to test whether it was possible for human HOS TE85 cells to survive storage at ambient temperatures with no atmospheric control i.e. outside the incubator in a laboratory. As illustrated in figure 15, cells were seeded into 6-well plates ($1.4x10^4$, $4.0x10^4$ and $6.25x10^4$) and cultured in normal conditions (37°C, 5% CO_2, 95% humidity) for a full 24 hours before pausing to allow cells to attach to the tissue culture plastic and achieve up to 90% confluence. Following normal culture and a complete medium change, the plates were carefully transferred into a sealed plastic container and placed inside a cupboard in the dark to undergo pausing at ~20°C with no controlled atmospheric factors. At exactly 24 and 48 hours, the cells were imaged and transferred directly into normal culture conditions
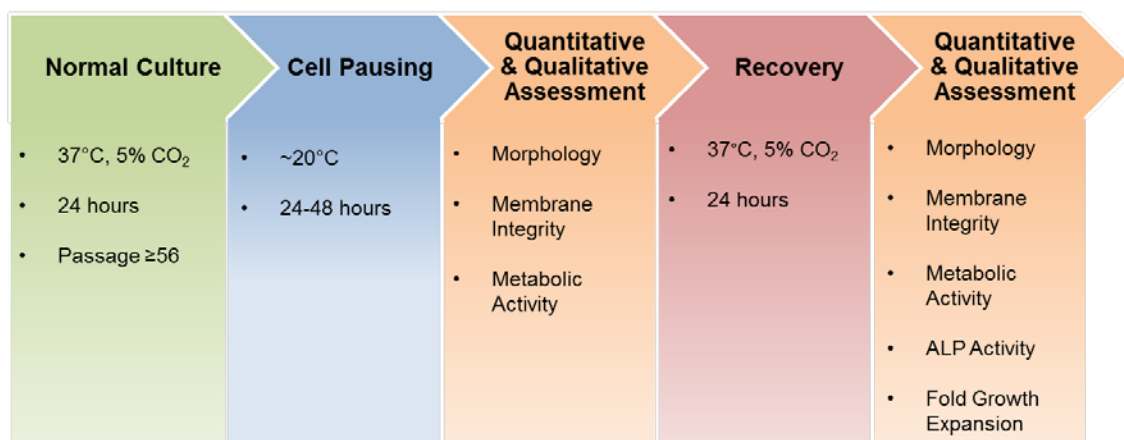
Figure 15: 24-48 hour HOS TE85 ambient pausing process - flow diagram

where they were recovered for a further 24 hours. A panel of viability and quality tests were performed before and after pausing/recovery, to examine the effects of an ambient environment on cell health and survival.

Changes in cell morphology, membrane integrity and metabolic activity were evaluated to determine the effect of pausing on viability. Cell health and quality were

observed by measuring the phenotypic osteoblast marker, ALP. Metabolite and fold

growth expansion assays were performed before and after pausing to test changes in

cell metabolite production and the cells ability to replicate. Testing the pausing

process using these parameters enabled further understanding of pausing HOS TE85

cells and highlighted some areas in need of further research.

4.2.2 HOS TE85 CELL MORPHOLOGY

HOS TE85 cells before and after pausing were imaged using an optical microscope. In

normal culture conditions, HOS TE85 cells had a fibroblastic morphology when

attached to tissue culture plastic (figures 16A and 16B). Some cells were notably

suspended in the medium, as they were smaller and more spherical in shape. During

pausing, most cells remained attached and retained their original morphology;

however, it was clear that more cells became suspended in the medium when exposed

to hypothermic temperatures (figures 16C and 16E). After pausing, cells were placed

into a 37°C incubator for 24 hours to allow suspended cells to reattach to the tissue

culture plastic. The cells that were capable of reattachment regained their extended,

adherent appearance; however, some cells remained suspended in the medium

(figures 16D and 16F).

4.2.3 HOSTE85 CELL MEMBRANE INTEGRITY

Trypan blue exclusion, propidium iodide and DAPI/AO staining were all methods used

to determine total live cell number and viability by membrane integrity after pausing

for both 24 and 48 hours. Cells cultured in normal conditions maintained high viability

(≥98%) independent of seeding density and time paused (figure 17A, 24 hour pause;

figure 17B, 48 hour pause). For paused cells, there was a significant decline in viability

(p≤0.05) after recovery when cells were seeded at the lower densities (1.3×10^4 and 4.0×10^4 cells/cm^2) compared with 6.25×10^4 at 24 hours (figure 17A) and 48 hours (figure 17B) pausing. For this reason, 6.25×10^4 cells/cm^2 was the seeding density chosen for all subsequent experiments. The total number of live cells retrieved following 24 and 48 hours of pausing at room temperature and recovery at 37°C increased sequentially over 3 days, whether stored at 37°C or paused in ambient conditions (figure 18A, Trypan blue exclusion; figure 18B propidium iodide staining). The only diversion from this pattern was a decrease in live cell number measured after 48 hours pausing and recovery as measured by Trypan blue (figure 18A). A significant difference between cell yields was found on day 3 between non-paused cells and cells paused for 48 hours and recovered for 24 hours in normal culture conditions (Trypan blue p≤0.01, propidium iodide p≤0.05). Viability determined by propidium iodide staining showed no significant difference between control cells and cells immediately after pausing (figure 19A). Although as seen in figure 19B, viability was significantly reduced when cells were paused for 48 hours and then recovered at 37°C (p≤0.01) for 24 hours.

4.2.4 HOS TE85 CELL METABOLIC ACTIVITY

Pausing cells for 24 hours is associated with significantly lower reductive metabolism compared with non-paused controls (p≤0.05); shown in figure 20A. In contrast, a significant reduction was not observed following 48 hours of pausing. After the recovery period (37°C, 5% CO2, 95% humidity) the percentage of PrestoBlue reduction became similar to the control (figure 20B). The same pattern was observed when the number of metabolically active cells was derived from the calibration curve (figure 12);

the number of metabolically active cells significantly decreased ($p \leq 0.05$) after pausing

for 24 hours (figure 21A) and resumed to that of the control after the recovery period

(figure 21B).
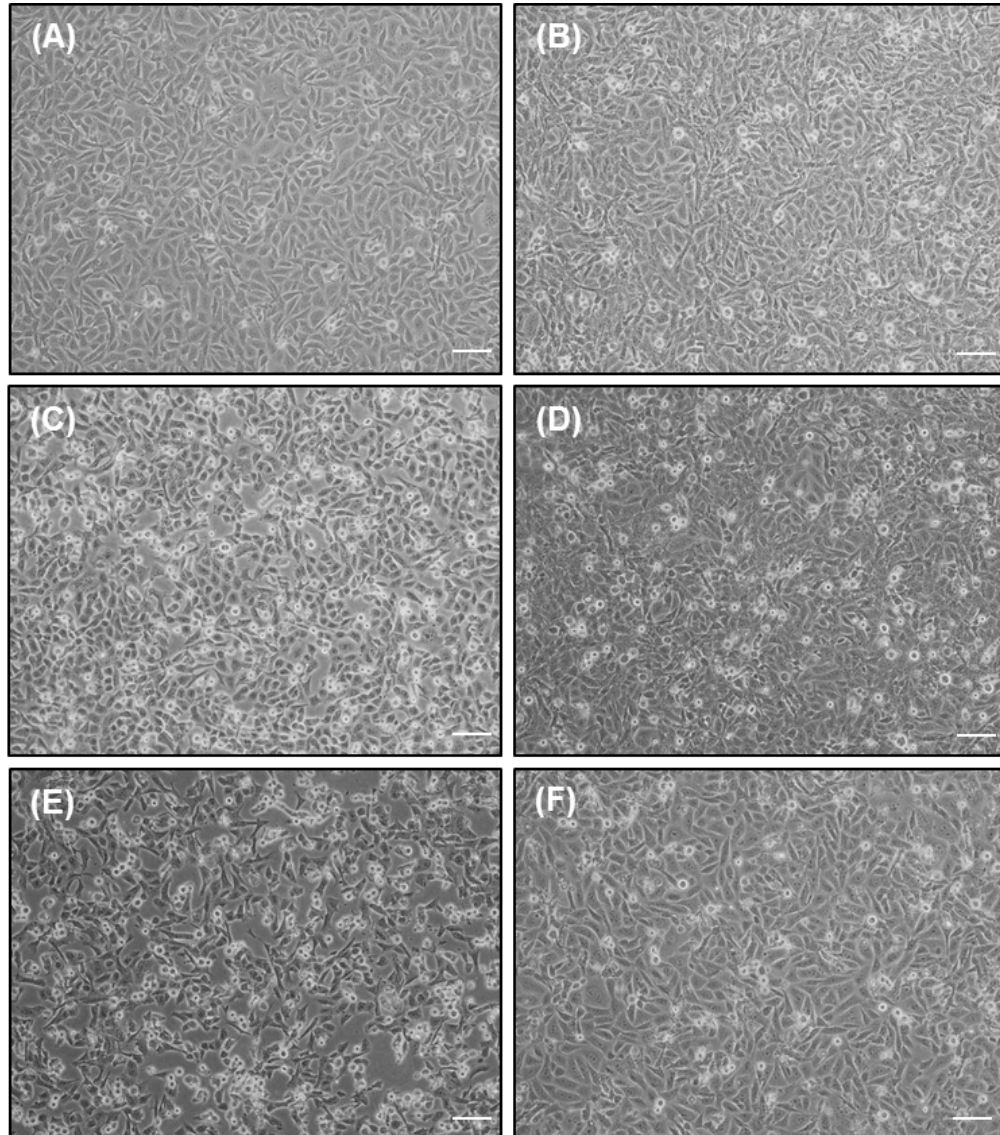
Figure 16: HOS TE85 cells paused in ambient conditions (no atmospheric control) for 24–48 hours (n=3). (A) Cells seeded at 6.25×10^4 cells/cm^2 and cultured in normal culture conditions (37°C, 5% CO2 and 95% humidity) for 24 hours prior to pausing, to achieve 80–90% confluence. **(B)** Cells after 48 hours in normal culture conditions **(C)** Cells immediately after pausing for 24 hours in ambient conditions **(D)** Cells paused for 24 hours and recovered for 24 hours in normal culture conditions **(E)** Cells immediately after pausing for 48 hours at ambient conditions **(F)** Cells paused for 48 hours and recovered for 24 hours in normal culture conditions. (Scale bar = 100 microns).
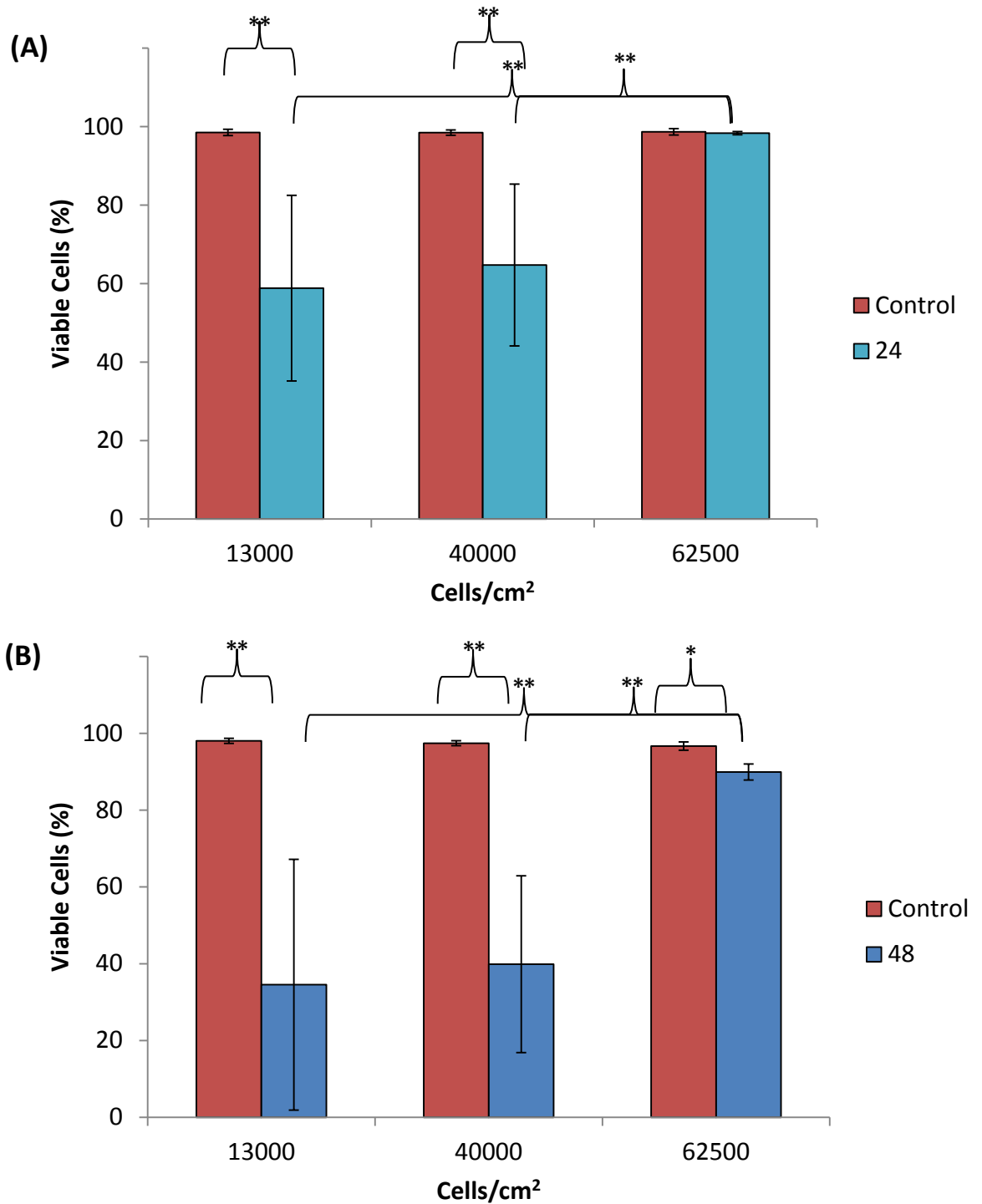
Figure 17: Effect of seeding density and pausing on percentage viability of HOS TE85 cells before and after 24–48 hours pausing at ambient temperature and recovery for 24 hours in normal culture conditions (n≥3). Viable cells were determined by trypan blue exclusion and DAPI/acridine orange staining **(A)** Cell viability before and after pausing for 24 hours when seeded at various densities **(B)** Cell viability before and after pausing for 48 hours when seeded at various densities. Error bars show the standard deviation of the mean between independent experimental repeats. A two-way ANOVA was used to measure significance between viability and seeding density together with the Bonferroni post-hoc test (*indicates p≤0.05; **indicates p≤0.01)
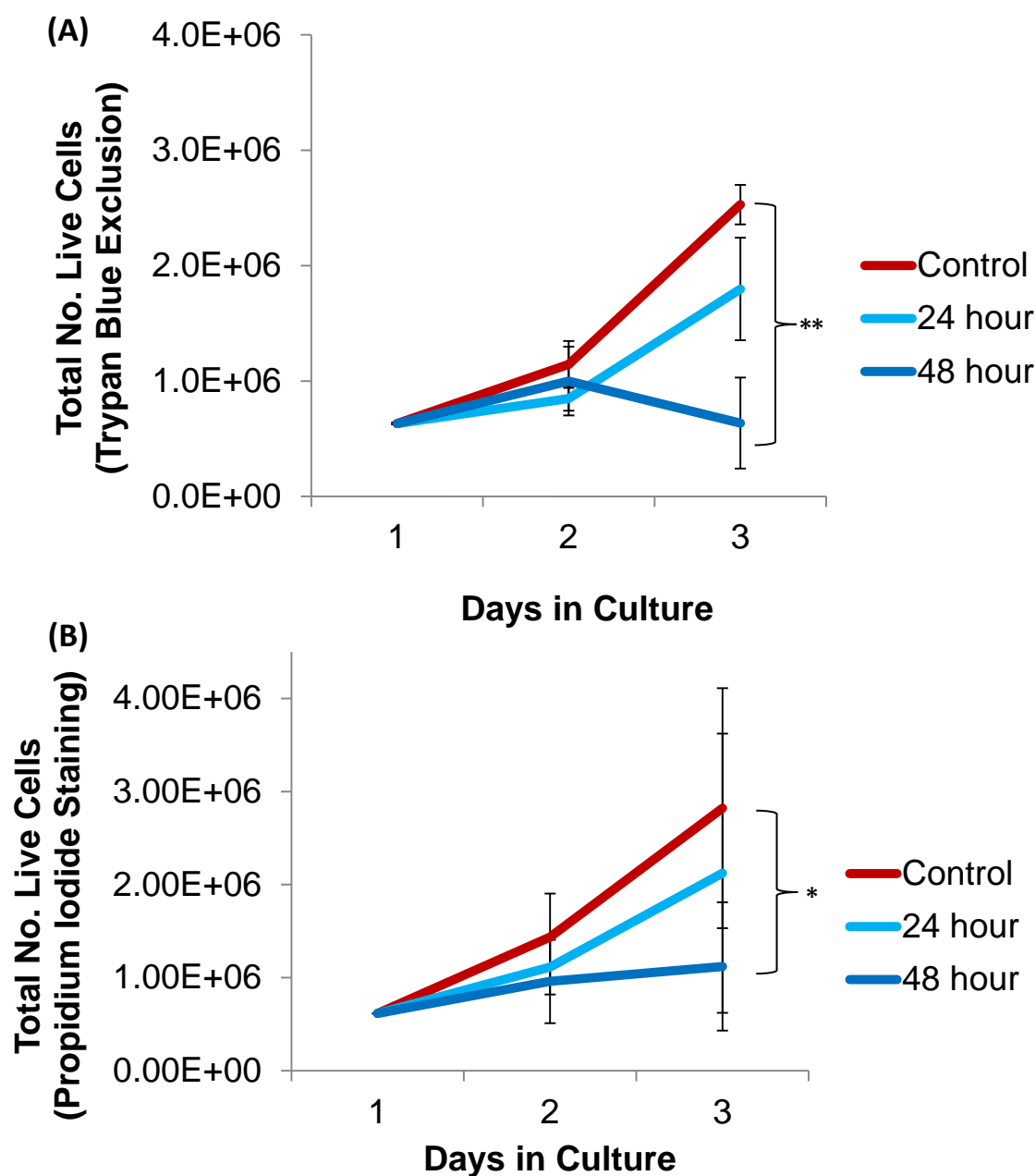
Figure 18: Total number of live HOS TE85 cells following 24–48 hours pausing at ambient temperature determined by membrane integrity. Cells were seeded at 6.25×10^4 cells/cm^2. Control cells were cultured at 37°C, 5% CO_2 and 95% humidity for 2 days. Paused cells were kept at ambient temperature for 24–48 hours (no atmospheric control) and recovered at 37°C, 5% CO_2 and 95% humidity for 24 hours. (Day 1) Original seeding density (Day 2) Live cell number after 24 hours culture in normal conditions (control) or immediately after pausing (Day 3) Live cell number after 48 hours culture in normal conditions (control) or after pausing and recovery **(A)** Total live cell number determined by trypan blue exclusion (n=3). Error bars indicate standard error between independent experimental repeats **(B)** Total live cell number determined by propidium iodide staining (n=3). Error bars indicate the interquartile range between independent experimental repeats. A two-way ANOVA test was used as a measure of significance between paused and non-paused cells alongside Tukey's range test to determine which data points were significant (* indicates p≤0.05/**indicates p≤0.01).

(A)

(B)
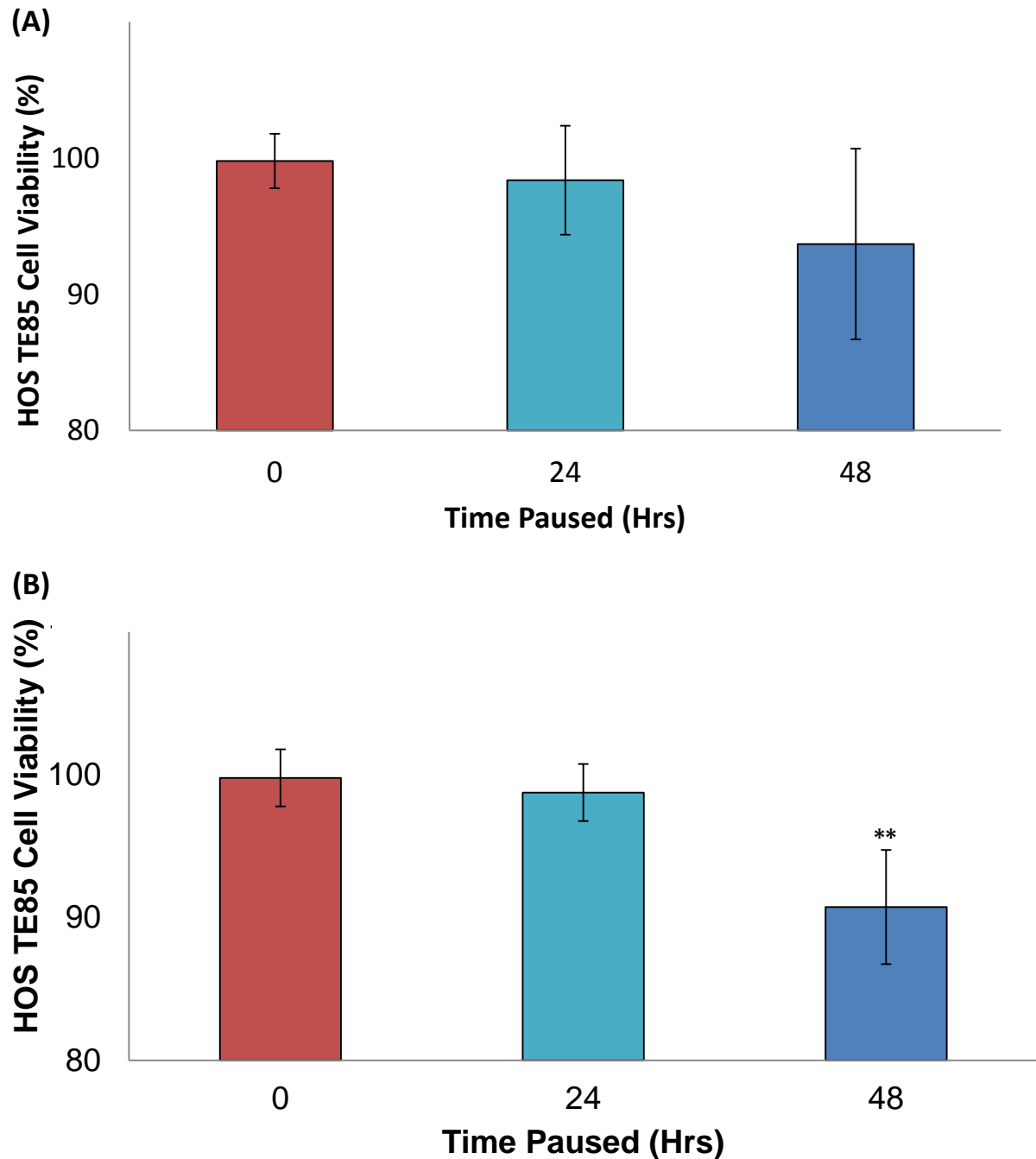
Figure 19: HOST TE85 cell viability after 24–48 hours pausing at ambient temperature determined by propidium iodide staining. Cells were seeded at 6.25×10^4 cells/cm^2 and paused for 24-48 hours at ambient temperature with no atmospheric control (n=3). Non-paused cells seeded at 6.25×10^4 cells/cm^2 and cultured for 24 hours in normal culture conditions were used as the control (0 hours paused). **(A)** HOS TE85 cell viability immediately after 24–48 hours pausing **(B)** HOS TE85 cell viability after 24-48 hours pausing and 24 hours recovery in normal culture conditions. Error bars show the interquartile range between independent experimental repeats. A two-way ANOVA was used as a measure of significance between paused and non-paused cells alongside Tukey's range test to determine which data points were significant (** indicates $p \leq 0.01$).
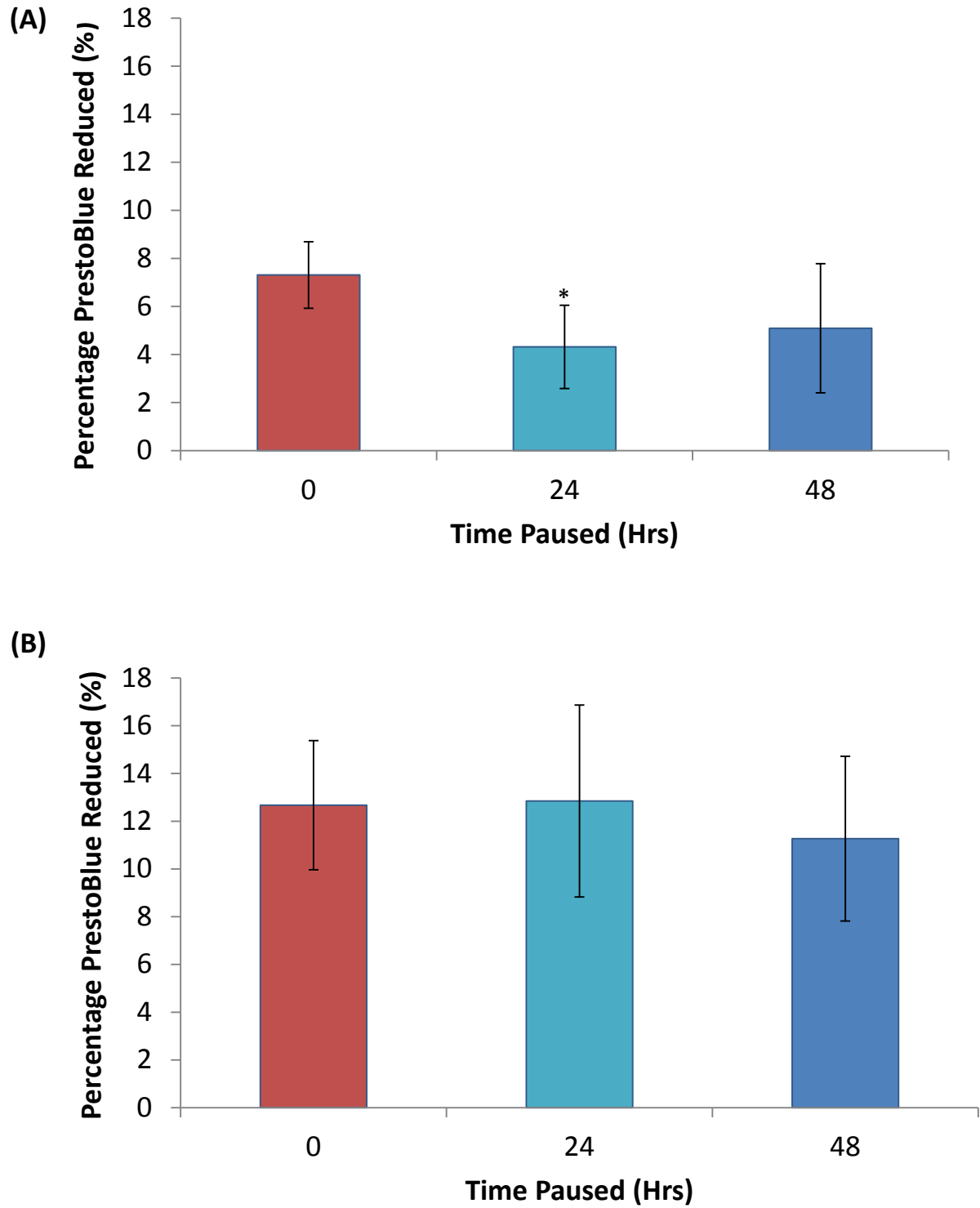
Figure 20: Percentage of PrestoBlue viability reagent reduced by HOS TE85 cells following pausing for 24–48 hours at ambient temperature with no atmospheric control (n=7). Non-paused cells seeded at 6.25×10^4 cells/cm^2 and cultured for 24 hours in normal culture conditions were used as controls **(A)** Percentage of PrestoBlue viability reagent reduced following 24–48 hours pausing **(B)** Percentage of PrestoBlue viability reagent reduced after pausing and recovery for 24 hours at 37°C. Error bars show the standard deviation between independent experimental repeats. A one-way ANOVA was used as a measure of significance between paused and non-paused cells together with Tukey's post-hoc test (*indicates p≤0.05).

(A)

(B)
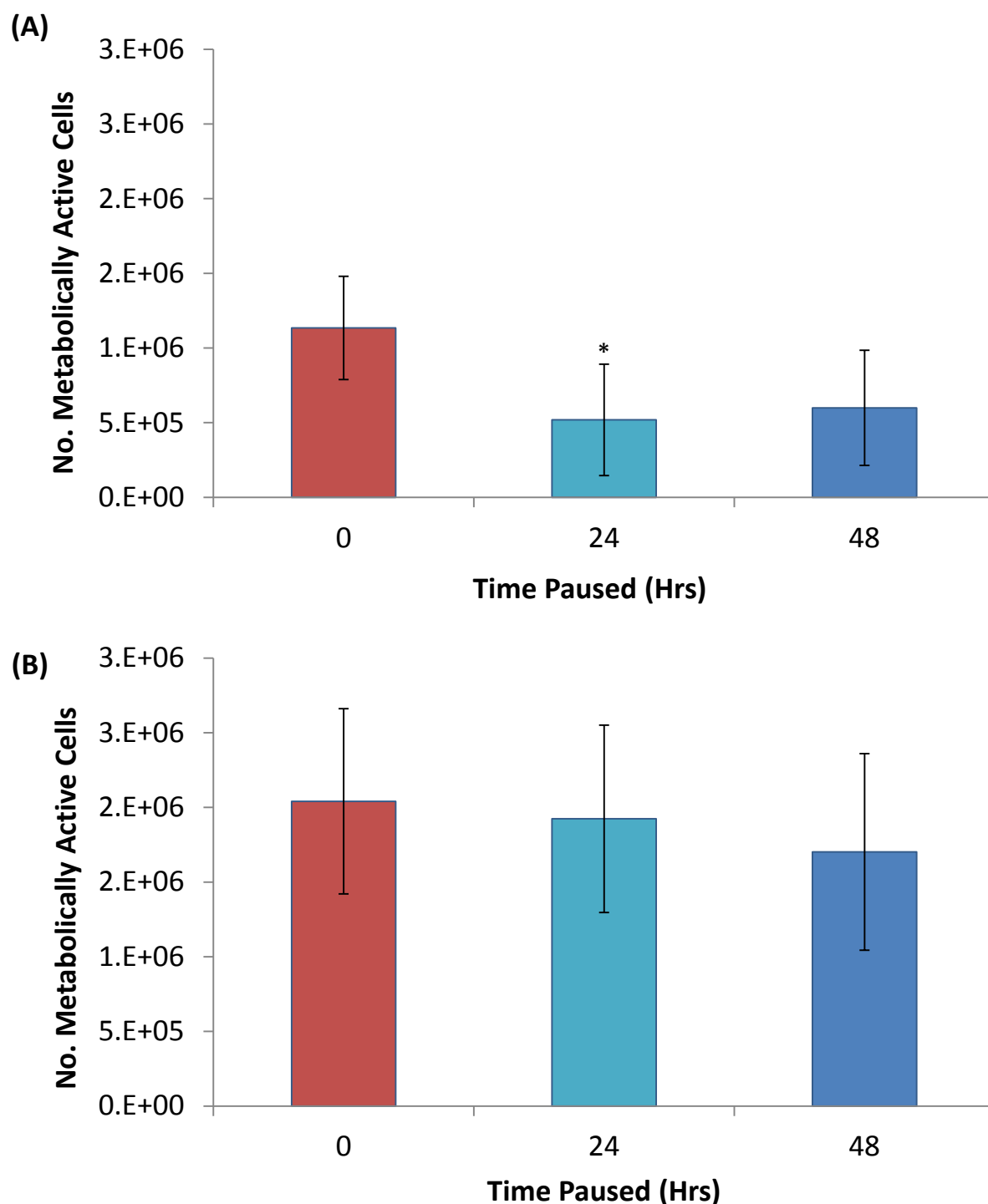
Figure 21: Number of metabolically active HOS TE85 cells following pausing at ambient temperature for 24–48 hours, determined by PrestoBlue fluorescence (n=7). Non-paused cells seeded at 6.25×10^{4} cells/cm^{2} and cultured for 24 hours in normal culture conditions were used as controls **(A)** Number of metabolically active cells immediately after 24–48 hours pausing **(B)** Number of metabolically active cells following 24–48 hours of low temperature exposure and recovey at 37°C for 24 hours. Error bars represent standard deviation between independent experimental repeats. A one-way ANOVA was used as a measure of significance between paused and non-paused cells together with Tukey's post-hoc test (* indicates $p \leqslant 0.05$).

4.2.5 HOS TE85 CELL METABOLITE PRODUCTION

Cell metabolite production was measured using a Nova Bioanalyser. 1mL of medium per sample was taken throughout normal HOS TE85 culture and immediately after pausing for 24 and 48 hours, as well as following recovery for 24 hours in normal culture conditions. Figure 22A shows the glucose, lactate and ammonium concentration in the medium from non-paused HOS TE85 cells cultured in normal conditions over a 48 hour period. A gradual decrease in glucose during culture is observed as it was used up during glycolysis to produce pyruvate. The energy released from this pathway is used to form ATP, a coenzyme used for energy transfer. By-products of glycolysis include lactacte and ammonium, which are seen to increase gradually throughout culture. Immediately after pausing for 24 (figure 22B) and 48 hours (figure 22C), there seems to be no difference in metabolite level compared with that before pausing. However, after 24 hours recovery in normal culture conditions, the glucose level falls and lactate and ammonium levels begin to increase.

4.2.6 HOS TE85 CELL FOLD GROWTH EXPANSION

After 24–48 hours pausing and a subsequent 24 hour recovery period, HOS TE85 cells were serially passaged 5 times every 48 hours to assess fold growth expansion over time. The total number of live cells was determined by propidium iodide staining. No significant difference was found between paused and non-paused cells. However, a significant difference in fold growth was found between passages 1 and 5 (24 hour data; figure 23A) and between passage 1 and 5 and 2 and 5 (48 hour data; figure 23B).

4.2.7 HOS TE85 CELL BONE SPECIFIC ALKALINE PHOSPHATASE ACTIVITY

As an assessment of quality, HOS TE85 cells were tested for ALP activity before and after pausing for up to 48 hours. A trend towards reduced activity was observed after 48 hours pausing; however, this trend was insignificant due to variation between independent experimental replicates being high (figure 24).

4.2.8 DISCUSSION

The first section of this chapter introduces the experimental process of pausing human cells and addresses its feasibility over a 24–48 hour time frame in terms of membrane integrity, metabolic activity, phenotype and the ability of cells to replicate following pausing. Seeding density and its effects on cell survival during ambient storage were also explored, in order to obtain information on the optimum conditions for pausing. As previously explained, the definition of a viable cell is somewhat controversial, with no clear guidelines as to what assays or parameters must be measured and achieved for a cell to be viable. Many researchers have solely used membrane integrity as an indicator of cell viability; however, a combination of membrane assays and intracellular health via metabolic assays will provide more information on the cell as a whole. Consequently, a panel of viability assays has been performed. These initial experiments have demonstrated that HOS TE85 cells can maintain membrane integrity and the ability to metabolise and proliferate after pausing in ambient conditions for up to 48 hours. Furthermore, no change in ALP activity was evident before and after pausing, suggesting that the phenotype of HOS TE85 cells does not change over this short time frame. Fold growth expansion after pausing for 24 and 48 hours returned to that of the control after 2–3 passages. This extended lag phase may reflect the cells

adjustment to culture conditions after pausing and recovery time from the stresses incurred during hypothermia. For example, before using cryopreserved cells for experimental use, it is standard practice to culture and passage them at least once to ensure cells are viable and able to grow and proliferate as normal.

Typically, HOS TE85 cells have an extended, adherent morphology when cultured in normal conditions with few cells suspended in the medium. Qualitative data, by means of images, indicated a clear increase in the number of suspended cells when HOS TE85 cells were paused in ambient atmospheric conditions. Suspended cells became more spherical, which may be due to the disassembly of the cytoskeleton from cold induced-stress on cellular lipid membranes and composition (Sonna, *et al.* 2002). Compromised cytoskeletal structures during pausing may disable cells to adhere to a surface and could explain the increase in suspended cells with pausing time. Upon recovery in normal culture conditions, most suspended cells were able to adhere to tissue culture plastic and regain their original fibroblastic structure; proving that some cells have the ability to recover from cold stress in terms of attachment and morphology. Cells which were unable to attach may have had irreversibly impaired cytoskeletal structures. Like the osteoblast cells, microglial cells following exposure to 20°C for 80 minutes displayed rounded and amoeboid cell shapes (Diestal, *et al.* 2010). Upon warming to 37°C, the microglial cells demonstrated elongated characteristics similar to the HOS TE85 cells. That cells can regain their original morphology after cold stress is promising, as permanent changes may impact cell behaviour, function, motility and migration.
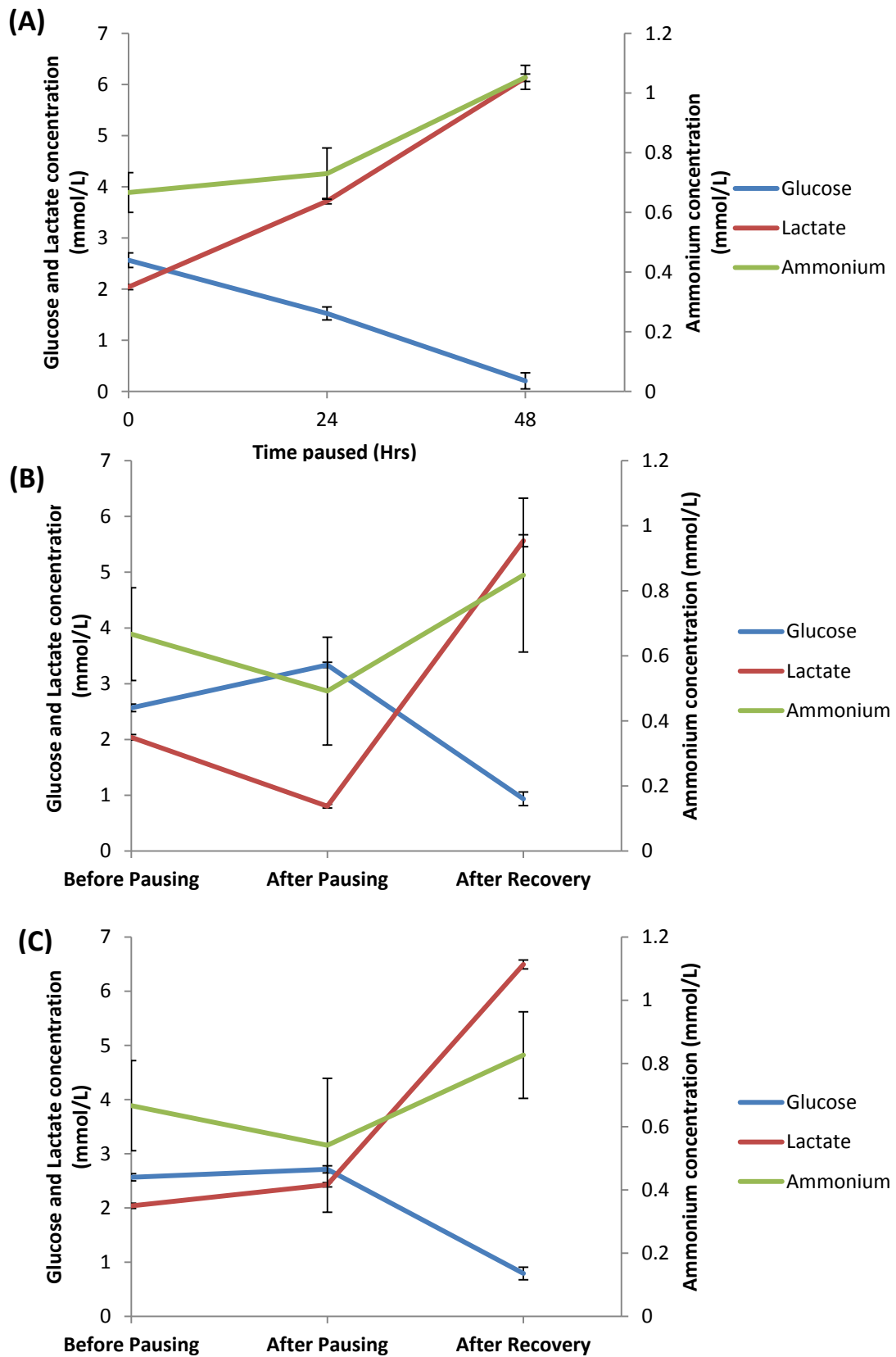
Figure 22: Glucose, lactate and ammonium concentration (mmol/L) in HOS TE85 cell medium during normal culture, pausing (~20°C) and following recovery at 37°C for 24 hours (n=3). The concentrations of each metabolite were measured in 1mL of medium using the Nova Bioanalyser **(A)** Control culture for up to 48 hours **(B)** 24 hours paused **(C)** 48 hours paused.
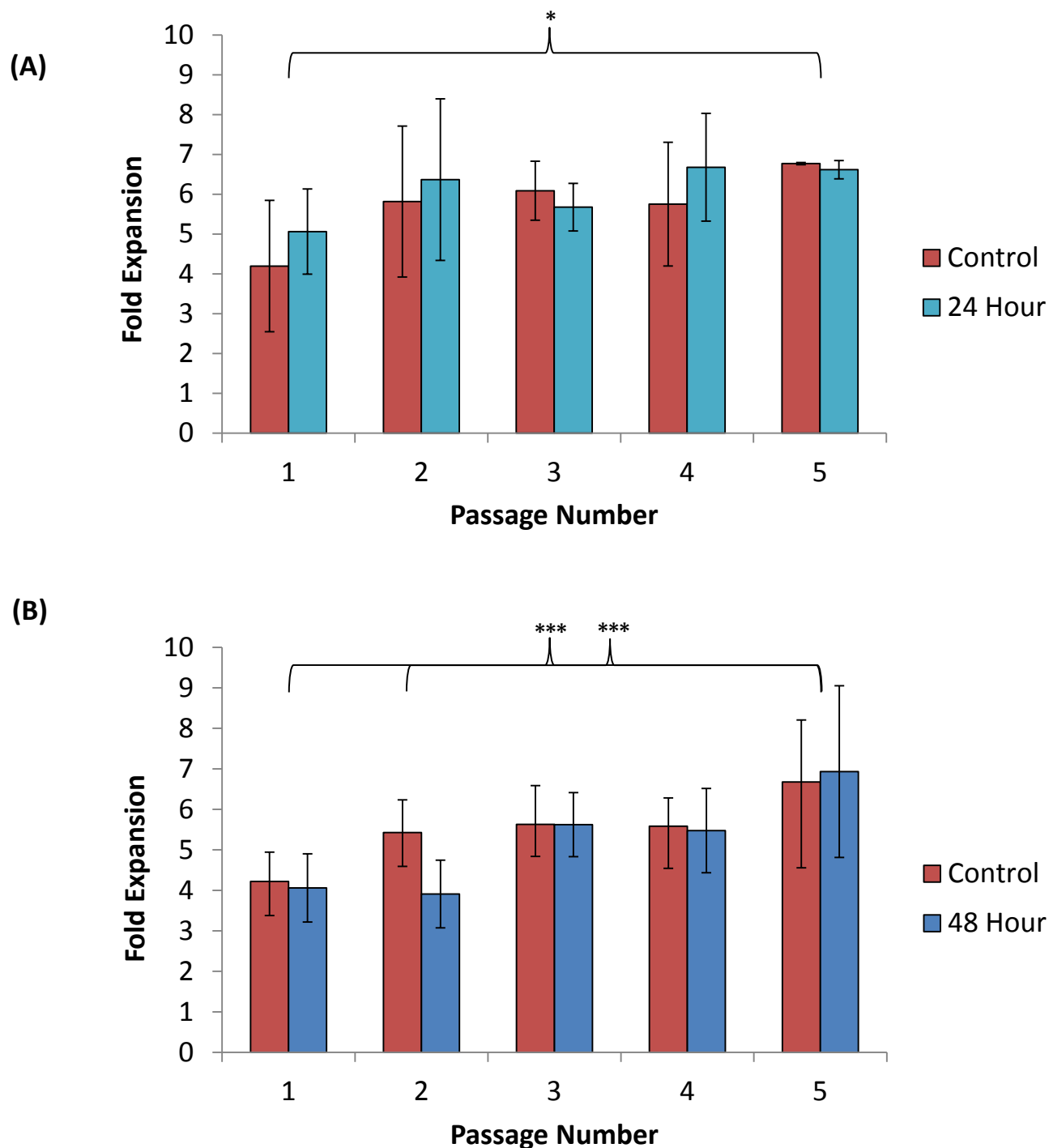
Figure 23: Fold growth expansion of HOS TE85 cells after 24–48 pausing at ambient temperature (n=3). Cells seeded at 6.25×10^4 cells/cm^2 were paused for 24–48 hrs in ambient conditions (no atmospheric control) and recovered at 37°C for 24 hours. Cells were passaged 5 times every 48 hours and the number of live cells was determined by propidium iodide staining **(A)** Total number of live cells paused for 24 hours after each passage **(B)** Total number of live cells paused for 48 hours after each passage. Controls consisted of non-paused cells. Error bars show error between independent experimental repeats. A two-way ANOVA was used as a measure of significance between paused and non-paused cells alongside Tukey's post-hoc test (* indicates p≤0.05; ***indicates p≤0.001).
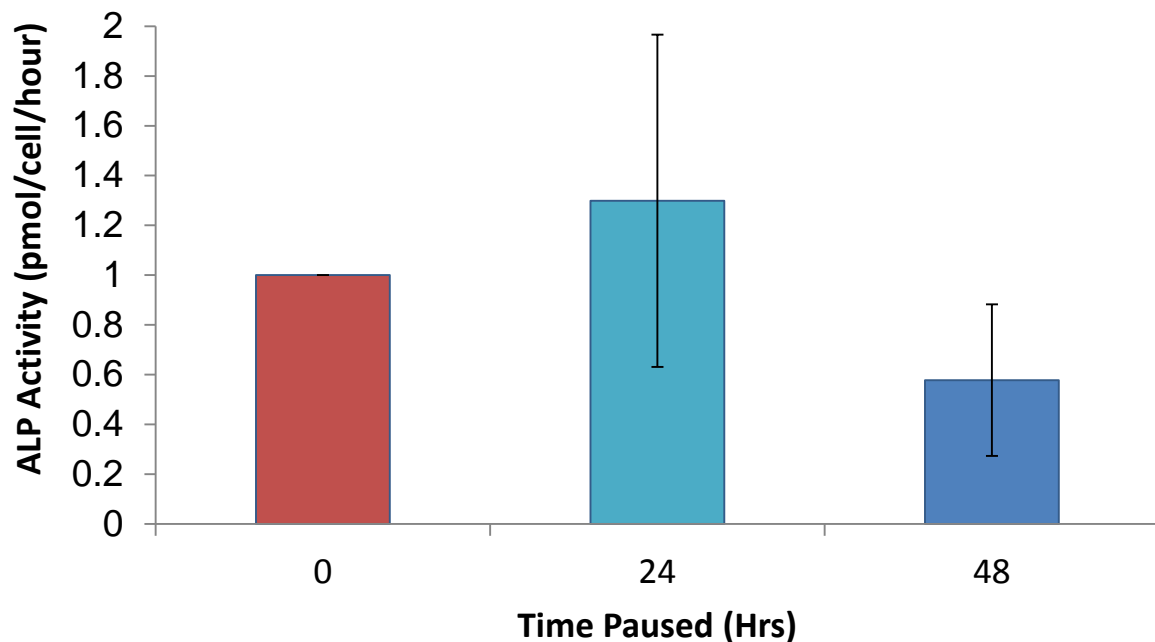
Figure 24: Alkaline phosphatase activity of HOS TE85 cells following pausing for 24–48 hours. Cells were seeded at 6.25×10^{4} cells/cm^{2} and paused for 24–48 hours in ambient conditions and recovered at 37°C for 24 hours (n=3). ALP activity was normalised to the control (0 hours paused). Bars show the amount of substrate reduced in pmol/cell/hour. Error bars show the interquartile range between independent experimental repeats. A one way ANOVA was used as a measure of significance between paused and non-paused cells. No difference was found between the non-paused cells or cells paused for 24 or 48 hours.

A panel of viability assays were performed to obtain a clear understanding of the effects of pausing on HOS TE85 cells. Methodologies for examining viability are extremely diverse across the field and vary between research groups and scientists (table 3). These many approaches generate useful information, yet on their own they are limited, as they only provide a snapshot of the whole picture and results can differ between methodologies. For example, viability assessments via trypan blue exclusion and propidium iodide staining produced differences in live cell number and viability. Whilst each assay provided different endpoints, which supports the use of multiple testing methods, the same overall conclusions were drawn (figure 18). It is also

important to note that methods that work via the same mechanism may be performed differently; trypan blue is a manual counting method using a haemocytometer and propidium iodide methods are generally used with an automated cell counter. Variations in analytical techniques can affect reproducibility (Archibald, *et al.* 2016); therefore, it is always best practice to ensure similar patterns of results are achieved with different protocols before drawing conclusions on one set of data.

Due to the extent of cell types tested in cell pausing experiments (CHO cells, human hepatocytes, hMSCs) and the different responses between cell types; it is of great importance that a panel of cell viability assessments is performed. In addition, standardising methods of viability and long-term quality of cells used for therapy is essential if results are to be compared amongst research groups. As well as assays that measure external parameters, such as the cell membrane, examination of the internal systems of the cell can also indicate health and viability during and following preservation. Previous work on low temperature cell pausing has mainly used membrane integrity as a defining factor for viability (Mathew, *et al.* 2002; Janssen, *et al.* 2003; Hunt, *et al.* 2005; Heng, *et al.* 2006). Using membrane integrity alone can be misleading as cells that are no longer viable may still retain an intact membrane; the cell may be compromised in terms of vitality and metabolic activity or could be undergoing the early stages of apoptosis, leading to false negative results. Alongside a membrane integrity assay, is it valuable to use a colorimetric assay to quantify cell metabolic and proliferative activities (Mathew, *et al.* 2004; Ginis, *et al.* 2012). Few studies have used metabolic assays to measure broad enzymatic activity during cell pausing (Mathew, *et al.* 2004; Ginis, *et al.* 2012). Hence, PrestoBlue viability reagent

was chosen in this instance, which uses the reducing power of living cells (arbitrarily thought to be from mitochondrial enzymes (O'Brien, *et al.* 2000)) to quantitatively measure cell proliferation. Similar to the MTT and Alamar Blue assays, PrestoBlue can rapidly measure the cells' reducing capacity, which is dependent on oxygen consumption through metabolism, in as little as 10 minutes. The percentage of PrestoBlue viability reagent (resazurin) reduced was lower in HOS TE85 cells during low temperature exposure compared with the control (figure 20A). This effectively demonstrated cell 'pausing' behaviour with an increase in enzyme activity when cells were returned to normal conditions (figure 20B). This reduction however, was only significant after 24 hours pausing. An insignificant reduction in enzyme activity following 48 hours pausing may have been an artefact of measurement variance or the cells successfully adapted to low temperature storage with increased pausing time. Live cell numbers immediately before and after pausing were similar, which suggests proliferation had paused as expected. An elevation in reducing power suggests the cell cycle was restarted once cells were warmed to 37°C, as live cell number returned to that of non-paused cells cultured for 24 hours.

One clear pattern throughout the results so far is a decrease in the number of live cells and viability with increased pausing time. Interestingly, this reduction is only significant after the cells have been recovered in normal culture conditions for 24 hours (figure 19B). It could be that the longer the cells are paused, the more sensitive they become to the rewarming process, possibility attributable to an increased level of ROS production. Another explanation is the phenomenon of delayed onset cell death or the cell death continuum, whereby the stresses from cooling and warming cells

continue to impact cell survival upon their return to physiological conditions (Corwin, *et al.* 2009). Delayed onset cell death is described as ischemia-reperfusion injury in hypothermic organ systems for transplantation. Although hypothermia offers protection against ischemia and reduces the demand for energy-dependent processes (Kanemoto, *et al.* 2009), it also causes cold induced damage, which is most encountered during the reperfusion step. Rauen *et al.* (1999) demonstrated lipid peroxidation in rat hepatocytes and liver endothelial cells, which led to apoptosis, specifically during the rewarming period from 4°C to 37°C. The extent of apoptosis was explained to be dependent on the duration of cold incubation; this could explain the significant difference in viability after 48 hours pausing and 24 hours recovery when compared to the control and 24 hours pausing.

During low temperatures, cellular processes that use ATP-dependent energy systems will slow, and how slow depends on the severity of the temperature (please refer to the Q_{10} equation in section 2.8.1). As a cell warms from hypothermic to normothermic temperatures, energy, metabolic and signalling pathways are kick started back into action, which explains why apoptosis or cell death is unlikely to be observed during or immediately after pausing. This deterioration in cell viability with prolonged pausing remains a theme across previous low temperature preservation research (Hunt, *et al.* 2005; Heng, 2006; Rauen, *et al.* 1999; Ginis, *et al.* 2012).

Seeding density before low temperature exposure may influence the effectiveness of pausing. It is clear from these results that the highest seeding density (6.25×10^{4}), regardless of time paused, produced the best outcome in terms of cell membrane integrity (figure 17A, 24 hour pause; figure 17B, 48 hour pause). The lower seeding

densities chosen generated more variability between independent experimental repeats. A high seeding density means a higher confluence is reached before cells are exposed to low temperatures. Cellular communication via gap junctions and paracrine and autocrine cell signalling may be enhanced with increased cell number, which may aid cell survival during hypothermic stress. Cell seeding densities are known to influence behaviour in rat bone marrow stromal cells which are stimulated to proliferate and differentiate by lower seeding densities (Kim *et al.* 2009); this is a potential drawback for cells during pausing as both mechanisms are energy dependent, which there is a shortage of during hypothermia. Cell interactions may further increase a cells' ability to attach to neighbouring cells or surfaces; critically important for the survival of adherent cell lines in culture. These findings suggest that optimised seeding density, level of confluence or time adhered to a surface may be an important prerequisite for effective pausing of other cell lines and therapeutically relevant cells. As cell therapies require large amounts of cells, between 10^6 and 10^9 per patient, depending on disease and prevalence (Mummery, 2005; Mason and Dunhill, 2009), it will be valuable to find the maximum number of cells/cm^2 that can be paused successfully with no considerable decline in viability or quality when space and nutrients are limited.

HOS TE85 cell metabolite production, immediately after pausing, including glucose, lactate and ammonium is similar to that before pausing (figure 22A–C). It is only once the cells are rewarmed to 37°C that glucose depletes due to the kick start in cell glycolysis and glutamine metabolism; this subsequently leads to the production of pyruvate and an accumulation of lactate and ammonium. A significant increase in

these metabolites is known to inhibit cell growth and limit protein production (Lao and Toth, 1997; Wagner, 1997; Gambhir, *et al.* 2003). Lacate is also associated with lowering the pH of the medium (Altamirano, *et al.* 2013) and has effects on cell osmolality (Cruz, *et al.* 2000). It is well known that high ammonium levels are toxic to cells and is therefore maintained below 0.05mM in human blood (Xie and Zhou, 2005). As the levels of these potentially toxic metabolites do not exceed that of the control at 48 hours (figure 22C), it seems that pausing cells does not pose any increased risk from metabolite toxicity. In fact, previous research with hMSCs has indicated that the inhibitory values for ammonium and lactate are above 2.0 and 20mmol/L, respectively (Schop, *et al.* 2009).

Again, the cells seem to exhibit pausing behaviour where metabolite consumption and production is considerably reduced when the surrounding temperature is suboptimal and resumes a pattern similar to the control upon warming. However, as culture continues past 48 hours, it is inevitable that the glucose level will eventually become exhausted and lactate and ammonium will reach potentially damaging levels. This will force cells to use alternative cellular pathways, which may influence cell metabolism and proliferation on recovery. Combating severe ammonium increases during culture is a technique used to achieve high cell densities and extend culture lifetime (Xie and Zhou, 2005). One method is to lower the glutamine level in culture to keep cell growth rate slightly below its maximum whilst significantly reducing ammonium production or alternatively to replace glutamine with a more thermally stable glutamine based di-peptide (Xie and Zhou, 2005). This may be a route for investigation if significantly longer time frames for pausing cells are required.

Research into low temperature pausing of cells has been highly centred on cell viability after hypothermic exposure with few studies examining changes in cell quality. One explanation for this is the difficulty in identifying quality attributes for every cell type tested. Being of osteosarcoma origin, ALP activity, a known phenotypic attribute of osteoblasts (Golub and Boesze-Battaglia, 2007) with great importance in bone mineralization (Ali, *et al.* 1996), was chosen as an indicator of cell quality before and after pausing. No difference was found between paused and non-paused cells after 48 hours; however, variability between independent experimental replicates was high. Previous investigations have found that ALP activity in HOS TE85 cells was approximately one third of that found in non-sarcoma osteoblasts (Clover and Gowen, 1994); whilst the ALP activity levels may be lower than what is typical of primary cells, this experiment still detects changes attributable to the pausing process by quantitatively measuring activity levels before and after pausing.

To measure cell quality, as well as phenotypic markers, monitoring the ability of a cell to grow and replicate is an important marker of health for any cell type. Fold growth expansion over a number of passages will ascertain if cells can successfully re-enter and progress through the cell cycle after pausing and maintain a growth rate similar to that before pausing. Results have demonstrated that following pausing for up to 48 hours, fold growth expansion resumes a fold growth potential similar to the control at passage 3 (figure 23A, 24 hour pause; figure 23B, 48 hour pause).

With regards to stem cells, the importance of assessing functionality and growth is essential if we are to use these cells for therapeutic purposes. Stem cells are known for their potent characteristic which may be altered during hypothermic stress. It is

therefore vital that cell characteristics are measured before preservation so that levels of growth, metabolic activity and proliferation can be compared to baseline levels. Cell quality or potency has currently been identified as an area in need of more research (Bravery, *et al.* 2013; Coopman and Medcalf, 2014) within the industry as cells not only need to be viable but they must retain their therapeutic function if used in regenerative medicine. In an *in vitro* setting, cell function is difficult if not impossible to measure, as until the cells are infused into a patient, there is no way of examining if they can treat or cure a disease.

To summarise, these initial results support evidence of cell pausing behaviour during storage at ambient temperature with suppression of enzyme activity at ambient temperatures and increased activity upon rewarming to 37°C. The fact that HOS TE85 cells can survive pausing at ambient temperatures for 48 hours shows that the process could be applied to other human cell types and possibly stem cells. It also implies that pausing may be effective for longer periods of time i.e. 48 hours or more.

4.3 REFINING THE PAUSING PROCESS

This section centers on the refinement of the pausing process, focusing on atmospheric control together with longer term pausing for up to 144 hours. An extended shelf life is of vital importance for cell therapies, as delays to transport and surgery can lead to extensions in the length of time paused. In the biotechnology or research industry, it would be useful to know how to store cells successfully for short-time periods if required during a power failure or other delays to research. If the HOS TE85 cells can survive and maintain function following ambient temperature pausing

for up to 144 hours, this should provide sufficient time for shipment, setbacks and transplant preparation.

Since HOS TE85 cells had been able to survive pausing for up to 48 hours, it was valuable to test if the cells could be stored just as successfully for longer time periods. If cells were to be shipped by air from one destination to another, the minimum amount of time required would be 48 hours. However, security checks and delays make this time frame unreliable. A pausing time frame between 72 and 144 hours is more ideal when taking all manufacturing, shipping and quality control systems into account. Research is limited covering the preservation of cells in hypothermic conditions for more than 48 hours. Therefore, it was important to test how long the HOS TE85 cells could survive for in pausing conditions in terms of the parameters tested for 48 hours.

4.3.1 OVERVIEW OF EXPERIMENTAL PROCEDURE

Cells were retrieved from the cryobank, thawed and cultured as detailed in section 3.4. HOS TE85 cells were seeded (6.25×10^4 cells/cm^2) into 6-well plates with 3mL of medium and incubated for 24 hours at normal culture conditions (37°C, 5% CO_2 and 95% humidity) to allow attachment and 90% confluence. Fresh medium was replaced and cells were paused at ambient temperatures with and without atmospheric control for up to 144 hours. For pausing with atmospheric control, cells were stored in a Heraeus HERAcell 150i CO_2 incubator (Fisher Scientific, Loughborough, UK) set to ~20°C, 5% CO_2 and 95% humidity. Cells paused with no atmospheric control were kept in a sealed plastic container placed in a cupboard in the dark in ambient conditions. Similarly, HOS TE85 cells were kept at 37°C with and without atmospheric control.

100

Cells with atmospheric control were stored in an incubator set at normal culture conditions and cells without atmospheric control were also stored in an incubator set to 37°C however, CO_2 was set to 0%. The effect of cell pausing was assessed qualitatively using optical light microscopy and quantitatively using a panel of viability assays and quality indicators. Cell assessments were performed before and immediately after cell pausing and following a 24–48 hour recovery period at 37°C, 5% CO_2, 95% humidity. The control for all circumstances consisted of non-paused HOS TE85 cells maintained under normal culture conditions.

4.3.2 HOS TE85 CELL MORPHOLOGY

When HOS TE85 cells were paused at ambient temperatures for up to 144 hours with atmospheric control, most cells remained attached and looked fibroblastic in appearance (figures 25A, control; figure 25B–D, cells paused with atmospheric control). Some cells were suspended, which was identified by their small, circular outlined appearance; however, the well remained 80–90% confluent with adhered HOS TE85 cells. Without atmospheric control, many more HOS TE85 cells were suspended in the medium and the amount of cells increased in a time dependent manner (figures 25E–H, cells without atmospheric control). Without CO_2 diffusion, MEM medium with supplements appears bright pink due to its alkaline pH; however, when cells were paused with atmospheric control, the medium was more orange in colour and hence neutral in pH.

4.3.3 HOS TE85 CELL MEMBRANE INTEGRITY

DAPI and acridine orange staining were used to determine viability and total live cell number by membrane integrity after pausing at ambient temperature, with and

without atmospheric control. When HOS TE85 cells were paused with atmospheric control, viability remained above 90% for all time points up to 144 hours (figure 26A).

Without atmospheric control, viability significantly decreased in a time dependent manner from 92%, 71% and 52% after 48, 96 and 144 hours pausing, respectively ($p \leq 0.01$). At all-time points (48–144 hours) a significant difference in viability was found between those cultured with and without atmospheric control ($p \leq 0.01$). Variability, shown as standard deviation, also increased with pausing time when cells were without atmospheric control. Cell yield when cultured with atmospheric control stayed between 1.2×10^6 and 1.4×10^6 cells/well when paused for up to 144 hours; however, viable cell yield significantly decreased over pausing time when atmospheric control was not present (figure 26B). Interestingly, cell yield was significantly higher after pausing for 96 hours with atmospheric control ($p \leq 0.01$) but was not significantly different to the control after 144 hours. Without atmospheric control, cell yield was significantly lower when cells were paused for 96 and 144 hours ($p \leq 0.01$); however, after 48 hours no difference was observed. Viable cell yield when paused for 48 hours with and without atmospheric control was not significantly different; however a significant reduction in the number of live cells was apparent when cells were paused for 96 and 144 hours without atmospheric control compared to those that had controlled CO_2 levels.

Cells stored for 24 hours at 37°C, devoid of CO_2 control, had significantly reduced viability (figure 27A) and live cell number (figure 27B) compared with cells in normal culture conditions.
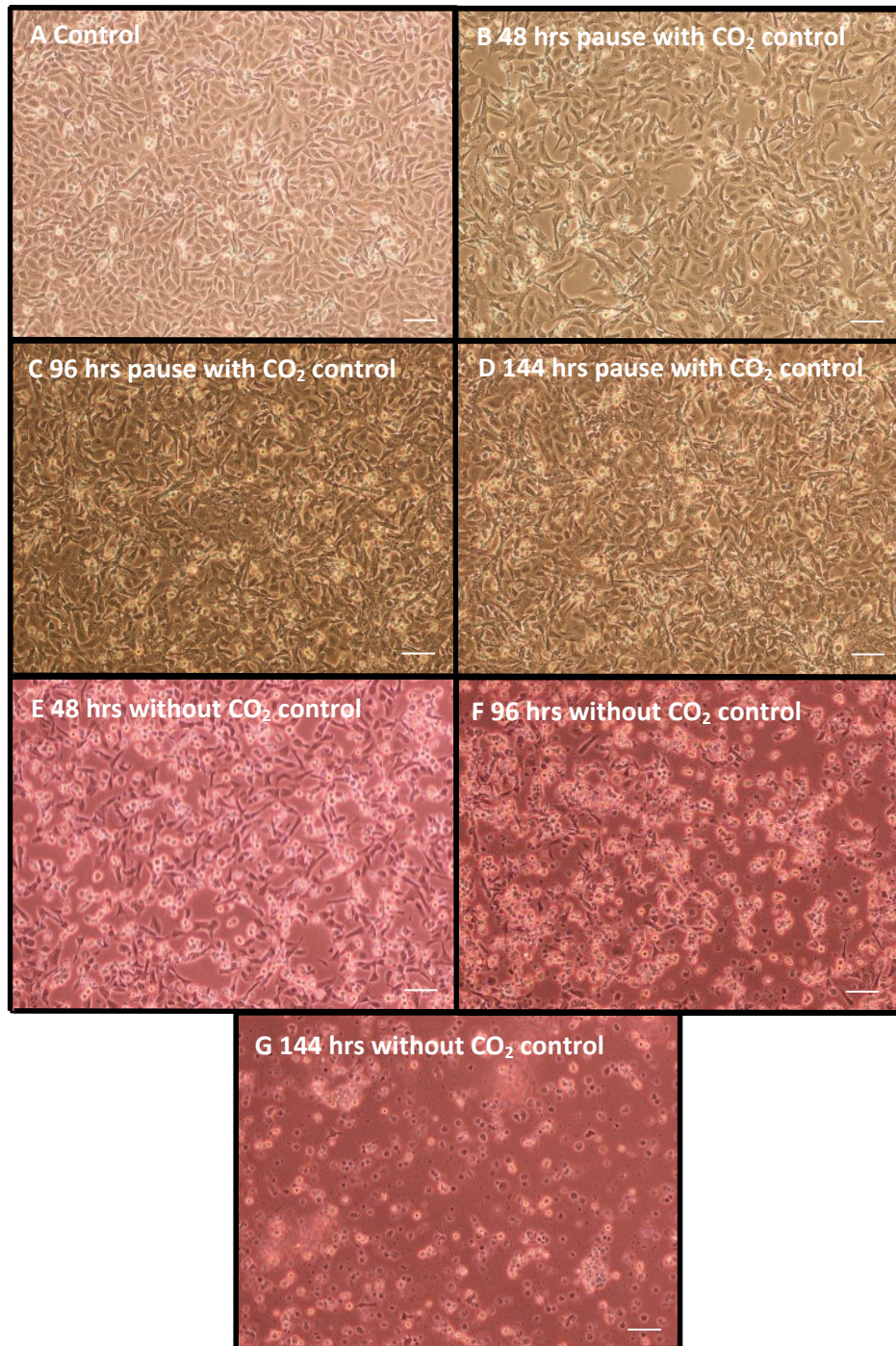
Figure 25: HOS TE85 cells paused at ambient temperature with and without atmospheric control for up to 144 hours (n=3). (A) Cells seeded at 6.25×10^4 cells/cm^2 and cultured in normal culture conditions (37°C, 5% CO_2 and 95% humidity) for 24 hours prior to pausing. **(B-D)** Cells after 48–144 hours pausing with atmospheric control. **(E-G)** Cells after 48–144 hours pausing without atmospheric control (Scale bar = 100 microns).
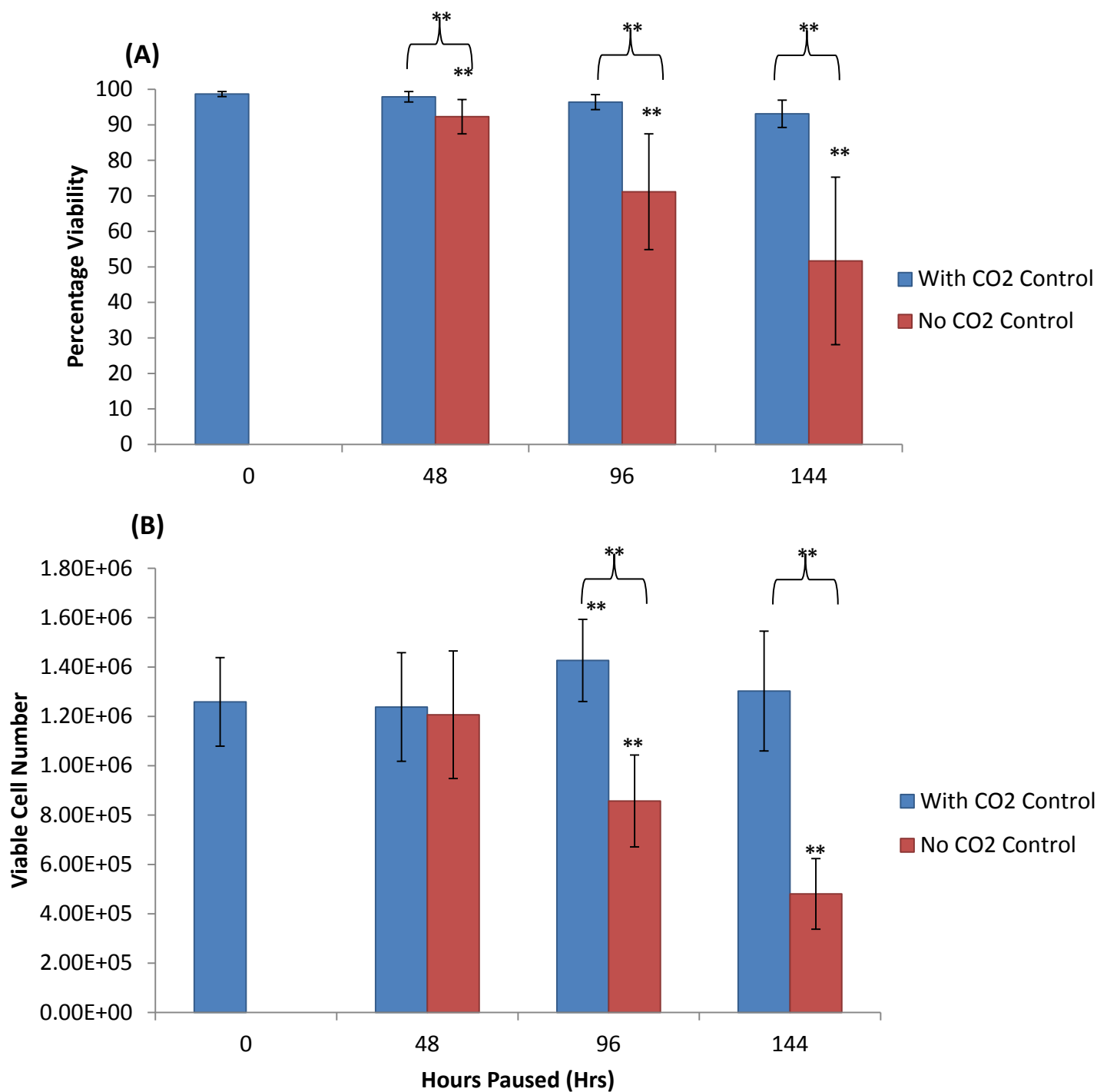
Figure 26: HOST TE85 cell viability and viable cell number after 144 hours ambient temperature pausing, with and without atmospheric control, determined by DAPI and acridine orange staining (n=4). Cells were seeded at 6.25×10^{4} cells/cm^{2} and cultured in normal conditions for 24 hours (control) cells were then paused at ambient temperature for up to 144 hours with and without atmospheric control. **(A)** Viability of cells immediately after pausing up to 144 hours with and without atmospheric control. **(B)** Viable cell number immediately after pausing up to 144 hours with and without atmospheric control. Error bars show the standard deviation between independent experimental repeats. A two-way ANOVA was used as a measure of significance between paused and non-paused cells as well as the Bonferroni post-hoc test cells. A significance symbol directly above the bar shows significance compared to the control (**indicates p≥0.01).
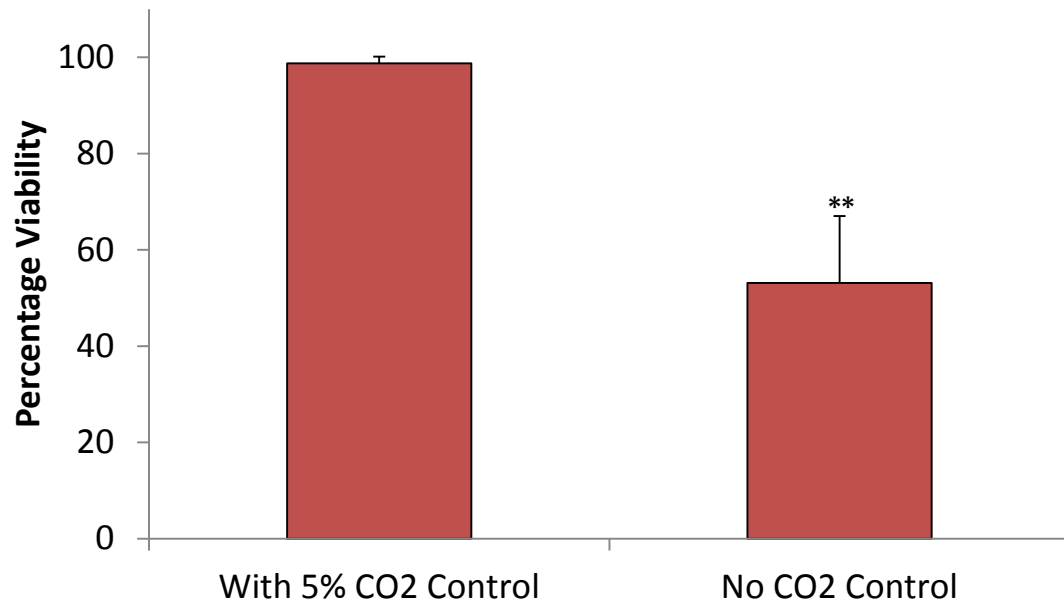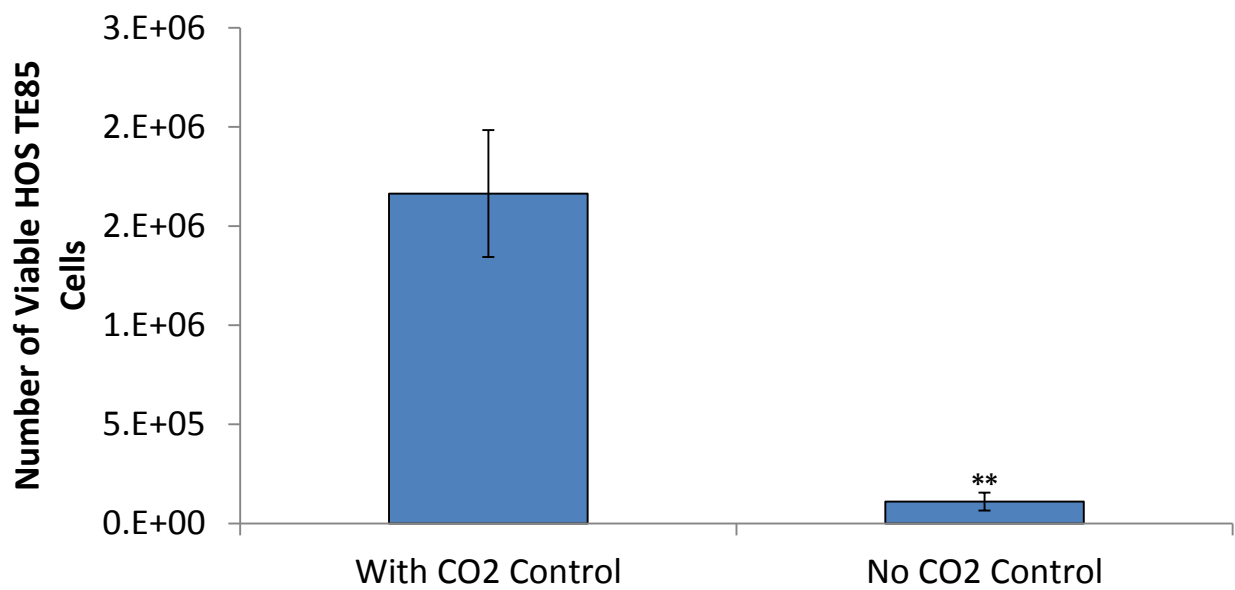
Figure 27: HOST TE85 cell viability and viable cell number after 24 hours culture at 37°C, with and without atmospheric control (n=3). Cells were seeded at 6.25×10^{4} cells/cm^{2} and cultured in normal conditions for 24 hours (control) before storage at 37°C with and with CO_2 control. (A) HOS TE85 cell viability (B) HOS TE85 cell viable cell number. A two tailed t-test was used as a measure of significance between cells with and without CO_2 control (**indicates $p \leq 0.01$).

4.3.4 MEDIUM PH THROUGHOUT PAUSING

1mL of HOS TE85 cell medium was taken before pausing and immediately after 48, 96 and 144 hours pausing with and without CO_2 control. A significant rise in pH is shown at all time-points (figure 28); however pH seems to plateau at pH 9, regardless of time paused.

4.3.5 HOS TE85 CELL FOLD GROWTH EXPANSION

At all-time points, cells paused under atmospheric control were successfully passaged 5 times, with a short lag phase between passages 1–3 and fold growth expansion returning similar to the control and reaching a plateau at passage 4–5 (figure 30). Without atmospheric control, cells paused for 48 hours had a similar growth expansion to cells paused with atmospheric control (figure 30A); however, cells paused for 96 and 144 hours could not be passaged further than passage 1 (figures 30B and 30C, respectively). Cell yield after pausing for 96 and 144 hours was lower than what was seeded and therefore gave a negative fold growth expansion number. Once again ALP measurements were variable but showed no significant difference between paused and non-paused cells (figure 29).
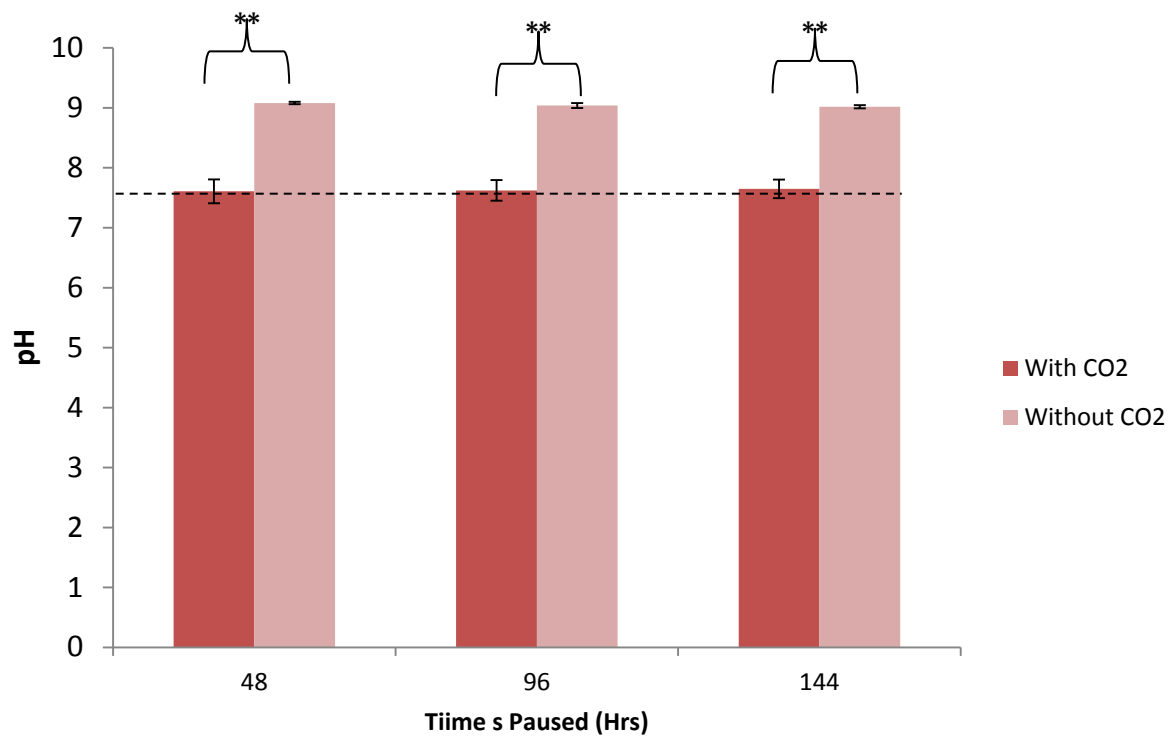
Figure 28: pH of the medium during pausing at ~20°C, with and without CO$_2$ control (n=4). The dotted line indicates the average pH of the medium before pausing (n=4) Error bars represent the standard deviation between 8 replicates from 4 independent experimental repeats.
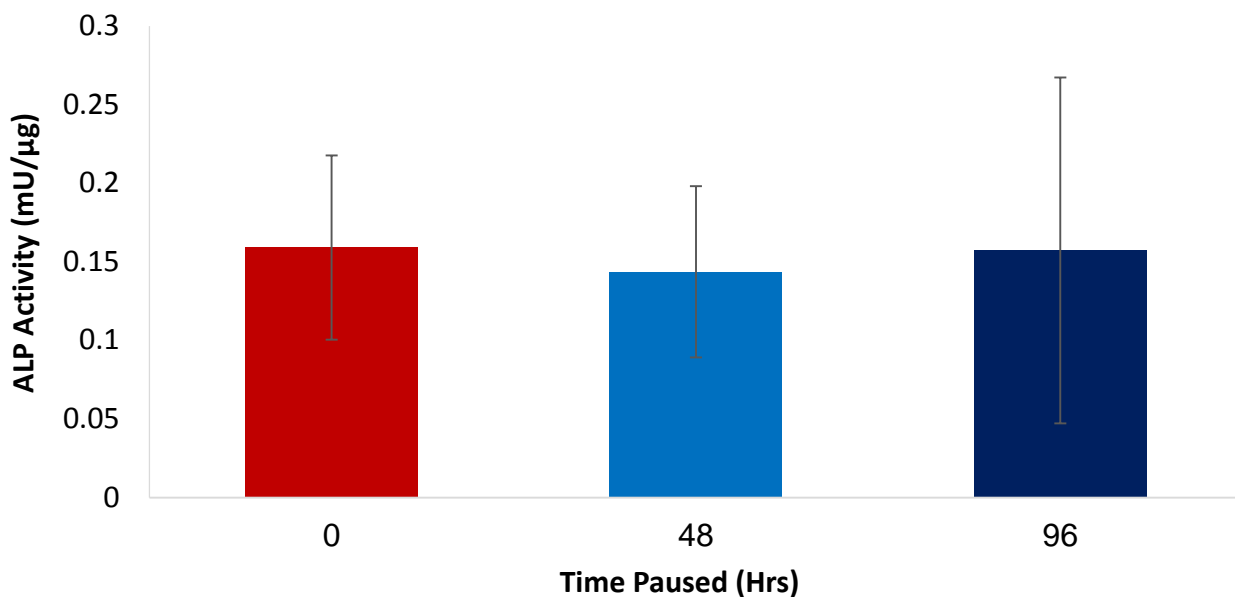
Figure 29: HOS TE85 ALP activity (mU/µg) following pausing at ambient temperatures for 48 and 96 hours (n=3). ALP activity levels were corrected for protein content (Bradford assay). A one way ANOVA found no significance difference between paused and non-paused cells whether paused for 48 or 96 hours.

(A)

(B)

(C)
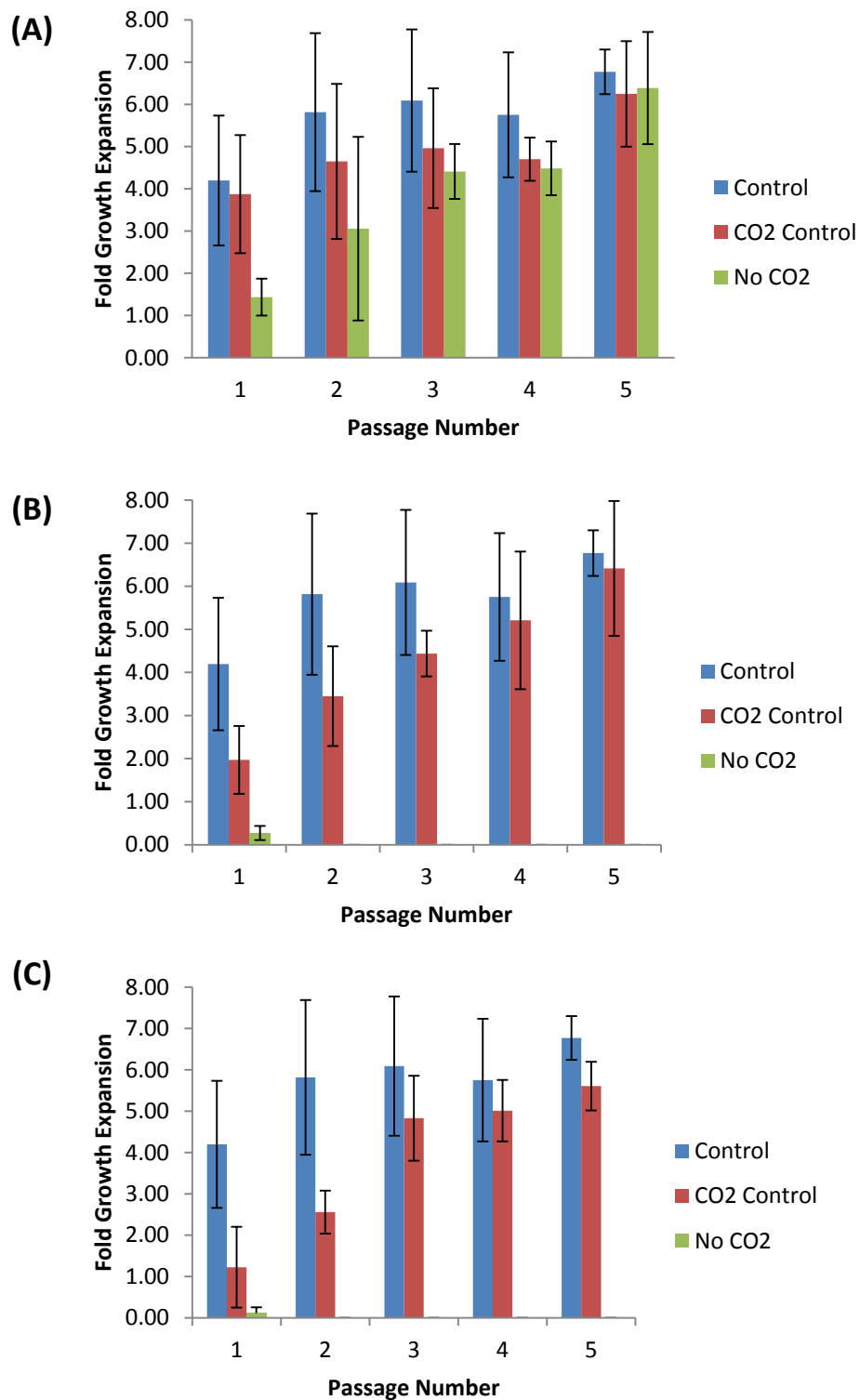
Figure 30: Fold growth expansion of HOS TE85 cells following 144 hours pausing at RT with and without atmospheric control (n=3). Cells seeded at 6.25×10^4 cells/cm^2 were paused for 48–144 hours at RT (with and without atmospheric control) and were passaged 5 times every 48 hours. The number of live cells was determined by DAPI and acridine orange staining. Controls consisted of non-paused cells. Error bars show the interquartile range between independent experimental repeats. **(A)** Fold growth expansion of cells paused for 48 hours **(B)** Fold growth expansion of cells paused for 96 hours **(C)** Fold growth expansion of cells paused for 144 hours.

It was important not to focus on temperature being the dominating factor for determining cellular health during pausing and recovery. Atmospheric components, particularly CO_2 diffusion levels in the medium, will impact on pH and need to be investigated as parameters that may require control during the pausing process. Work so far is 'proof of concept' that HOS TE85 cells can be successfully paused at ambient temperatures (viability ≥90%) in standard MEM medium with supplements for up to 144 hours, when CO_2 is controlled. With atmospheric control, confluence increased throughout culture (figures 25A-D) with more suspended circular cells when reaching 96 and 144 hours. This increase in suspended cells may be due to over confluence and hence limited space for attachment. The cells that are attached are no longer elongated or spread out on the tissue culture plastic and instead are tightly compact together. The fact that confluence increases during pausing with atmospheric control is an indication of some cell activity occurring during ambient storage, which is supported in the literature (Fujita, 1999). At 96–144 hours, no free space for growth is available which may accelerate cell signalling to inhibit growth and metabolic pathways. Once cells have reached the stationary phase, proliferation is greatly reduced or ceases entirely, especially if no medium change is performed as is the case here. Without CO_2 control, there are a considerably higher amount of cells suspended in the medium and this elevates with time paused for up to 144 hours. Viability significantly decreases with pausing time (figure 26A) and cells cannot be recovered for further passaging when paused for 96 hours or more (figure 30B). An explanation for this could be the continuing formation of ROS during hypothermic stress (Rauen and de Groot, 1998), which are a key mediator in cold induced apoptosis (Rauen, *et al.*

1999). More stress derived toxicity will be produced with time, together with an increase in waste products and nutrient deprived medium. Alongside ambient temperature investigations, an experiment was also performed culturing cells at 37°C with and without CO_2 control. The main point of this experiment was to take the focus off temperature and look at the effects of CO_2 and subsequently pH on cell viability and number over time. It is clear from figure 27 that without a controlled environment of 5% CO_2 during culture, cell viability (figure 27A) and number (figure 27B) significantly decrease, which highlights the importance of atmospheric factors. Clearly, an accumulation of stresses including suboptimal temperatures (≤35), atmospheric CO_2/O_2 fluctuations and a build of waste/lactic acid will have a negative impact on cell health.

The pH of the medium is dependent on the balance of dissolved CO_2 and HCO_3, which means changes in gaseous CO_2 can impact medium pH (figure 31). Figure 28 shows the pH of the medium increase from an average of 7.4 up to ~9 during ambient temperature, irrespective of the time paused. It seems once the CO_2 level has depleted, pH is maintained at a very alkaline pH 9 for up to 144 hours.

$$CO_2 + H_2O$$
$$H_2CO_3 \rightleftharpoons HCO_3^- + H^+$$
$$NaHCO_3 \xrightarrow{H_2O} Na^+ + HCO_3^-$$
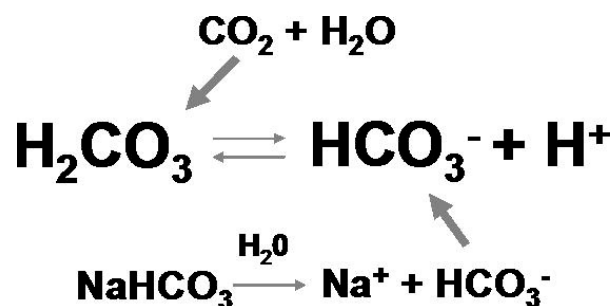
Figure 31: Regulation of pH by CO_2 concentration from the incubator and bicarbonate concentration in cell culture medium. Taken from Swain, 2010.

It is clear that a stabilised, neutral pH is an important factor for pausing, perhaps more so than temperature. In fact pH ≥8.2 is known to be toxic to cells and can induce cell death (Zetterberg and Engström, 1981). An alkaline pH has been shown to interfere with metabolism and promotes lactate production (Zwartouw and Westwood, 1958). A high pH can also increase the influx of certain nutrients (Eagle, 1973) which will upset the osmotic balance of the cell. Apart from detrimental effects, an alkaline pH can promote growth, which is more noticeable in confluent over sparse cultures (Froehlich and Anastassiades, 1974). Cells in alkaline conditions are shown to be morphologically larger and more spherical (Lie, 2012); this is observed in figures 25E-G. Research into an alternative buffer system to maintain a neutral pH such as HEPES (Good, *et al.* 1966) as well as additional anti-oxidants may improve pausing at ambient temperature.

Extending the shelf life of paused cells will be an important challenge for the processing and distribution of cells for therapy; one which could be addressed with tailored medium or refinement of pausing parameters such as temperature, cell density, storage vessel and atmospheric factors. Results so far indicate that cells paused for 96 hours do not attach to tissue plastic or proliferate like control cells (figure 30B). However, 72 hours still provides a long enough time frame for the transport and preparation of cells for therapy. It may be that further medium optimisation, with a focus on stabilising pH, as well as storage apparatus, will help preserve cells successfully for that time frame.

ALP is an enzyme typically secreted by osteoblasts and is used here as a hallmark of osteoblast phenotype; this will assess whether paused cells can remain

undifferentiated, which is of great importance for stem cells. Experiments to measure ALP secretion after 96 hours pausing were performed (figure 29). ALP activity was normalised to protein content using a BSA protein assay as the number of cells collected for each sample varied considerably. No difference in ALP activity was observed between cells paused for 48 or 96 hours. Unfortunately, due to the small number of cells derived from 144 hours pausing and the inability of cells to proliferate after this time frame these cells were not tested for ALP activity.

Keeping mammalian cells in culture medium, outside of an incubator, without any atmospheric control may be a more ideal approach for preserving cells during transport from manufacturing site to medical facility. However, as cells are adapted for culture at 37°C with controlled CO_2, O_2 and humidity levels like inside the body, it was unknown whether the cells were able to survive this suboptimal environment. Overall, results so far have shown potential for the ambient temperature pausing of HOS TE85 cells.

4.4 PAUSING TEMPERATURE FLUCTUATIONS

Ambient temperature cannot be defined to a certain degree and is variable depending on the time, place and country it is measured. Understandably, this inability to control atmospheric factors is not ideal if using ambient temperature pausing in the cell therapy setting. As has been revealed, cells are sensitive to temperature and pH changes, but the extent to which cells can withstand elevations and reductions in temperature from 37°C, is currently unknown. It is important to identify the limits of temperature changes that cells can withstand during a short-term pausing procedure. As it would be impossible to test all temperature change scenarios, only the extremes

were examined, which included ambient temperature pausing (18–22°C) for 48–96 hours with a either a cold shock at 4°C or an increase to 37°C for 3 hours after 24 hours normal culture. Whilst it is known that temperature is only one of the stresses that cells encounter during hypothermia, with gas diffusion, pH and vibration being other significant factors, here the focus is on temperature fluctuations.

4.4.1 OVERVIEW OF EXPERIMENTAL PROCEDURE

Cells were obtained, passaged and seeded into 6-well plates as described in section 3.7.1. Before pausing, the medium was replaced with freshly warmed standard MEM medium. Plates of cells were immediately placed into sealed plastic containers in a dark cupboard in the laboratory. Following 24 hours, the plates were moved either to a cold room set at 4°C or an incubator set at 37°C, 5% CO_2 and 95% humidity. For both cases, a temperature recorder was kept near to the plates to monitor the temperature fluctuations taking place (figure 32, 4°C; figure 33, 37°C). Cells were left in these conditions for 3 hours only and were then transferred back to ambient conditions for the rest of the pausing time frame (48 or 96 hours). The morphology of the cells was examined using an optical light microscope. Images were taken at random in each well of the plate, before and after pausing as well as after each temperature deviation. Membrane integrity was measured using DAPI/AO staining using the NC-3000.

Samples of cells were taken for ALP analysis immediately after 24 hours normal culture and 48 and 96 hours pausing. Alongside this assay, the same cell sample was used in the Bradford assay to quantify cell protein content and correct the ALP activity based on this measurement. Fold growth expansion assays were also performed after each time point to examine HOS TE85 cell proliferation rates for up to 5 passages.
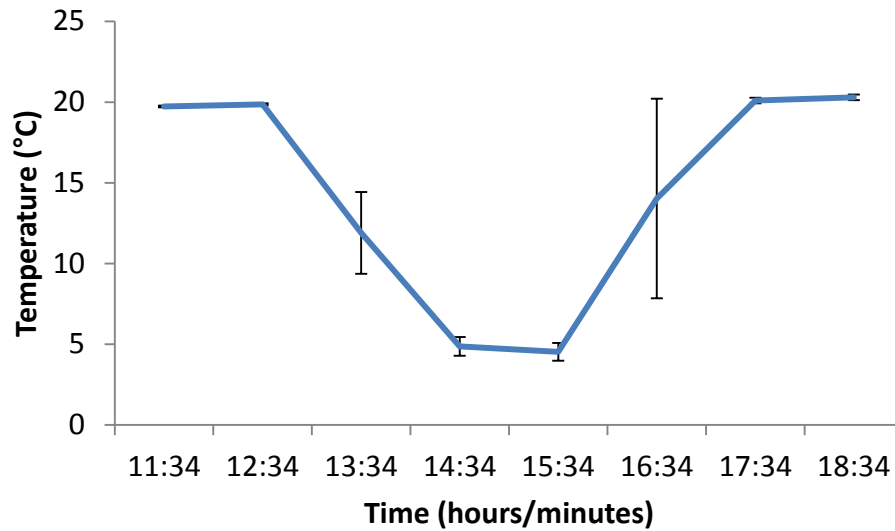
Figure 32: Temperature deviation from ambient ~20°C to ~4°C and back to ambient conditions over a 7 hour period (n=3). This graph shows the drop in temperature from ~20°C experienced by cells during a period of severe cold shock for 3 hours and their return to ambient conditions. These measurements were taken using a modular signal recorder data logger. Error bars show the standard deviation between independent experimental repeats.
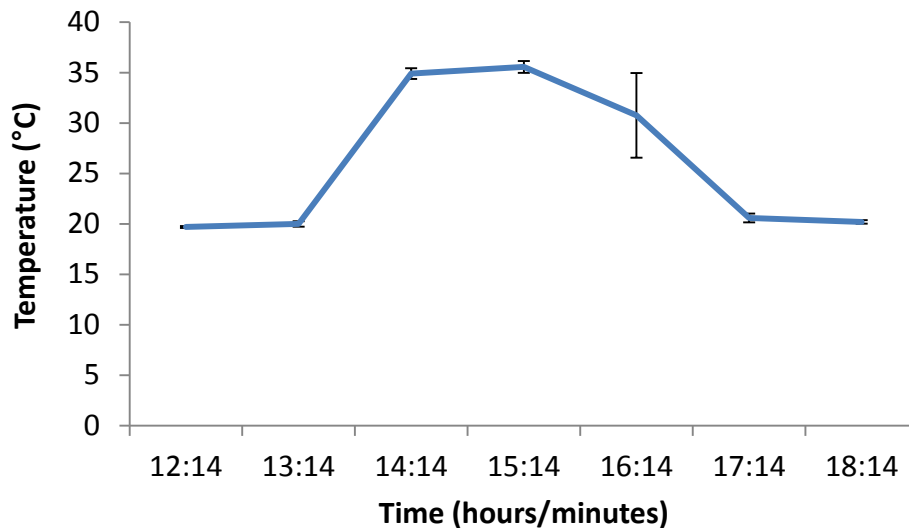
Figure 33: Temperature deviation from ambient ~20°C to ~37°C and back to ambient conditions over a 7 hour period (n=3). This graph shows the increase in temperature from ~20°C experienced by cells during a period of normal culture conditions for 3 hours and their return to ambient conditions. These measurements were taken using a modular signal recorder data logger. Error bars show the standard deviation between independent experimental repeats.

It is clear from figure 16, that when cells are introduced to pausing conditions, confluence is decreased and cells begin to detach from tissue-culture plastic. When the temperature deviations were introduced, more cells seemed to be suspended in the medium when stored at 37°C for 3 hours, than when cells were cold shocked (4°C) or kept in ambient conditions throughout the experiment (figure 34).
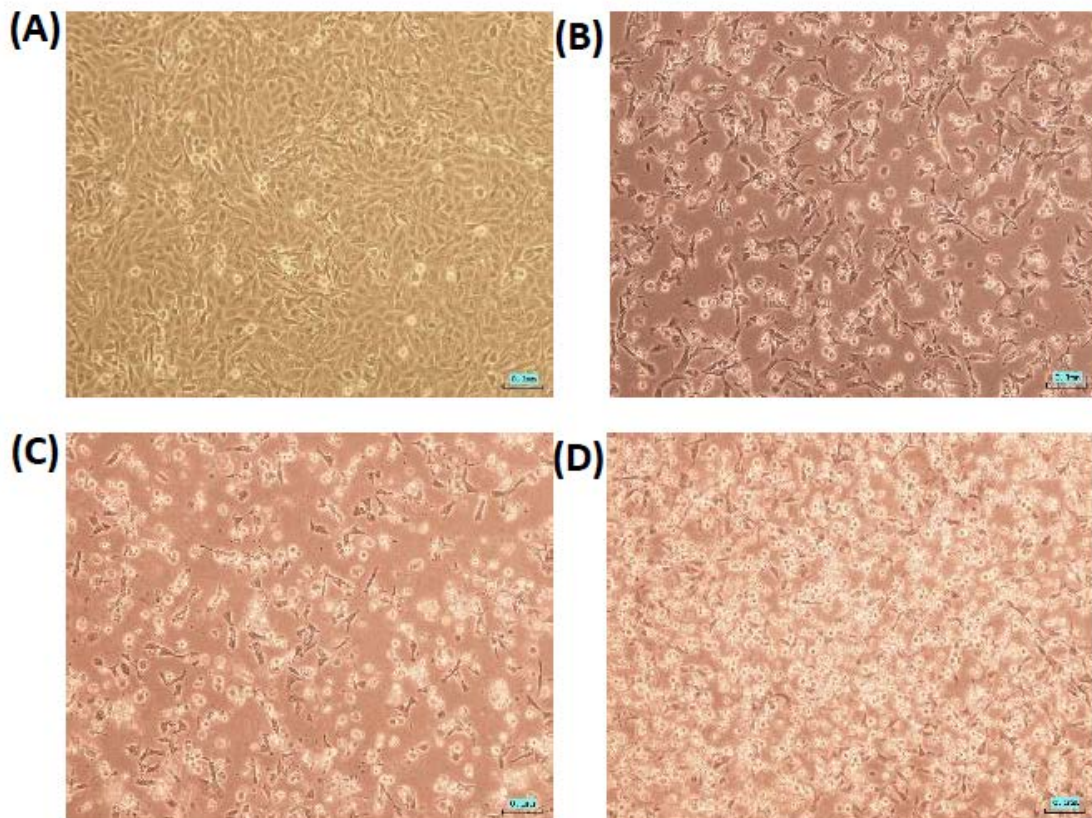
Figure 34: HOS TE85 cell morphology during pausing at ~20°C with extreme temperature deviations (n=3). (A) Control – cells cultured in normal conditions for 24 hours **(B)** Cells immediately after pausing for 96 hours **(C)** Cells that were subject to 4°C for 3 hours, 24 hours into pausing for 96 hours **(D)** Cells that were subject to 37°C for 3 hours, 24 hours into pausing for 96 hours. Taken by Joseph McHale.

4.4.3 HOS TE85 CELL MEMBRANE INTEGRITY

A deviation in temperature from 20–4–20°C for 3 hours only, causes a significant decline in membrane integrity (p≤0.01) at the end of the 48 hour pausing period compared to cells that have not experienced a temperature deviation (figure 35). This significant drop in viability is even more pronounced after 96 hours (p≤0.01). A deviation in temperature from 20–37–20°C appears to have less of an effect than 4°C; however, this decline in cell membrane integrity is still significant (48 hours p≤0.05; 96 hours p≤0.01). Temperature deviations whether at 4°C or 37° did not significantly affect live cell number before and after pausing (figure 36).
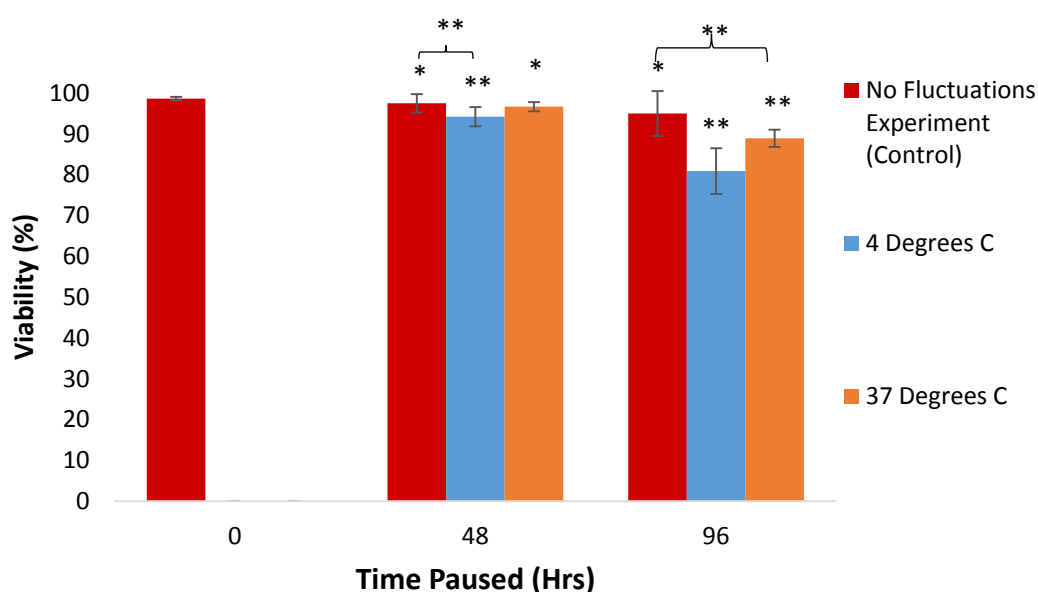
Figure 35: HOS TE85 cell viability by DAPI/AO staining when subjected to temperature deviations of 4°C and 37°C for three hours during the pausing process for 96 hours (n=3). A two-factor ANOVA test was used to test for significance alongside the Bonferroni post-hoc test (* indicates p≤0.05;**indicates p≤0.01). Significance stars without brackets refer to significance between the specific bar and the control; significance stars with brackets refer to significance between the two indicated sets of data. Error bars show the standard deviation between independent experimental repeats.
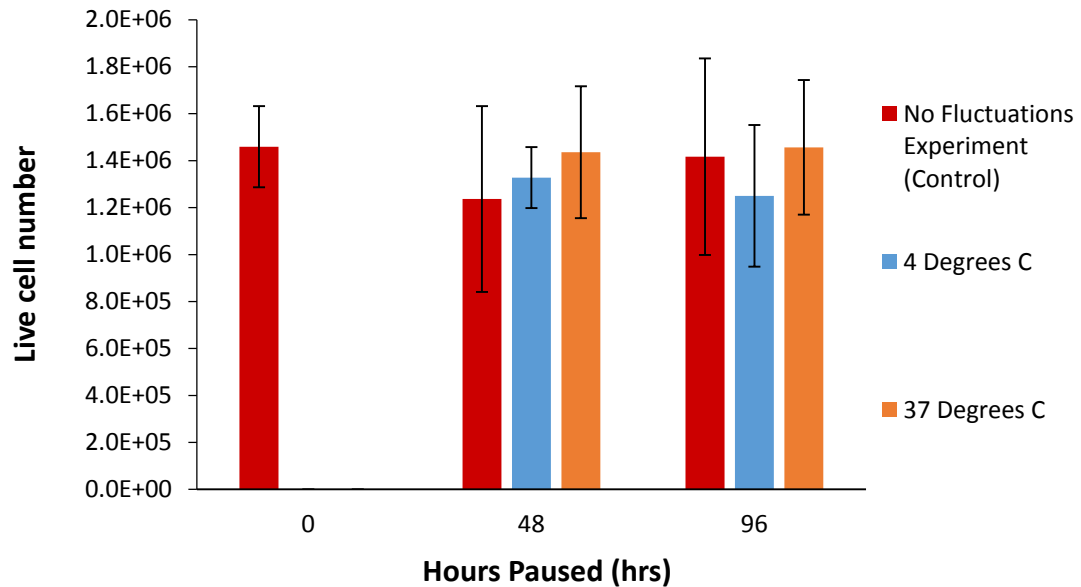
Figure 36: HOS TE85 live cell number by DAPI/AO staining when subjected to temperature deviations of 4°C and 37°C for three hours during the pausing process for 96 hours (n=3). A two-factor ANOVA was performed and no significant differences were identified. Error bars show the standard deviation between independent experimental repeats.

4.4.4 HOS TE85 CELL ALP ACTIVITY

ALP activity was corrected for the protein content in each sample, which varied considerably, so that ALP activity could be expressed in mU/μg. No significant difference in ALP activity was observed at both 48 and 96 hours between cells that had been subjected to temperature deviations (4°C and 37°C) and cells that were either not paused or were paused at 20°C without a temperature deviation (figure 37).

4.4.5 HOS TE85 FOLD GROWTH EXPANSION

A temperature drop to 4°C for 3 hours led to a significant reduction (p≤0.05) in fold growth expansion on the first passage compared with non-paused control cells. This decline was similar to the reduction observed when cells were paused at 20°C throughout for 48 hours (figure 38). Upon the second passage, the decrease in growth expansion was still significant; however, from passage 3 onwards cells seemed to

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return to their normal expansion behaviour. In contrast, a temperature deviation to 37°C for 3 hours seemed to promote increased proliferation on passage 1 after pausing for 48 hours compared with cells maintained at ~20°C. From passage 3 and above, no significant difference in fold growth expansion was observed. When paused for 96 hours in all three scenarios (20°C, 20–4–-20°, 20–37–20°) the fold growth results are significantly different from the control on passage 1 and cells are unable to reattach to tissue culture plastic and consequently grow and proliferate.
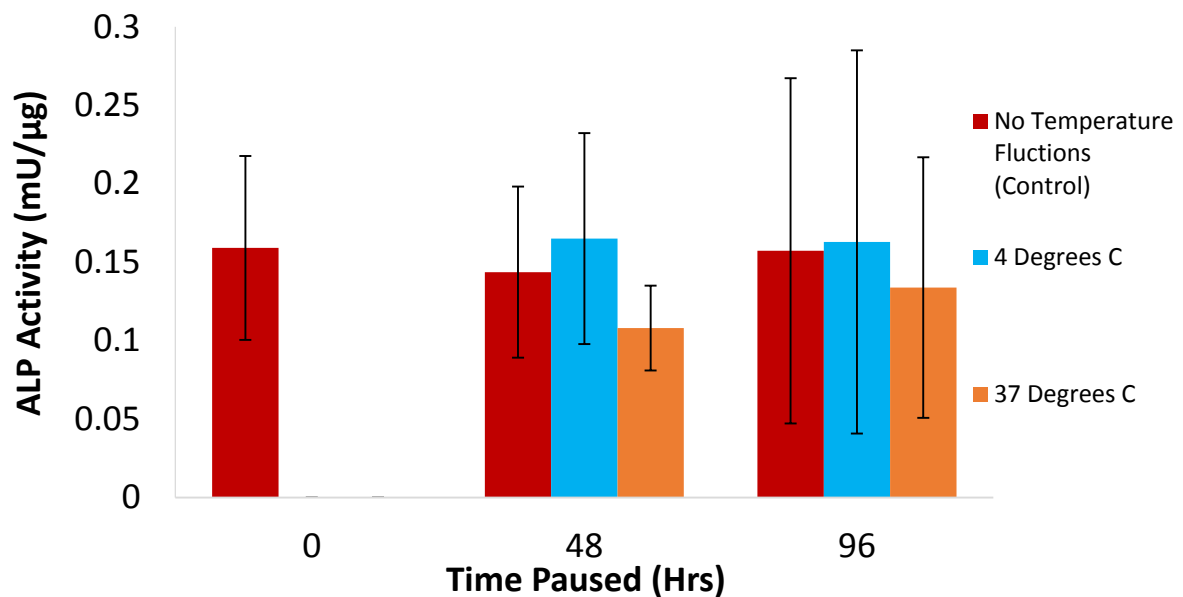
Figure 37: HOS TE85 ALP activity (mU/µg) when subjected to temperature deviations of 4°C and 37°C for 3 hours during the pausing process for 96 hours (n=3). ALP activity levels were corrected for protein content (Bradford assay). A two-factor ANOVA found no significance differences between cells subject to temperature deviations and cells kept at ambient temperature throughout pausing. Error bars show the standard deviation between independent experimental repeats.
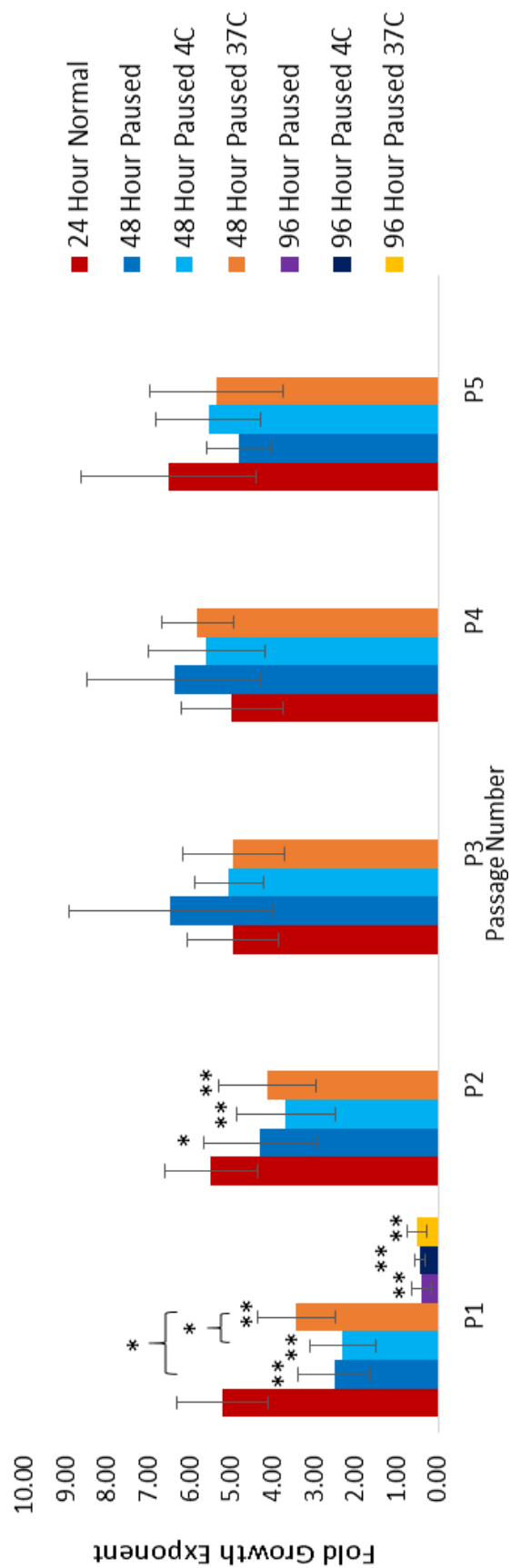
Figure 38: HOS TE85 cell fold growth expansion when subjected to temperature deviations of 4°C and 37°C for 3 hours during the pausing process for 96 hours (n=3). A three-factor ANOVA test was used to test for significance (**indicates $p \leq 0.01$, * indicates $p \leq 0.05$, significance stars without brackets refer to significance between specific bars and the control for the same passage number, significance stars with brackets refer to the significance between the two indicated sets of data). Error bars show the standard deviation between independent experimental repeats.

4.4.6 DISCUSSION

Due to the difficulty in defining ambient temperature and the risk of temperature fluctuations during transport, some likely scenarios of temperature variations were tested during cell pausing. This gave an indication of the effect changes in temperature might have on cell survival and provided avenues of investigation and direction for the subsequent chapters. The aim of these experiments was to test if HOS TE85 cells can survive temperature deviations during pausing in ambient conditions. As it would not be possible to test all temperature fluctuation scenarios, two extremes were examined (4°C for 3 hours; 37°C for 3 hours). During transport from one location to another, temperature will inevitably fluctuate, and it is unknown how cells will cope in this circumstance. By finding the limits of temperature change cells can withstand, a temperature range can be defined so that cells that have been kept inside this range for a short period of time will be viable and able to function therapeutically. For cell therapies, it is critical that manufacturers are able to prove that their products will retain their effectiveness upon delivery to the medical site. The first scenario tested was pausing cells at ambient temperature for 48 and 96 hours, and then introducing a temperature deviation of 37°C for 3 hours. In this particular case, cells may be transported in an ambient environment and put back inside an incubator set for culture at normal conditions on arrival at the hospital or medical site. The cells may then be taken out of the incubator to be prepared for surgery. During the 3 hours at 37°C, the rewarming process will begin together with the initiation of energy dependent processes, which may cause damage due to ROS formation (Meng, 2003). The second situation is a temperature deviation at 4°C, which represents a scenario in which cells are put in cool storage before preparation for infusion. This will make

energy-dependent mechanisms even slower, and may have effects on membrane composition. Cells will then enter a rewarming phase upon their return to 20°C for the rest of the pausing period. These deviations are examples of extreme shifts in temperature which will promote pathways such as apoptosis and necrosis (Mathew, *et al.* 2004), may hinder the stability of cellular membranes and may disturb internal cellular processes. Changing the temperature for 3 hours was chosen to give time for the temperature of the cells to drop to 4°C or rise to 37°C and for the cells to be at these temperatures long enough for an effect to be observed. Additionally, as the cells were in medium, it also took time for the liquid to reach equilibrium with the surrounding temperature. As temperature readings were only taken hourly, this may have exaggerated the slowness of the temperature change. As the point of this experiment was to test fluctuations in temperature on paused HOS TE85 cells, it was not vital that the cells were kept at 4°C or 37°C for a solid 3 hours, and as all experiments were performed consistently, it meant that a conclusion could be drawn. Additionally, cell suspensions have been specified a shelf life at ambient temperature for 3 hours before expiry (EMA, 2013). For cells that experienced a temperature deviation at 37°C, the temperature fluctuation seemed to have a more pronounced effect on viability as the pausing length increased (48 hours $p \leq 0.05$; 96 hours $p \leq 0.01$). One explanation for observing dead cells later on in pausing i.e. 96 hours, is the DAPI/AO staining method chosen to measure viability. This test will only count a cell as dead if the membrane is damaged or is not intact so that the DAPI can penetrate the cell and stain the internal DNA. Dead cells that still have an intact membrane or cells undergoing apoptotic processes will not be detected until later on when their membrane integrity is finally lost. At both time points, the temperature deviation at

4°C yields much lower viability than the deviation at 37°C, compared to the control cells paused with no temperature deviation. Although these decreases in viability are significant, viability does not drop below 70% (stated by the FDA to be the threshold above which cells are considered useable in a cell therapy setting). This is a promising result for the use of pausing for cell products, as if a robust osteosarcoma cell line can survive extreme temperature fluctuations such as these, the more sensitive and complex stem cells at least have the potential to survive a small range of temperature.

No significant change in live cell number was apparent over the 3 experiments for either deviation or time period paused. Due to the high variation between experiments, it may be that more data would highlight a significant difference as observed with viability.

Similar to results so far, ALP activity between non-paused cells and those subject to temperature deviations did not significantly change, even when corrected for the amount of protein in each sample. Whilst the variability between replicates for each ALP experiment was minimal, high variability was observed between the average data points for each repeated experiment (figure 29 and 37). One explanation for this variability could be the difference in the number of cells that managed to survive the pausing process between each experiment. The Bradford protein assay was used to measure protein concentration in each sample; however, after correcting for the difference in protein content, there were still high levels of variability. The variability may have resulted from the small sample size of cells used in the fluorescence assay; 10µl is an extremely small sample for cell culture and is limited in terms of accuracy with pipetting at such small volumes. Increasing the sample size to 20µl, for example, may have reduced variability; however, this may have affected the number of

replicates that could have been used for each data point. Whilst every effort was made to ensure each experiment was performed the same, there may have been some differences in the solutions used (e.g. date first opened/used), incubation time and light exposure, which may have affected the results of this highly sensitive fluorescence assay. For future work, ALP staining and cell imaging may be a more reliable indicator of changes in ALP expression before and after pausing.

A temperature deviation of 4°C during ambient pausing causes the fold growth capacity of cells at passage 1 to drop significantly ($p \leq 0.01$); this result is also true for cells maintained at ambient temperatures throughout the 48 hours (figure 38). Keeping cells at 37°C for 3 hours during pausing similarly reduces fold growth at passage 1 ($p \leq 0.01$). For cells paused for 96 hours, both temperature deviations cause a significant reduction in growth expansion over the first passage, similar to when cells are kept at ~20°C without a temperature change. Most cells were unable to reattach when seeded for a second time and therefore the fold growth expansion result was negative. Cells which experienced exposure to 4°C during pausing seem to have a reduced fold growth expansion ratio on passage 1 and 2 compared with cells paused without a temperature fluctuation. An explanation for this may be that severe cold shock at 4°C will slow internal cellular processes and signalling pathways so much that the time it takes for cells to resume entry and progression through the cell cycle is extended during the recovery period from 4°C to ~20°C.

In contrast, cells which experienced a deviation to 37°C during pausing seemed to have a slightly higher fold growth ratio on passage 1 than those which had not been through a temperature change from ~20°C. It seems that a rise in temperature to 37°C

for 3 hours only, may boost growth and cell cycle progression during the first passage. However, this mild increase in fold growth is no longer observed from passage 2 onwards. It is unclear whether this finding is true due to some variability between independent experimental repeats and more data may be required to clarify this pattern.

For these experiments, the temperature deviation occurred 24 hours into the pausing process. This time point was chosen as this provides enough time for cells to enter into their 'pausing' state and performing the deviation at the same point in the process ensured consistency. If the temperature deviation had occurred earlier or later during pausing, a different result may have been obtained. For example, changing the temperature for 3 hours at 12 hours into pausing may have damaged cells earlier on and given more time for cells to enter apoptotis and disassemble leading to lower cell viability at both 48 and 96 hours. The specific time of temperature change during pausing might play a critical role in whether a cell remains safe and effective to use for therapy; further studies may need to be performed to improve our understanding of this matter.

To summarise, cells were able to survive temperature deviations for 3 hours at either 4°C or 37°C. Proliferative rates over the first passage were affected by temperature change, exposure at 4°C decreasing fold growth expansion and 37°C promoting fold growth expansion, compared with cells maintained at ~20°C. This suggests that a small range of temperature needs to be defined for cell pausing to limit declines in cell health. Overall, fold growth expansion patterns remained unchanged after passage 1.

As these were extreme cases of temperature variation, the fact that cell viability remained above 70% for all time points tested is promising.

4.5 CONCLUSION AND OVERALL SUMMARY

Overall, a test system has been developed to investigate the potential of pausing human cell types and to improve and optimise process parameters. Firstly, the pausing system was designed and refined for *in vitro* investigation. Initially, a human osteosarcoma derived cell line (HOS TE85) was chosen to test the pausing process. Being of osteosarcoma origin, the cells were an abundant source due to their highly proliferative nature and were robust in design, an ideal model cell line. As discussed in the literature review, data is severely limited for mammalian cell pausing at ambient temperatures and being able to successfully store a human derived cell line such as HOS TE85 at room temperature for short time periods, demonstrates that complex mammalian cells can survive hypothermic stress.

Pausing for up to 48 hours only was selected for early investigations to gauge the potential for prolonged storage of HOS TE85 cells in ambient conditions. A rewarming step immediately after pausing at normal culture conditions meant we could examine the viability of cells after their return to physiological conditions, as would occur during potential cell therapy procedures.

A clearer understanding has been achieved of the additional work required to understand the cellular interactions and metabolic processes which take place during pausing. Work so far is 'proof of concept' that HOS TE85 cells can be preserved in standard MEM medium by entering a phase of suspended animation during pausing at ambient temperatures for up to 144 hours with CO_2 control. Live cell numbers

immediately before and after pausing were similar, which suggests the rate of proliferation slowed severely during pausing as expected. It seemed the cell cycle was restarted once cells were warmed to 37°C as live cell number returned to that of non-paused cells cultured for 24 hours.

Cell deterioration with increased pausing length is a theme amongst all results so far. It may be that further medium optimisation may be necessary such as the addition of anti-oxidants to help combat ROS formation from cellular stress during hypothermia. The buffering agent HEPES may be necessary to stabilise pH. As demonstrated throughout this chapter, a stable pH, perhaps more so than temperature is important for successful pausing. This may be the key for effectively extending pausing length as currently pausing for ≥96 hours leads to cells which are unable to attach to a surface and proliferate when devoid of atmospheric control.

Overall, cell pausing in an ambient environment for up to 96 hours has delivered promising results and has provided new avenues for investigation which will be explored in chapter 5, centring on medium optimisation for enhanced pausing.

CHAPTER 5: MEDIUM ADDITIVES FOR CELL PAUSING

5.1 INTRODUCTION

Standard medium, which has been used thus far throughout experiments, is optimised to support cell culture at 37°C and is not equipped in terms of composition for falls in surrounding temperatures. Nevertheless, cells have retained viability above 70% and have demonstrated proliferative behaviour following pausing for up to 96 hours. When pausing past this time length, HOS TE85 cells are impaired due to damaged membrane integrity and their inability to reattach to tissue culture plastic. Most standard culture medium is developed for the default setting of most cell culture incubators (37°C, 5% CO_2 and 95% humidity) and relies on phosphate and bicarbonate buffers to regulate pH. However, in hypothermic, open air systems these buffers lose their ability to maintain pH as CO_2 escapes. A loss of protons means the pH becomes more alkaline and pink in colour, indicated by phenol red. Medium for pausing needs to be able to withstand the uncontrolled atmospheric environment by maintaining pH and ionic balance so that cells can survive suboptimal conditions.

The use of specialised medium for cell pausing has evolved from methods used to preserve whole organs. ViaSpan® was the first solution formulated for the hypothermic transport of the pancreas, kidney and liver by Belzer and Southard in the late 1980s. This lead to the development of many other intracellular-like solutions including EuroCollins, Celsior (SangStat Medical Corporation) and Custodiol (Odyssey Pharmaceuticals, Inc.), which sought to manage hypothermic stress factors. Most recently, BioLife Solutions®, known for their production of cGMP biopreservation

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media products for cells, tissues and organs created the well-known hypothermic storage media HTS-FRS, as well as the freeze media Cryostor. The apoptotic and necrotic pathways endured during and post-pausing are most probably caused by a multitude of stressors including ionic and oncotic imbalances, pH fluctuations, the production of free radicals and a decrease in energy sources such as glucose.

This chapter will explore in detail, preservation solution design, in order to extend pausing length and obtain higher viability post-thaw for ambient temperature pausing. The composition of media must be tailored to the temperatures cells will be exposed to and consequently the osmotic and molecular challenges they will face. For example, the constitutions of cryogenic and hypothermic media are considerably different as cells will encounter varying forms of stress during each of these methods of preservation. As discussed previously, cryogenic medium such as CryoStor is focussed on mitigating stress during the freezing and thawing procedure, and is pre-formulated with DMSO. HTS-FRS on the other hand, is directed towards stress-related events that occur during the chilling and warming of cells and includes components that scavenge free radicals and balance the cells ionic composition. Thus being, the ideal composition for media during ambient temperature pausing will be different again. Although there are no tailored media currently on the market for ambient cell storage, there is no doubt that companies such as BioLife Solutions® are developing these products. As identified in chapter 4, a medium formulation specialised for pausing may be the key for the prolonged and effective storage of mammalian cells in ambient conditions. Several media will be tested for ambient temperature pausing, including standard

osteoblast medium with additives and commercial media with proven use in biologic and medical research.

Additionally, since osteoblast cells are adherent, and so far have been paused when attached to tissue culture plastic, their potential for pausing in suspension will be investigated. When considering the logistics of cell therapy, cells attached to a surface are not ideal when transporting from one site to another. Further to this, since the number of cells required for a cell therapy may be in the order of trillions and the surface of area of the largest T-flask is only 175cm^2, it would be impractical to transfer this number of single or multi-flasks. Mono-layer cells also require retrieval off the surface before they can be administered to the patient. Therefore, a suspended solution is more ideal. The challenge here is that for cells which are naturally anchorage-dependent, forcing them to become suspended for a time period of hours to days may add to the stress already encountered during low temperature pausing. It is also known that cells which are innately adherent have a tendency to attach to each other via cell adhesion molecules (Alberts, *et al.* 2002) if they are not provided with an appropriate surface to attach to and form a monolayer. This in turn creates the risk of aggregate formation which in terms of cell infusion paves the pathway for numerous regulatory issues. Therefore, the second half of this chapter will explore the potential of storing HOS TE85 cells in a single-cell suspension at low temperature with the help of various anti-clumping agents.

5.2 TESTING VARIOUS MEDIUM FORMULATIONS FOR PAUSING

Various media were examined during low temperature pausing for up to 144 hours. Amongst those tested were standard MEM medium with additives and commercial

media as described in table 10, with standard MEM as the control. It is well known that pH fluctuations occur in suboptimal conditions; a major contributing factor to the deterioration of cells during low temperature storage. In normal cell culture, the ideal pH of medium is ~7.4. As demonstrated by results in chapter 4 and previous studies, pH increases when there is no CO_2 control in the ambient environment. This is due to the buffering system used, typically sodium bicarbonate, which depends on a constant diffusion of 5% CO_2 in the medium to maintain optimum pH (Swain, 2010). When cells are removed from the tightly controlled environment of an incubator, CO_2 is lost and pH becomes alkaline. To combat this, the alternative zwitterion buffering agent HEPES can be used which does not rely on a constant supply of CO_2. HEPES has been shown to be non-toxic to cells at concentrations below 25mM (Adams, 1990) and will therefore be used at 25mM in pausing experiments to achieve maximum buffering capacity. As bicarbonate is still required for some cellular systems (Adams, 1990) the HEPES will act as an additive to standard MEM medium. Other zwitterionic buffer agents have been used in cell culture such as TRICINE (N-[Tris-hydroxymethyl]-methyl)glycine and TES (N-[Tris-hydroxymethyl]methyl)-2-aminoethansulphonic acid; however, HEPES was chosen for these experiments due to its usage in commercial media e.g. HTS-FRS.

Cells have an innate capacity to deal with various forms of cellular stress in the form of glutathione, α tocopherol, ascorbic acid, and β carotene as well as anti-oxidant proteins such as superoxide dismutase, catalase and glutathione peroxidase (Das, 1998). Oxygen free radicals, if left to manifest, eventually reach the nucleic acids of the cell, causing DNA strand breaks, protein degradation and lipid peroxidation (Das, 1998).

These anti-oxidant components act by quenching oxygen derived free-radicals before any vital elements of the cell are damaged. Additional glutathione in the medium has long been known to increase outcome during organ preservation (Baust and Baust, 2007) by enhancing the anti-oxidant capacity of the organs upon recovery (Southard and Belzer, 1995). The water soluble vitamin E analogue is also recognised for its extensive anti-oxidant properties (Tomasetti and Neuzil, 2007) and has been demonstrated as an efficient anti-oxidant in HTS-FRS. Due to its well-known use as an anti-oxidant and the fact that it has been incorporated in commercialised products, Trolox has been added to standard MEM medium in these experiments alongside HEPES as a potential option for ambient temperature pausing.

Owing to its use and success as a medium for hypothermic preservation (2–8°C), HTS-FRS will be tested here as a possible option for ambient pausing. Although it is optimised for between 2–8°C, its composition is designed to combat events which occur during the chilling and warming of cells.

Plasma-Lyte A is an isotonic crystalloid solution which closely mimics normal plasma osmolality, electrolyte content and pH and was developed for use in resuscitation after trauma and hypovolemic shock (Dutton and Howard, 2007; Rizoli, 2011). Blood components such as platelets, granulocytes and haematopoietic cells (Valbonesi *et al.* 1995; Burger *et al.* 1999; Lightfoot *et al.* 2001) have been successfully stored in Plasma-Lyte A at low temperatures. Since it is approved for use *in vivo*, it is tested here as an option for ambient cell preservation. As it is restricted for use by registered practitioners only, an in-house formulation (Pseudo-Lyte A) was made based on the manufacturer's composition (see appendix A).

This section will investigate various media options in order to promote cell survival post-pause using HOS TE85 cells and hopefully highlight a commercial solution or media additive to bring forward in experiments with hMSCs.

5.2.1 OVERVIEW OF EXPERIMENTAL PROCEDURE

The aim of this experiment was to test various media (standard media with additives or commercialised solutions) against standard MEM medium for pausing HOS TE85 cells in ambient conditions. By adding elements to media, which can potentially help combat the onset of apoptosis or necrosis during ambient temperature storage, an increased pausing length may be achieved together with improved viability upon warming and the ability of cells to reattach and proliferate after ≥96 hours pausing. Table 10 provides information on the media used in these experiments as well as their composition. Cells were cultured and passaged as described in section 3.2.1. HOS TE85 cells were seeded ($6.25x10^4$ cells/cm^2) in standard MEM medium and cultured for 24 hours in normal conditions to allow attachment and 90% confluence. Following this, the standard medium was removed and replaced with 3mL/well of each medium described in table 10. Well-plates were then transferred into a sealed plastic container and placed in the cupboard in the laboratory (ambient pausing) or in a cold room set to 2–8°C (hypothermic pausing).

To keep consistent with previous experiments and to simulate likely storage and transport conditions, pausing at both temperatures was performed in the dark. Additionally, HEPES has been shown to be sensitive to light (Jayme and Gruber, 2006), which can impact the effects of the buffering agent.

Table 10: Various media used throughout pausing experiments. The table details the acronyms and the supplier of each media used throughout this thesis.

Media	Acronym	Supplier
Standard MEM medium	MEM	Fisher Scientific
Standard MEM medium + 25mM HEPES	MH	Fisher Scientific
Standard MEM medium + 25mM HEPES + 1µM Trolox	MHT	Fisher Scientific
Hypothermosol -FRS	HTS	BioLife Solutions USA (UK provider Quest Biomedical)
Pseudo-lyte A	PLA	Made in in-house

Pausing was carried out for up to 144 hours at ~4°C and ~20° as before with morphology, viability, and fold growth expansion tests carried out at 96 and 144 hours. Due to the variability and non-significant data encountered with previous ALP assays, the assay was not performed for medium additive experiments. Measurements of medium pH were also taken throughout the experimental process.

5.2.2 HOS TE85 CELL MORPHOLOGY

HOS TE85 cells paused in ambient conditions for 96 hours in standard MEM were detached in large numbers, with some cells having a dark and grainy appearance (figure 39A); there was a further increase in the number of suspended cells at 144 hours (figure 39F). In standard media with pH control, more attached fibroblastic looking cells are apparent compared to the control, with an elongated structure and the refraction of light around the membrane (figure 39B, 96 hours; figure 39G, 144 hours). Confluence is well maintained; however, there are clear spaces between attached cells. Suspended cells can be identified by their round and plump shape. There seems to be no obvious increase in the number of suspended cells between 96

and 144 hours in medium with HEPES (figures 39B and 39G, respectively) and HEPES plus Trolox (figures 39C and 39H). Cells in HTS-FRS appear well spaced out on the surface (figures 39D, 96 hours; figure 39I, 144 hours) however; instead of an elongated structure they are shorter and more spherical with a dark centre. Although detached cells can be identified, there are less compared to those in standard MEM medium. Cells in PLA are also less fibroblastic in appearance (figures 39E, 96 hours; figure 39J, 144 hours) than cells in standard MEM and are more clumped together. Furthermore, the number of attached cells in PLA seems minimal. No difference in morphology can be identified between cells paused for 96 and 144 hours in HTS-FRS and PLA.

When paused at 4°C, all cells in MEM regardless of time point appear to have lost their ability to attach and elongate along a surface (figure 40A, 96 hours; figure 40F, 144 hours); however, it was hard to focus on the detached moving cells to obtain a true image. In medium with pH control, cells are mostly suspended at 96 hours with a clear round membrane (figure 40B and 40C), this clarity is reduced at 144 hours (figure 40G and 40H). On the other hand, the majority of cells in HTS are attached to tissue culture plastic (figure 40D, 96 hours; figure 40I, 144 hours). Whilst adherent, these cells still lack the fibroblastic appearance of control cells and are short and plump with clear membranes. In PLA, there is a mix of suspended and adherent cells which seem to be clumpier compared to cells in other types of media (figure 40E, 96 hours; figure 40J, 144 hours). No change in morphology was observed between time points for cells in HTS and PLA.

5.2.3 HOS TE85 CELL MEMBRANE INTEGRITY

Cells paused in ambient conditions, in MEM medium alone, have an average membrane integrity of 78% and 55% after 96 (figure 41A) and 144 hours (figure 41B) pausing, respectively. With the addition of HEPES to standard medium, viability was significantly improved after pausing for 96 hours (MH and MHT = 93%, p<0.001) and 144 hours (MH = 82%, p<0.001; MHT = 84%, p<0.001). The use of HTS compared with the control also produced a significant improvement in viability following 96 and 144 hours ambient temperature pausing (97% and 95%, respectively, p<0.001). There was however, a significant reduction in membrane integrity to 70% for cells kept in PLA compared with the control, after 96 hours (p<0.05) and a further reduction to 65% after 144 hours. No significant difference was found in viability between MH, MHT and HTS at 96 hours however, significance was proved between MH and HTS at 144 hours (p<0.05). All other media except PLA significantly improved viability immediately after ambient temperature compared to when using standard MEM medium.
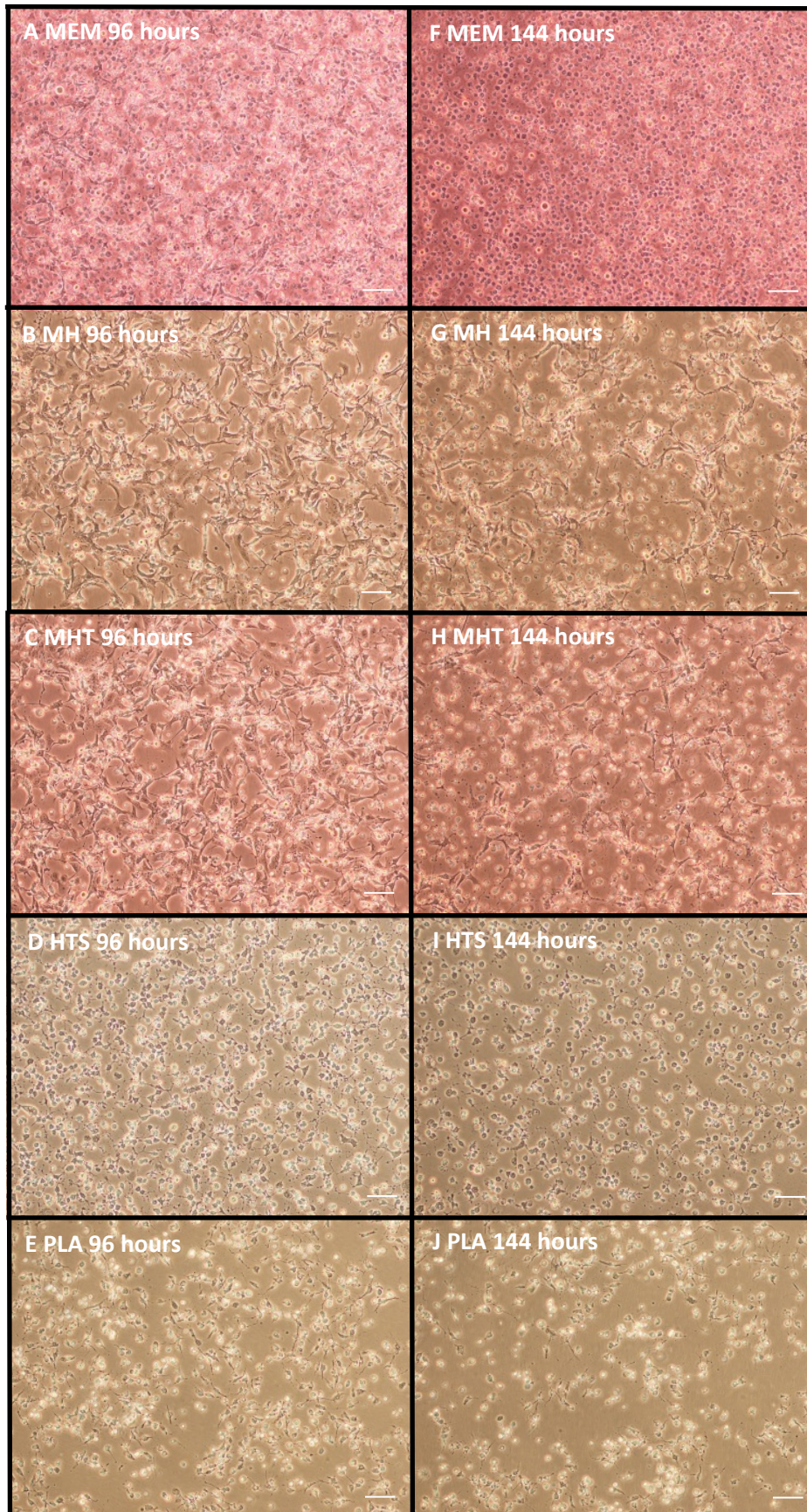
Figure 39: HOS TE85 cell morphology after pausing in ambient conditions for 96 and 144 hours in various media (n=3). HOS TE85 cells were seeded at 6.25×10^4 cells/cm^2 and cultured in normal culture conditions (37°C, 5% CO_2 and 95% humidity) for 24 hours. Media was then replaced with either standard MEM medium (MEM), standard medium with HEPES (MH), standard medium with HEPES and Trolox (MHT), Hypothermosol-free radical solution (HTR-FRS), or pseudo-lyte A (PLA). Images were taken immediately after 96 or 144 hours pausing (scale bar = 100 microns).

Figure 40: HOS TE85 cell morphology after pausing at 2–8°C for 96 and 144 hours in various media (n=3). HOS TE85 cells were seeded at 6.25x10^4 cells/cm^2 and cultured in normal culture conditions (37°C, 5% CO$_2$ and 95% humidity) for 24 hours. Media was then replaced with either standard MEM medium (MEM), standard medium with HEPES (MH), standard medium with HEPES and Trolox (MHT), Hypothermosol-free radical solution (HTR-FRS), or plasma-lyte A (PLA). Images were taken immediately after 96 or 144 hours pausing (scale bar = 100 microns).

In chilled conditions, all medium tested against standard MEM with no additional components significantly enhanced viability following pausing for 96 (figure 42A) and 144 hours (figure 42B) (p<0.001). No difference was found between the membrane integrity of cells when paused in MH or MHT. Pausing with HTS at 4°C yielded the highest viability (96 hours = 96% and 144 hours = 88%); viability was significantly improved at 86 hours compared to MH and PLA (p<0.05). The difference in membrane integrity between MH, MHT and PLA was considered non-significant.

5.2.4 HOS TE85 CELL FOLD GROWTH EXPANSION

Cells stored in standard MEM for 96 hours at ambient temperature were successfully passaged once and then failed to reattach and grow past P1 (figure 43A). With the addition of HEPES and HEPES and Trolox to the medium, cells were able to adhere to tissue culture plastic and progress through the cell cycle to proliferate and increase in number through subsequent passages (P2–5). There was no significant difference between the fold growth expansion of cells in MH or MHT medium. Interestingly, cells paused in the commercial medium HTS were unable to attach and proliferate past P1, likewise for cells in PLA. Following pausing for 144 hours, only media with the addition of HEPES was able to support cell attachment and growth for up to 5 passages (figure 43B). There was no significant difference in fold growth between cells in media with HEPES and media with HEPES plus Trolox. Cells in MEM, HTS and PLA were not capable of attachment and therefore very few or no cells were counted at P1 and hence no further passaging.

Cells kept at 4°C, regardless of pausing media produced a negative fold growth expansion number on P1 (<1) and therefore could be passaged no further (appendix B).
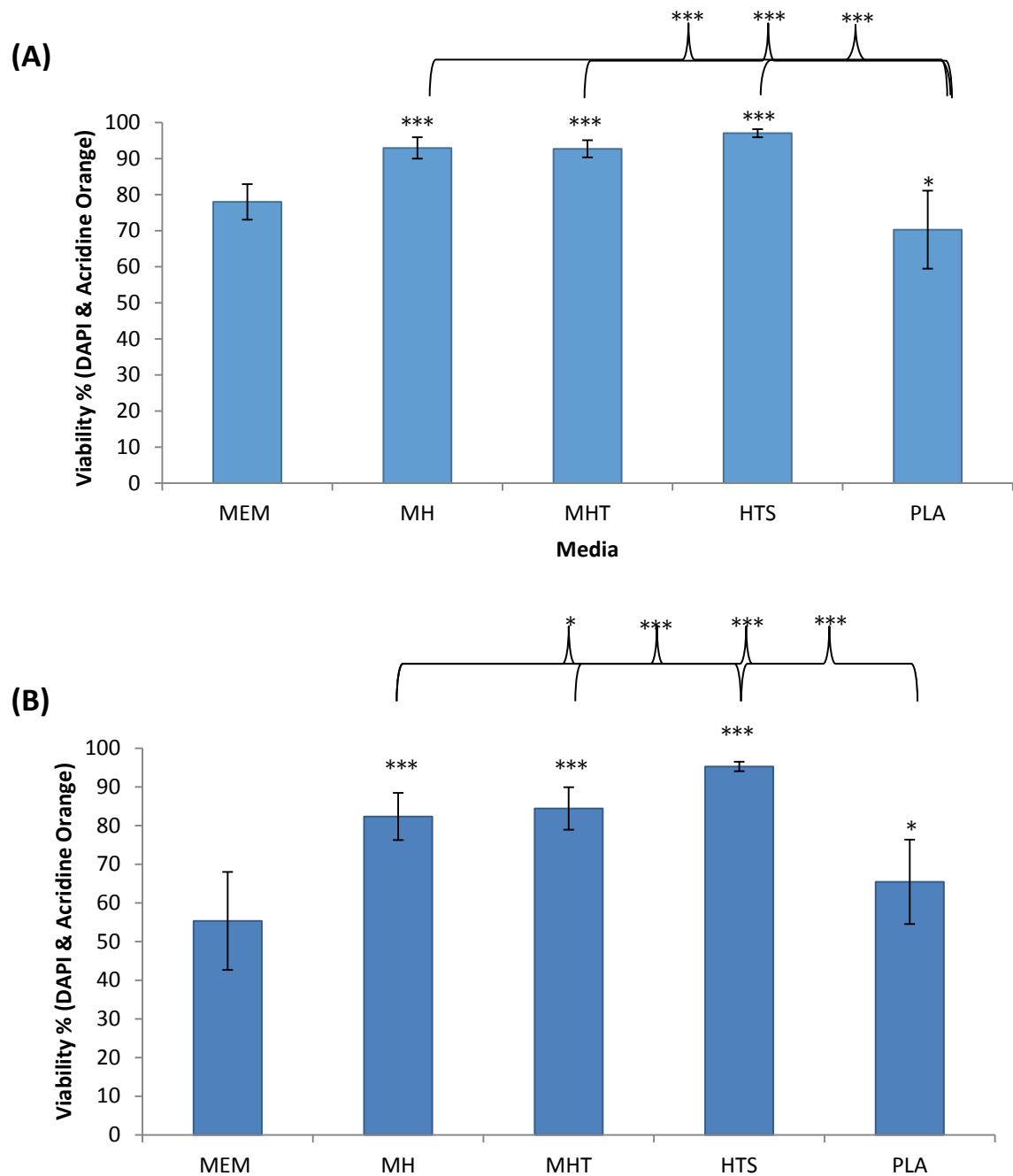
Figure 41: HOS TE85 cell viability (%) measured by DAPI/AO staining when paused at ambient temperature for 96 and 144 hours, in various media (n=3). (A) Cell viability immediately following a 96 hours pause **(B)** Cell viability immediately following a 144 hours pause. A one-way ANOVA was used to test for significance alongside Tukey's range test to determine which data points were significant. (* indicates $p < 0.05$; *** indicates $p < 0.001$). Stars show significance compared with the control (MEM) unless specified by brackets. Error bars show the standard deviation between independent experimental repeats.
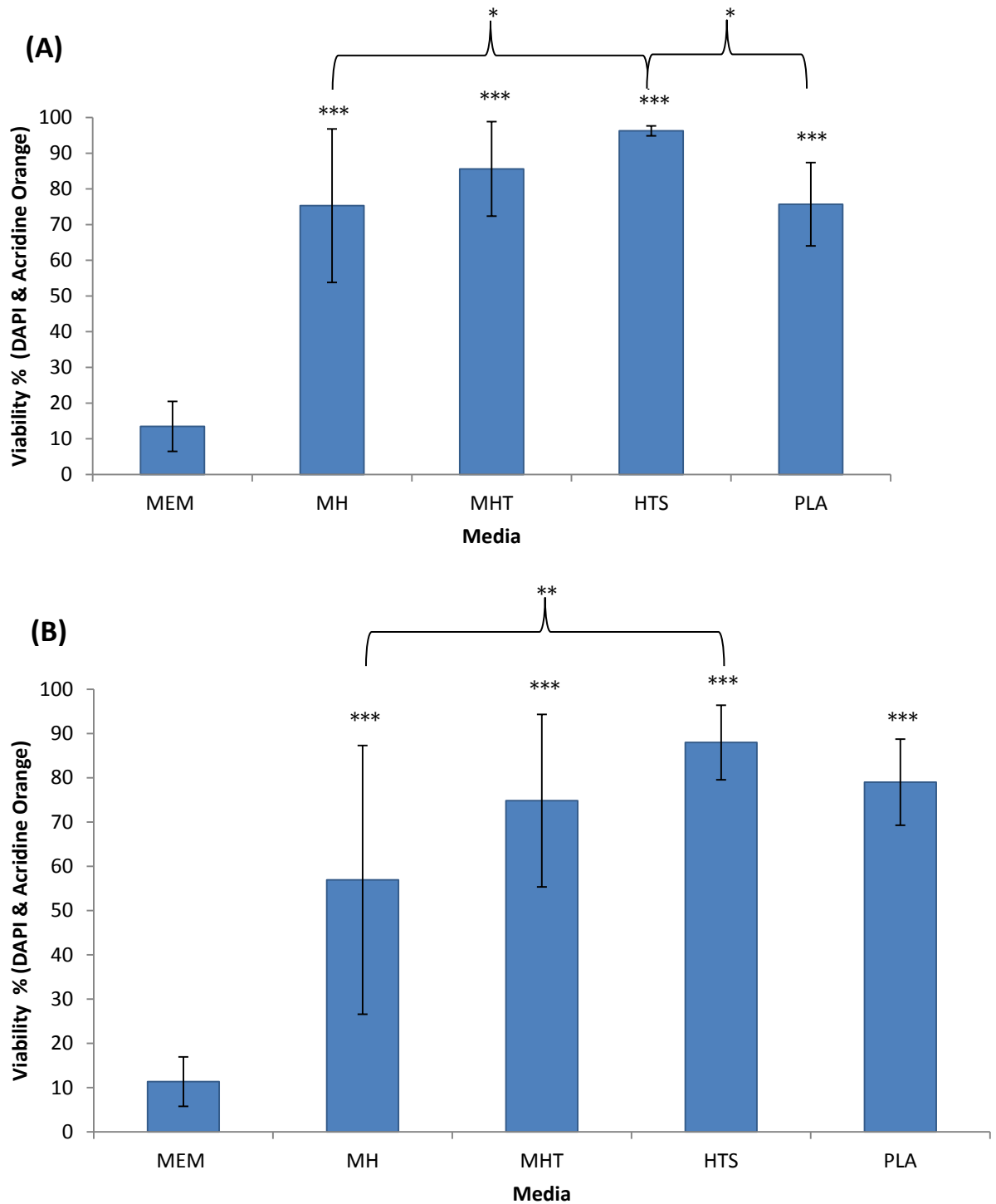
Figure 42: HOS TE85 cell viability (%) measured by DAPI/AO staining when paused at 4°C for 96 and 144 hours in various media (n=3). (A) Cell viability immediately following a 96 hour pause **(B)** Cell viability immediately following a 144 hour pause. A one-way ANOVA was used to test for significance alongside Tukey's range test to determine which data points were significant. (* indicates $p<0.05$; *** indicates $p<0.001$). Stars show significance compared with the control (MEM) unless specified by brackets. Error bars show the standard deviation between independent experimental repeats.

5.2.5 MEDIUM PH MEASUREMENTS

Medium pH was measured after 96 and 144 hours of cell storage at 4°C, ambient temperature and 37°C. For all three temperatures (figure 44A, 4°C; figure 44B, ~20°C; figure 44C, 37°C), pH increased from an average starting point of 7.4 to 9, 8.6 and 8.8, respectively, when cells were kept in MEM medium (figure 44). However, the media MH, MHT and HTS managed to maintain medium pH between 7.4 and 7.6 during 96 and 144 hours in ambient conditions. The pH change of the medium when HEPES buffer was added is clearly shown in figure 39A-B by the colour change of the medium (pink to orange); this was the case regardless of temperature. Similarly, medium at 4°C, with the addition of HEPES, kept pH rising above 7.6. HTS was also successful at maintaining a neutral pH at chilled temperatures. At 37°C, pH was kept slightly more acidic with MH, MHT and HTS at 7.2, 7.4 and 7.3, respectively. PLA, at all temperatures, lowered pH to an average of 6.7.
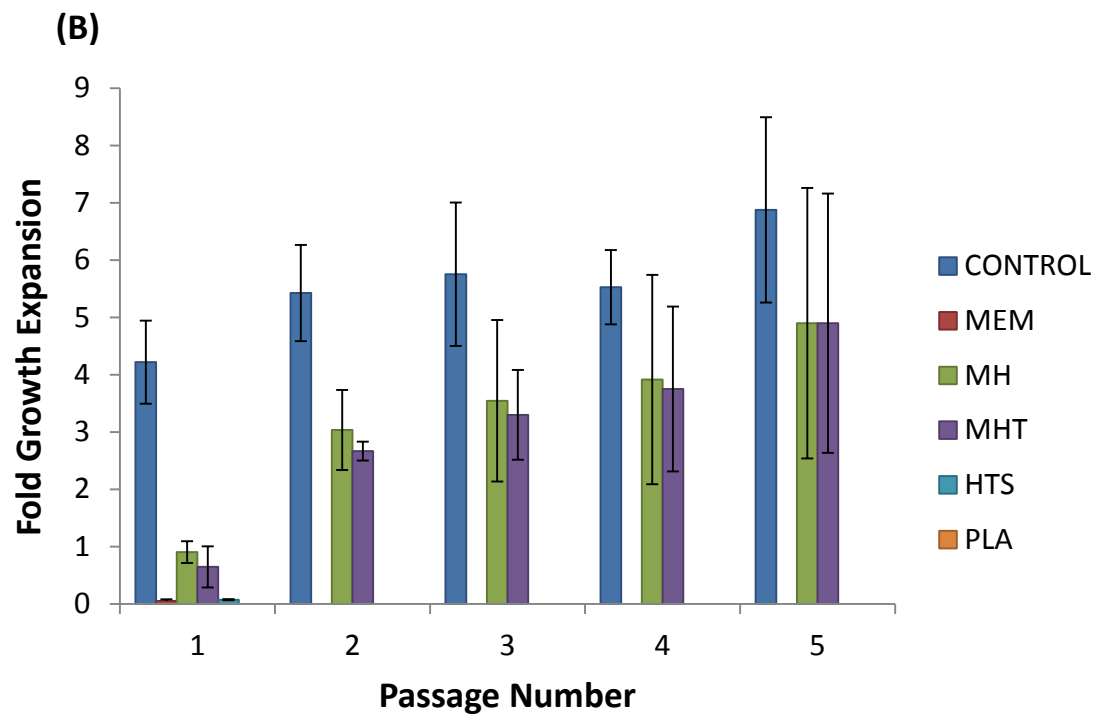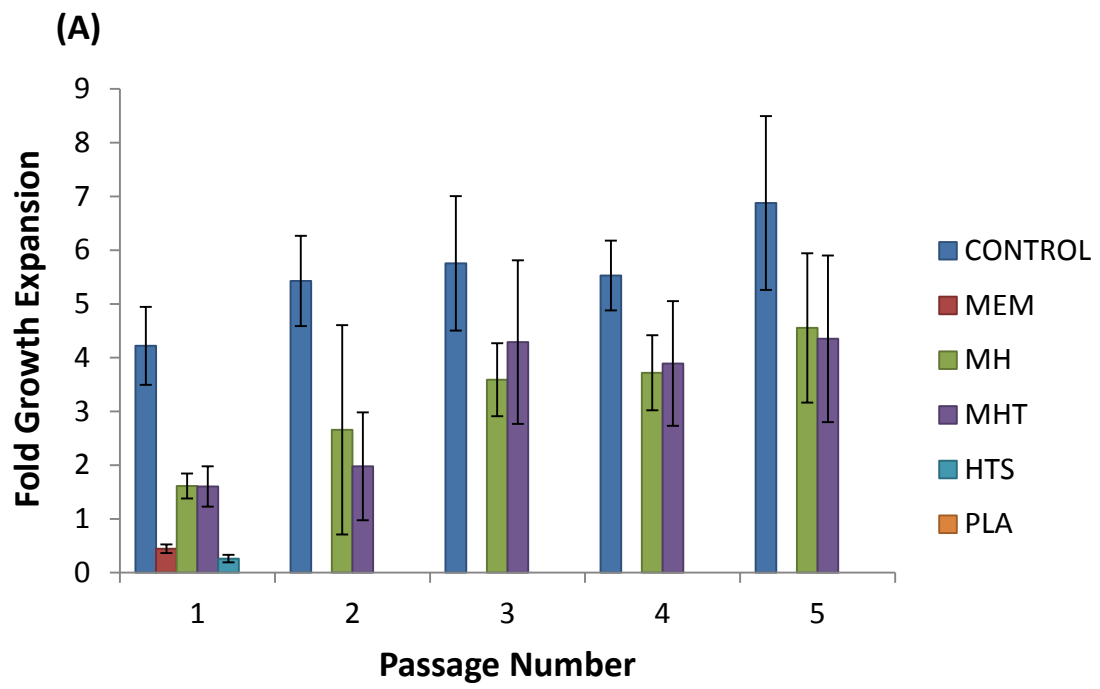
Figure 43: HOS TE85 cell fold growth expansion when paused at ambient temperature for 96 and 144 hours in various media (n=3). (A) Following a 96 hours pause **(B)** following a 144 hours pause. A three-factor ANOVA test was used to test for significance. Error bars show the standard deviation between independent experimental repeats.
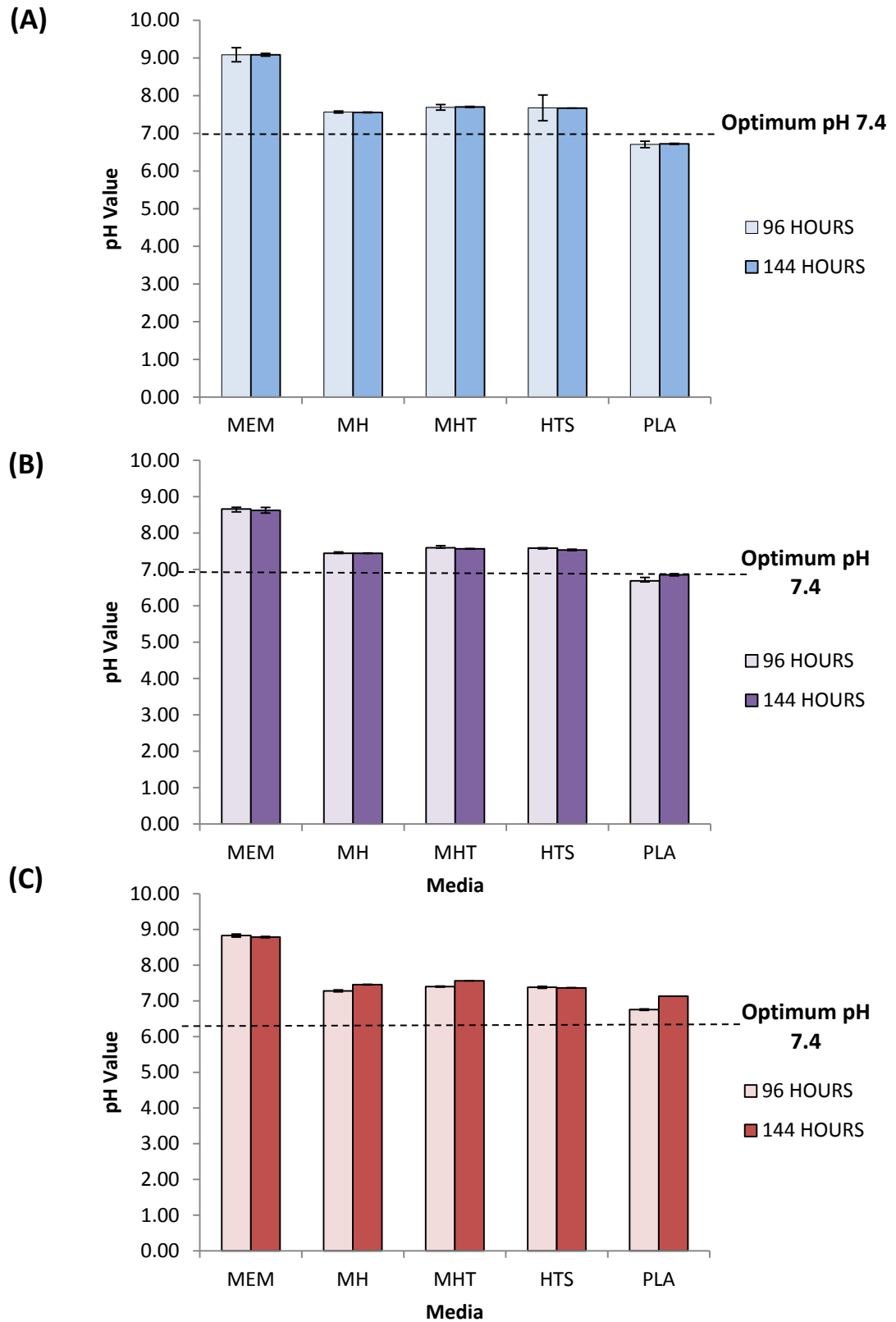
Figure 44: pH measurements following 96 and 144 hours of HOS TE85 cell pausing in various media (n=3). (A) Paused at 4°C **(B)** paused at ambient temperature **(C)** paused at 37°C. Error bars show the standard deviation between independent experimental repeats.

Results demonstrate the core importance of atmospheric control, in particular CO_2 and pH stability for successful cell pausing at ambient temperatures. In order to maintain ≥90% viability immediately after pausing, atmospheric control was crucial. This was highlighted again during fold growth expansion experiments when cells that had undergone pausing for 96 hours or more in standard MEM medium were unable to be passaged further than P1. As the buffering system in MEM medium uses sodium bicarbonate, which is dependent on CO_2 diffusion from the atmosphere, it works well in a controlled incubator; however, in ambient conditions pH becomes more alkaline (~8.6) and cells are less able to maintain ionic homeostasis, especially in hypothermic conditions where energy dependent systems slow down and cells are subject to cold induced damage. Recent results have suggested that alternative buffer systems such as HEPES may be the key to stabilising pH to physiological conditions (~7.4) during ambient temperature pausing. Considering the commercial medium HTS also uses HEPES as its main buffer system, it was a suitable choice for the hypothermic preservation of cells either at 4°C or ambient temperatures.

During ambient storage, the viability of HOS TE85 cells was significantly improved with the addition of HEPES to the medium ($p < 0.001$). The pH, instead of rising to above 8, remained at ~7.4 for both MH and MHT, which is recommended for optimal cell culture. This demonstrates just how crucial the stability of pH is during cell preservation and as explained in section 4.3.5, a pH of ≥8.2 is known to be toxic to cells by upsetting their ionic and oncotic balance and can result in death. It was hypothesised that cells may require additional support in terms of anti-oxidants to help quench the free radicals produced during the cellular stress that occurs during

pausing. Therefore, the synthetic anti-oxidant Trolox was added to MEM medium, along with HEPES. Although there was significant improvement in terms of viability and fold growth expansion with MHT compared to MEM without additional supplements, the difference between the MH and MHT was not significant. This highlights that the increased viability and the ability of cells to attach to a surface and repopulate is more than likely to be from the addition of HEPES alone and not the additional anti-oxidant. One reason behind this lack of effect could be the small concentration of Trolox used in experiments, 1µM. As the commercial solution HTS-FRS uses the same concentration, it was thought that this was an ideal amount of anti-oxidant to add to the medium, as high concentrations can have toxic effects on cells. It may be that the concentration of Trolox required for pausing in standard MEM needs to be higher to combat the stress experienced during ambient or chilled conditions.

From the viability results alone, HTS comes out on top in terms of preserved membrane integrity. The majority of cells in HTS during pausing for 96 and 144 hours were mostly attached to tissue culture plastic and although they appeared less elongated than control cells, they looked typically healthy with clearly outlined membranes. Interestingly, regardless of time paused, cells in HTS were unable to reattach to a surface and subsequently did not grow and proliferate. At P1, few live cells or no cells at all were counted due to being washed away during the passage procedure and were therefore not replated for further passaging. This was also the case for cells paused in PLA.

It seems that HTS, designed for storage at 2–8°C is inadequate for ambient temperature storage. However, what is of great interest is that cells paused at 4°C in

HTS lose their ability to adhere to a surface and proliferate when transferred back to normal medium and culture conditions. Whilst viability remains high after 96 and 144 hours pausing (>90%), cells are damaged in terms of their attachment efficacy. In fact, none of the media used to support pausing at 4°C were successful in retaining cells capable of attachment and proliferation past the first passage (see appendix B). One explanation for this could be the cell line used, HOS TE85. All cell types will perform differently under stress and cancer derived cell lines are known to behave differently to some primary cell lines (Strander, 1986). BioLife Solutions® have provided successful viability results for the preservation of human dermal fibroblasts in HTS for 5 days, which is the most relevant example for HOS TE85 cells but of course these cells are not cancer derived. Being of a cancerous phenotype and therefore robust in nature, it would be sensible to hypothesise that this particular cell line would be able to recover from hypothermic stress and this is true when stored in pH controlled medium. Since HTS is protein and serum free, this could be the reasoning behind the lack of ability to reattach to tissue culture plastic. While the mechanism is not fully understood, FBS is known to assist in cell growth and attachment. The change in medium, from MEM to HTS back to MEM, could also be an additional stress for the cells. Once they have adjusted to the ionic and oncotic environment of one cellular solution, changing to another will further affect the properties of the cell membrane and may lead to osmotic swelling and leakage. Hypothermia has also been shown to reduce the expression of β1 and β2 integrins, which bridge cell-cell and cell-extracellular matrix contact (Rowin *et al.* 2001). Overall, these findings further supports the use of standard medium with additives for the storage and transport of cells, as a change in medium formulation will affect cell acclimatisation. Although in

terms of morphology and membrane integrity cells may appear viable, this does not guarantee they will perform therapeutically when infused into the patient. This highlights the need for more potency assays and testing whether the cells can endure complete changes in medium or whether additional components to original medium may reduce the amount of stress a cell experiences during storage and transport.

Whilst PLA has its uses medically, in terms of membrane integrity and attachment it is the least favourable option in terms of the chilled and ambient pausing of HOS TE85 cells. Morphologically, the cells appeared to be clumpier in PLA than the other media used, possibly due to the use of human serum albumin (hSA) and a vast majority of cells were suspended. It is unclear why cells could not reattach to tissue culture plastic following pausing in this medium; however, the change in serum type could have affected the cells membrane and its ability to recover. PLA was made in-house and hSA was the choice of serum used at 5% (w/v), as this is consistent with other medical protocols; FBS would not be infused into a patient for ethical and regulatory reasons.

To summarise, it is clear that the addition of HEPES to medium is required for cells to be able to survive pausing in ambient conditions for up to 144 hours and ensures attachment and cell proliferation for multiple passages. The anti-oxidant Trolox at 1µM does not improve cell recovery any further than HEPES alone and thus will not be used for future experiments. The concentration needed to elicit the desired effect would have to be optimised accordingly. Immediately after pausing cells in HTS, cells appear healthy from their morphology and viability however, this success is not followed through to cell attachment and proliferation, possibly due to the effects of a

complete change in medium. Pausing in PLA was not as effective in terms of cell viability and attachment compared with the other media tested in these experiments.

5.3 HOS TE85 CELL ADAPTATION TO PAUSING IN SUSPENSION

One of the main challenges for using adherent cell types for cell therapy is the difficulty in achieving a single cell suspension. With the exception of HSC, most cells are anchorage-dependent and need to be cultured on a surface, which allows for cellular attachment and spreading. For research purposes, culturing cells in a monolayer is useful, as it allows for easy morphological assessment and attachment tests can be considered a measure of viability. In a clinical setting however, cells will need to be detached from a surface, in a single cell suspension, so they are ready for patient administration. So far, experiments have been performed on cells attached to tissue culture plastic as a monolayer and it is unknown how these cells will tolerate the stress of pausing with the additional stress of being suspended in medium. Being adherent in phenotype, attached cell lines have a tendency to stick to each other, forming clumps or aggregates of varying size. The number and size of these aggregates will cause areas for concern from a regulatory point of view, with a risk of pulmonary embolism, most recently demonstrated in patients after intravenous delivery of adipose-tissue derived stem cells (Jung *et al.* 2013). Due to these concerns, it was important to assess how the typically adherent HOS TE85 cells would cope when forced to stay in suspension rather than attached to a flat surface. Firstly, cells were stored in suspension for between 24–48 hours at various temperatures to examine how these cells respond and recover when not attached to a surface. Secondly,

methods for achieving and maintaining a single-cell suspension were explored and discussed.

5.3.1 OVERVIEW OF EXPERIMENTAL PROCEDURE

Cells were seeded into ULA plates (6.25×10^4 cells/cm^2) in standard MEM medium and stored for between 24–48 hours at 4°C, ambient temperature or 37°C, with no atmospheric control. ULA plates have a covalently bound hydrogel layer which acts to deter cell attachment (SigmaAldrich, 2016). Cells were assessed morphologically during and post-storage for up to 48 hours. Viability in terms of membrane integrity was also examined after stored cells were transferred to normal tissue culture plastic and cultured in normal culture conditions for 24 hours.

5.3.2 HOS TE85 MORPHOLOGY

Cells kept at 4°C harboured the least amount of clusters or aggregates (figure 45A) compared with the other temperatures; these cells were also not very efficient in terms of cell attachment upon recovery (figure 45B). The cells that were capable of attachment however, did proliferate and following 48 hours of normal culture, 50% confluence was reached (figure 45C). When kept at ~20°C for 24 hours, the cells formed grape-like clusters (figure 45D), but were not as tightly compact as cells kept at 37°C. Cells in ambient conditions also formed dense circular patches when reseeded back onto tissue culture plastic (figure 45E) and 70–80% confluence was reached after 48 hours of normal culture (figure 45F). During storage in suspension at 37°C for 24 hours, tightly compact clusters of cells can be observed (figure 45G). When transferred back to normal adherent well-plates, these aggregates settled and attached to the surface of the plate. Following 24 hours recovery in normal culture conditions, the

cells had spread out resulting in circular clusters of cells in a monolayer (figure 45H).

After 48 hours, the cells had spread further, resulting in 90–100% confluence and no

obvious dense patches of cells (figure 45I).
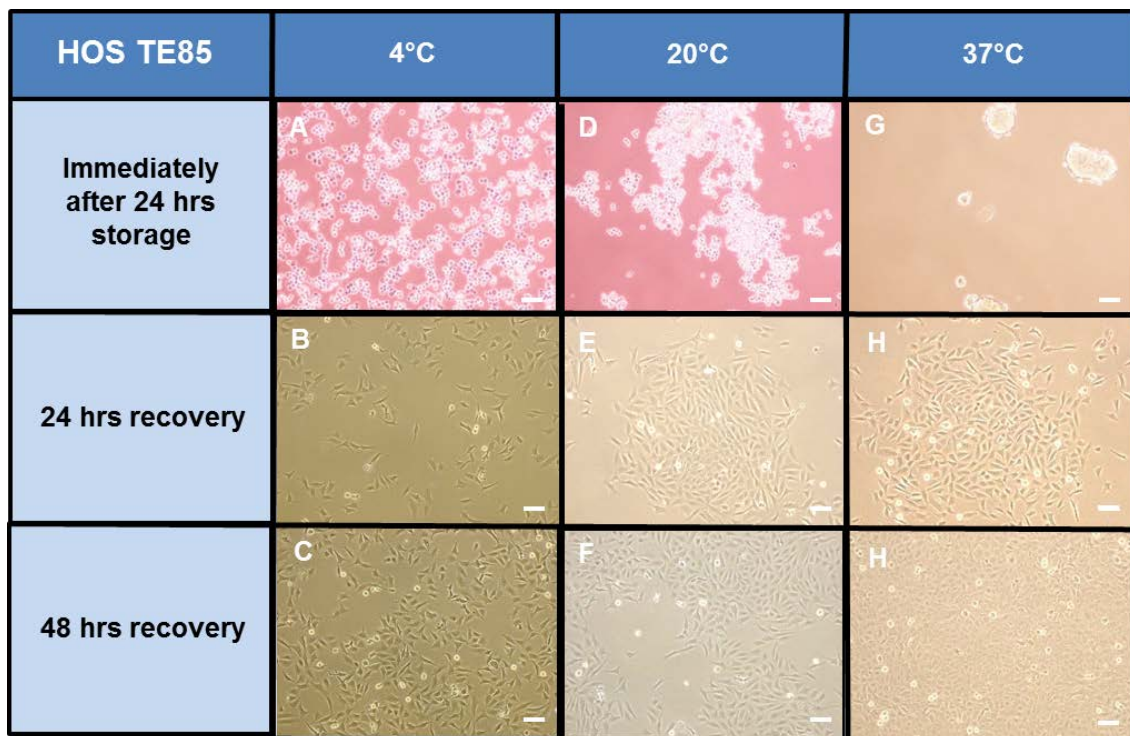
Figure 45: HOS TE85 cells stored in ULA plates at 4°C, ambient temperature and 37˚C (no atmospheric control) for 24 hours and recovery following normal culture for 24-48 hours (n=3). Cells were cultured in ultra-low attachment plates and images were taken immediately after 24 hours storage at each temperature and at 24 and 48 hours post-storage in normal culture conditions (37°C, 5% CO_2 and 95% humidity). Scale bar = 100 microns.

5.3.3 HOS TE85 VIABILITY

In terms of intact cell membrane integrity (figure 46), measured via trypan blue staining, there was no significant difference between the percentage viability of cells in ULA plates before or after pausing for 24 hours at 4°C, ~20°C or 37°C (86%, 96% and 83%, respectively). Storage for 48 hours, at all temperatures, significantly reduced viability compared with that measured before pausing, although no significance was observed between ambient temperature and 37°C. On the other hand, following 48 hours at 4°C, membrane integrity was significantly reduced compared with when cells were stored at warmer temperatures. To summarise, cell membrane integrity is lost when paused for ≥48 hours at 4°C, ~20°C or 37°C, which is similar to results with adhered HOS TE85 cells.
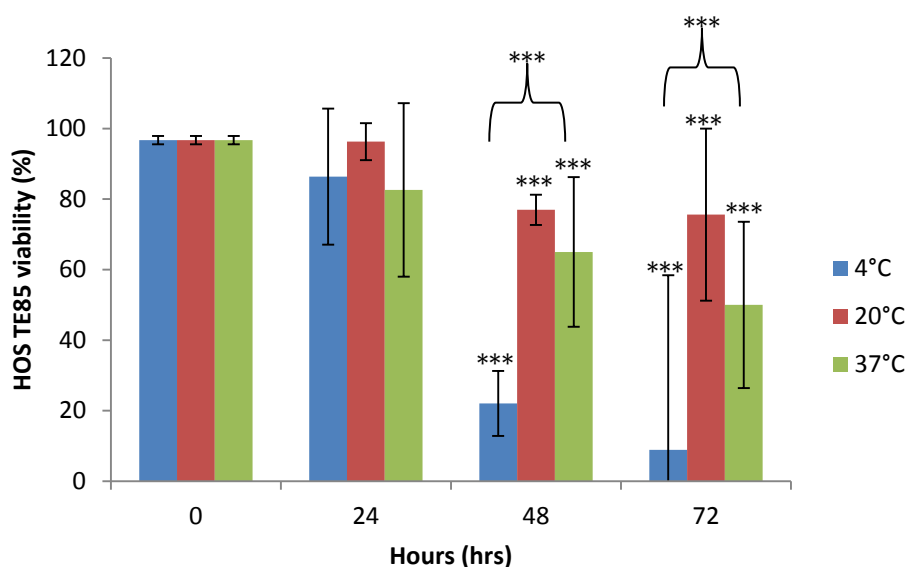
Figure 46: HOS TE85 cell viability, following storage in suspension at 4°C, ambient temperature and 37°C, for up to 48 hours with no atmospheric control (n=3). Cells were cultured in ultra-low attachment plates and kept at various temperatures for 24 and 48 hours. Viability (via Trypan blue exclusion) was determined after transfer to normal tissue culture plastic and 24 hours of normal culture at 37°C, 5% CO_2 and 95% humidity. A two-way ANOVA was used to determine significance alongside Tukey's range test. (***indicates $p < 0.001$). Stars show significance for all three temperatures compared with the control (0 hours) unless specified by brackets. Error bars show the standard deviation between independent experimental repeats.

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5.3.4 DISCUSSION

Results show that HOS TE85 cells recover well from pausing in suspension for 24 hours, whether kept at 4°C, ~20°C or 37°C (viability ≥83%). This is a promising result, as it demonstrates that not only can HOS TE85 cells recover from the stresses of reduced temperature and lack of atmospheric gaseous control, but they can survive the additional trauma of being maintained in suspension. At ~20° and 37°C, morphological assessment (figure 45) showed the aggregates were able to settle and attach to tissue culture plastic, once under normal culture conditions. These aggregates formed circular dense patches of cells on the surface, similar in appearance to bacteria colonies. The occurrence of these colonies could be attributed to the 3D structure of the aggregate; when settled onto the surface, the cells did not initially form a monolayer as they were laying on top each other. The cells then migrated away from the dense centre of the aggregate, and after 24 hours, the cells managed to form a monolayer of circular patches. Following a further 24 hours, the cells became more spread out and confluent. The significant reduction in viability following storage at 48 hours is similar to that observed with adhered HOS TE85 cells; membrane integrity is reduced with an increased pausing time. The reasoning behind the lack of aggregate formation when cells were kept at 4°C could be explained largely by the effects of low temperatures on cell membrane stability and consequently their ability to adhere to other cells. To avoid cell clumping for flow cytometry procedures, it is recommended to keep cells between 0–4°C (Giloh, 1993) and this is the general consensus for managing the formation of aggregates in the cell biology community.

It seems that cellular responses to temperature in terms of aggregation are highly dependent on cell type. Red cell aggregation is favoured by lowering the temperature

(Neumann *et al.* 1987), which is explained by their increased resistance to hydrodynamic dispersion and increased blood viscosity at reduced temperature (Lim *et al.* 2010). Retinal cell aggregation is more effective at room temperature (Burger, 1977) and the level of protein aggregation is known to be elevated at higher temperatures due to the increased level of unfolded proteins and strength of interactions (Gramer, 2014). In 1976, Llyod, *et al.* found that fibroblast aggregates are smaller and looser at colder temperatures e.g. 2°C, than aggregates in warmer temperatures. The study went on to explain that fewer gap and adherens-type junctions are made between cells in the cold and concluded that although gap junctions are capable of maintaining the cohesion of aggregates, for more tightly compact spheroids, an energy-dependent process whereby the cytoskeleton becomes organised at localised regions of the plasma membrane is required (Llyod *et al*. 1976). Due to the energy dependency of this process, this may explain the more compact aggregates observed at the higher temperature of 37°C.

To summarise, an increase in temperature leads to increased cell aggregation in HOS TE85 cells, with tightly compact spheroids at 37°C. Upon recovery in normal culture conditions, cells kept at ambient or 37°C were able to reattach to a surface, proliferate and obtain 70–100% confluence after a couple of days in normal culture conditions. Cells kept at 4°C however, had less evidence of clumping but it seems the severe temperature drop had affected their ability to recover in terms of adherence capability and cells may have died by the phenomenon of anoikis due to loss of cell-cell, cell-matrix interactions. Nevertheless, the few cells that did attach were able to adhere

and proliferate. These findings only strengthen the reasoning behind researching ambient temperature pausing for cell applications.

5.4 MAINTENANCE OF A SINGLE CELL SUSPENSION DURING PAUSING

As shown in section 5.3, the formation of cell aggregates increases with temperature. As the size and number of cell aggregates formed at ambient temperature may cause regulatory concerns and possibly impact the functionality of the cells themselves, it was important to investigate if the HOS TE85 cells could be forced into a single cell suspension with the addition of an anti-clumping agent. Several agents, with known anti-clumping properties, were chosen for investigation:

- The polyethers PEG and PPG were chosen as potential anti-clumping agents due their history in the medical, food and pharmaceutical industry as well as their known usage as a surfactant. When these substances are absorbed or covalently linked to a cell surface, they repel cell aggregation by steric and electrostatic force (Hui *et al*. 1999).

- A chemically defined formulation with the name 'anti-clumping agent' from Gibco® was tested to reduce cell clumping in suspension culture, as Gibco® claim its composition is a more gentler approach than the harsh enzymes usually associated with cell disassociation products. Further to this, Gibco® anti-clumping agent is devoid of animal components; this avoids the addition of adventitious agents to the cell suspension and limits batch to batch variations.

- P68 is a non-ionic surfactant, free of animal components (ThermoFisher Scientific, 2016), which is generally used to control and protect against shear

forces produced during gas sparging in large-scale mammalian cultures (Gigout *et al.* 2008). Due to its demonstrated use in biotechnology and its manufacture in a cGMP compliant facility, it was an ideal candidate to test for obtaining a single cell suspension with HOS TE85 cells.

- EDTA is widely used a cell disassociation reagent and is also known for its uses as an anti-clumping agent. EDTA works as a calcium chelator; cell adhesion molecules such as integrins rely on calcium to make cell-cell, cell-cell matrix interactions (Sjaastad and Nelson, 1997).

- Benzonase® Nuclease was also chosen as potential anti-clumping agent for HOS TE85 cells. Benzonase is a genetically engineered endonuclease from the bacterium *Serratia marcescens* (Sigma-Aldrich, 2016). When cells lyse due to cell stress, DNA is released due to overdigestion with proteolytic enzymes; this endonuclease acts to remove these sticky DNA and RNA components and subsequently helps prevent clumping.

Tween-80, a polyethylene sorbitol ester, which has been widely used in biochemical applications as a surfactant and for emulsifying and dispersing substances in the medical and food industries, was also considered for its anti-clumping properties. However, due to its effects on HOS TE85 cell morphology, it was disregarded for future experiments (see appendix C).

5.4.1 OVERVIEW OF EXPERIMENTAL PROCEDURE

Firstly, various concentrations of the anti-clumping agents, described in table 6, were tested for their toxicity and anti-clumping effects by the addition of the agents to MEM medium during adherent HOS TE85 cell culture for 24 hours (appendix C).

Changes in morphology compared to the control (MEM with no additional anti-clumping agent) were used to help decide on the best high and low concentration of each anti-clumping agent to use in further experiments. The chosen concentrations are detailed in table 7, and these were added separately to standard MEM medium, to see if the number and size of clumps formed at ambient temperatures in suspension could be reduced. Cells were prepared as described in section 3.5.1. Once the centrifugation step was reached, media was aspirated away and cells were quenched in MEM medium with one of the additional anti-clumping agents and seeded (6.25×10^4 cells/cm^2) into 2 x wells of ULA plate. The cells were paused for 24 hours at 4°C and ambient temperature. Storage at 37°C was not tested in these experiments as cells would not be 'paused' and transported at this temperature. Following storage, cell morphology was examined as well as cell membrane integrity and the average number of aggregates formed, measured by the NC-3000.

5.4.2 HOS TE85 CELL MEMBRANE INTEGRITY

Figure 47 shows the viability of HOS TE85 cells following 24 hours of pausing or culture in the various anti-clumping agents. No significant reduction in viability was found between cells stored at ambient temperature in any of the anti-clumping agents (figure 47A) compared with the control (MEM medium). Cells kept at 20°C had ≥90% viability except 2% polypropylene glycol. At 4°C, (figure 47B) the only agent to have lower viability than the control is the low concentration of EDTA (0.1mM).

(A)

(B)
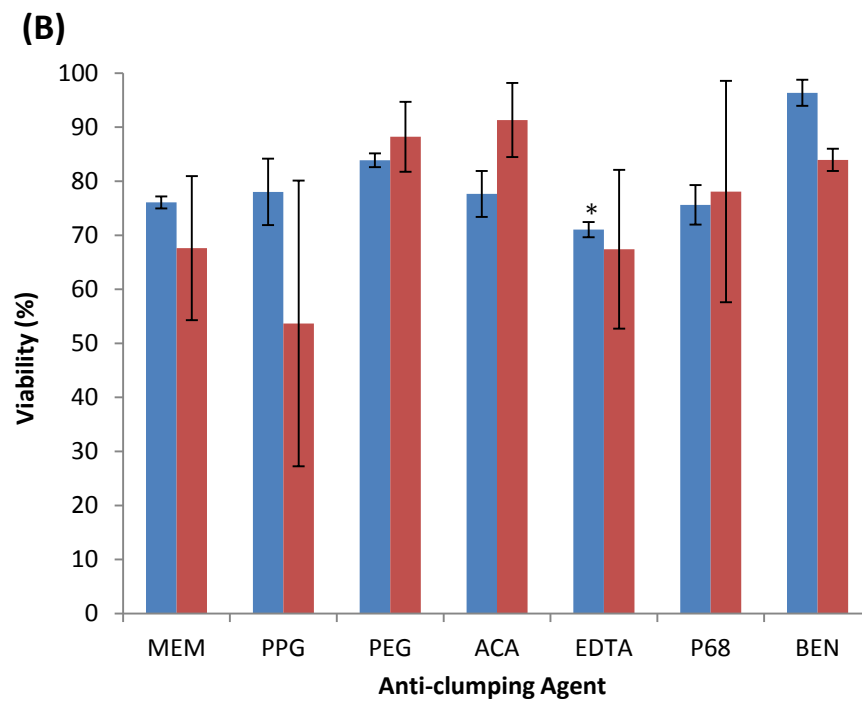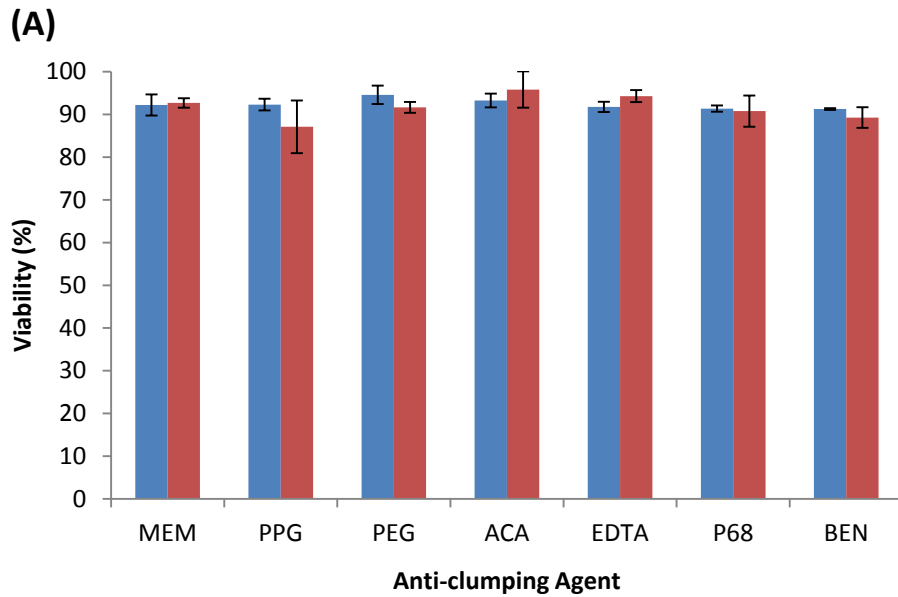
Figure 47: HOS TE85 cell viability determined by DAPI and acridine orange staining when paused for 24 hours at ambient temperature and 4°C with a high and low concentration of various anti-clumping agents (n=3). (A) Cells paused in ambient conditions **(B)** Cells paused at 4°C. Anti-clumping agents included polypropylene glycol (PPG), polyethylene glycol (PEG), anti-clumping agent (ACA), ethylenediaminetetraacetic acid (EDTA), pluronic-F68 (P68), benzonase® nuclease (BEN) and standard MEM medium with no added agent which acted as the control. Blue bars indicate the low concentration used and the red bars indicate the high concentration. A two-way ANOVA was used to determine significance alongside Tukey's range test. (*indicates $p < 0.05$). Stars show significance compared with the control (MEM). Error bars represent the standard deviation between experimental repeats.

(A)

(B)
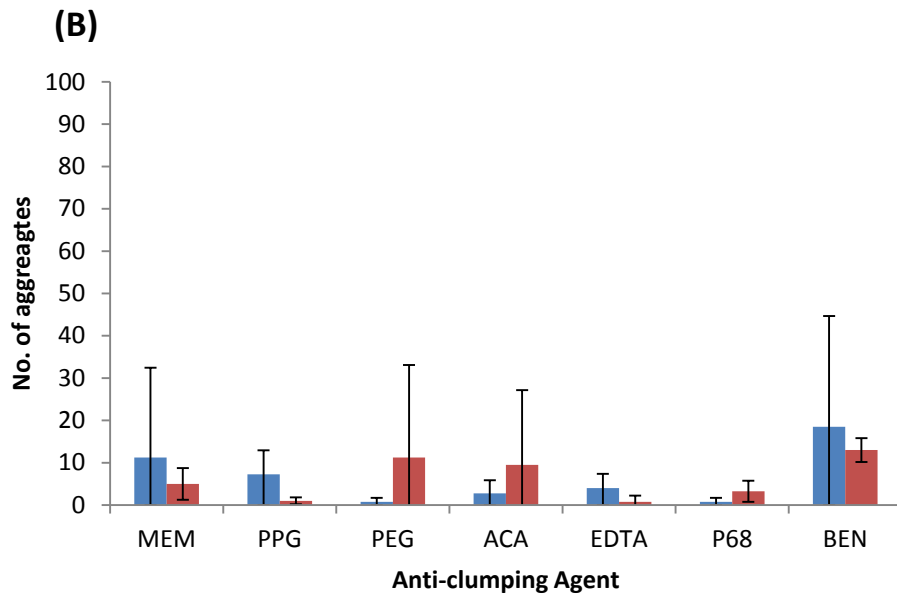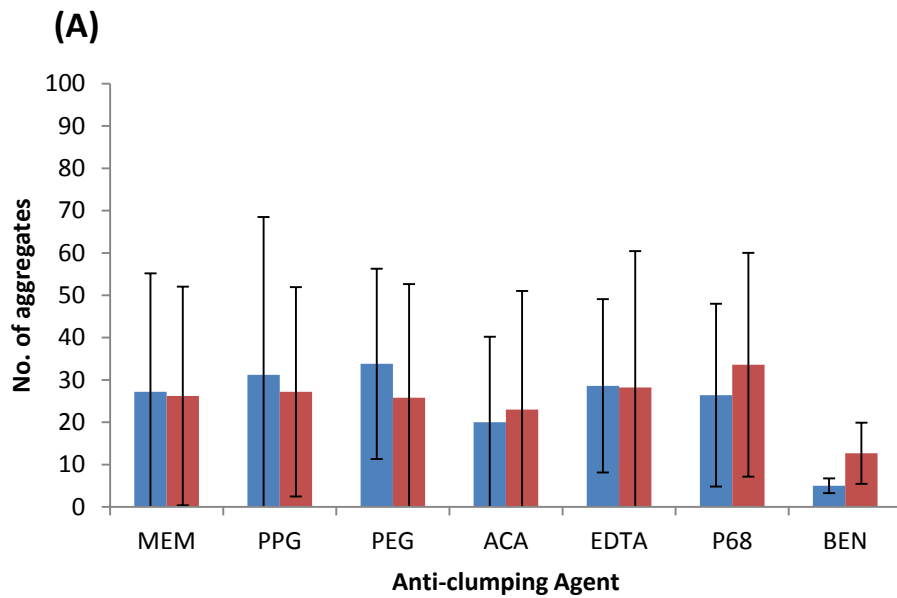
Figure 48: Number of HOS TE85 aggregates determined via the NC-3000, when paused for 24 hours at ambient temperature and 4°C with a high and low concentration of various anti-clumping agents (n=3). (A) Cells paused in ambient conditions **(B)** Cells paused at 4°C. Anti-clumping agents included polypropylene glycol (PPG), polyethylene glycol (PEG), anti-clumping agent (ACA), ethylenediaminetetraacetic acid (EDTA), pluronic-F68 (P68), benzonase® nuclease (BEN) and standard MEM medium with no added agent which acted as the control. Blue bars indicate the low concentration used and the red bars indicate the high concentration. Error bars represent the standard deviation between experimental repeats.

5.4.3 EFFECT OF VARIOUS ANTI-CLUMPING AGENTS ON HOS TE85 CELL AGGREGATION

The average number of HOS TE85 cell aggregates after pausing for 24 hours with various anti-clumping agents is shown in figure 48, estimated by the NC-3000. As the variation between experimental repeats was so large, no statistical analyses could be performed on these data. What can be drawn from these results however, is a trend towards increased cell aggregation at ~20°C (figure 48A) compared with the average numbers at 4°C (figure 48B).

5.4.4 DISCUSSION

When considering the logistics of cell storage and transportation, it is important to acknowledge that cells will need to be distributed in suspension. The reason for this is to ease the transportation process and to maximise the number of cells that can be delivered. This introduces a challenge for the shipment of cells as most therapeutically relevant cells are adherent in nature. As demonstrated in figure 45, the amount of clumping with HOS TE85 cells increased with temperature. At ambient temperature, if cells are unable to attach to tissue culture plastic, as in the ULA plates, they tend to attach to each other forming cell aggregates. Cell aggregation may be problematic during the administration of a cell product into a patient, as cell clumping has been associated with pulmonary embolism following cell therapy (Jung *et al.* 2013). In contrast, there is evidence that the delivery of MSCs in aggregate form prolongs cell survival once administered into the patient. The formation of 3D aggregates in this sense has been termed microenvironmental preconditioning and is thought to be a promising strategy to reduce cell apoptosis once *in vivo*, whilst maintaining cellular function (Sart, *et al.* 2014). Although research in this area is minimal due to the ethical

issues and need for human participants, it is important to investigate the potential of cell aggregate inhibition at ambient temperatures so at least an element of control can be introduced if required.

Least aggregation was observed when cells were kept at 4°C, irrespective of anti-clumping agent used, which may be why most cell pausing experiments in the literature have focused on pausing temperatures between 2–8°C. In 1965, Moscona explained that the formation of cell clusters declines at lower than optimum temperature (Moscona, 1965); at 25°C, neural retina cells and liver cells form minute clusters or a loosely cohering mass, similar to findings here.

The main point to highlight is the large variability in the number of aggregates between the three experiments. No statistical significance could therefore be drawn from these results. This indeed could have been attributed to user variability and it is possible that the level of cell dispersion by pipetting cell samples could have been of greater force during one experiment compared with another. Additionally, the variability encountered between experiments could have resulted from the method utilised by the NC-3000, which captured 13 random images of the cell sample on the glass slide. The distribution of aggregates may not have been equal and it may have been that the aggregates were confined to one area of the slide, skewing the true average number counted by the machine.

Although results so far have been unsuccessful in finding a suitable anti-clumping agent at ambient temperature which significantly minimises the formation and size of aggregates, it is clear from literature based evidence that cell clumping is highly dependent on cell type. As will be shown from results later in chapter 6, hMSCs do not

demonstrate the same level of clumping and aggregation in ambient conditions as the HOS TE85 cells. The purpose of osteoblast cells is to secrete and mineralise bone matrix to form new bone (NIH, 2016) which involves compaction, essential for bone hardness and strength. This inevitably requires cells to become in close in proximity with each other, which may explain their increased level of cell aggregation compared to the unspecialised hMSCs. It may be that a higher concentration of anti-clumping agent would be required for HOS TE85 cells to be stored successfully as a single cell suspension at ~20°C. Alternatively, due to the aggregates at ambient temperature only being loosely tied together, it may be that a small drop in temperature e.g. 15–18°C, is all that is needed to inhibit aggregate formation. Furthermore, from a clinical point of view, delivery of loosely attached cells through a needle and the shear stress associated with extensional flow (Aguado *et al.* 2012) may also help with cell dispersion upon delivery.

There are a variety of reasons why the anti-clumping agents tested here might have been unsuccessful at significantly reducing the amount of cell clumping during pausing at 4°C and ~20°C. Changes in pH may significantly affect the ability of the anti-clumping agents to work effectively. Most tried and tested methods to prevent cell aggregation have done so under normal culture conditions, usually in stirred tank bioreactors, where the pH is generally ~7.4. PEG solutions are known to be more stable at 4°C than those stored at ambient temperature (Brownstein, 1982) and do not considerably degrade with changes in pH. However, degradation is more prominent at warmer temperatures (Brownstein, 1992) which could explain the lack of effect of PEG at ambient temperature, and similarly for PPG. ACA is stated to be effective between

pH 6–8 and BEN and EDTA at pH 8; however, under ambient conditions it cannot be guaranteed that pH will not rise above 8 and is shown here to reach pH 9 (figure 44). This elevation may hinder the clumping prevention properties of these solutions. As is a running theme throughout this project, stable pH is of vital importance, not only for cellular health but also for the effectiveness of any additional components to cell media.

The recommended working concentration of P68 to use in culture is 0.1%, which was used throughout experiments. There is evidence that P68 can be incorporated into the cell membrane, demonstrated by measuring the uptake of a fluorescent derivative of P68 (Gigout *et al.* 2008). The level of uptake may affect the concentration of P68 in the cell culture medium and impact on its shear stress protection capabilities. Experiments using a slightly higher concentration of P68 in order to counteract the amount taken up by the cell would be required to test this hypothesis.

The size of a spheroid or aggregate is of great importance. Alongside the risk of vessel blockage upon administration, the larger a cell aggregate is, the harder it gets to supply oxygen and nutrients to the inside of the aggregate and for waste products to be removed. In 2014, FDA guidance for the development of therapeutic protein products was to strive to achieve aggregates of between 0.1–2 microns in diameter (FDA, 2014). There has since been no further guidance from the FDA on what size aggregates would be acceptable to deliver intravenously into a patient receiving cell therapy. The average size of single HOS TE85 cell determined by measurements from the NC-3000 throughout experiments is between 13–14 microns and as shown in figure 45, the size and compactness of osteoblast aggregates increases considerably

with temperature. Recent studies to improve stem cell survival and therapeutic action after transplantation have indicated that the formation of 3D MSC aggregates can lead to improved cell delivery, *in vivo* retention and hence function. Whilst cell aggregates may offer some form of protection during delivery and may help with differentiation capacity and therapeutic action, evidence of immunogenicity and embolism is still a risk and most probably dependent on aggregate size and number. There is a need for clarification of what size aggregates will be safe for cell therapy administration and how the level of aggregation can be controlled. Whilst only acknowledged here, maintaining a single cell suspension or aggregates of an approved size is an area in need of much research.

Factors which regulate cell aggregation can include cellular parameters such as the type of cell and its function, its developmental state and cell number as well as environmental influences such as the size and shape of the vessel the cells reside in, the composition of the medium and temperature. It was hoped that with an element of control of environmental factors, such as the addition of an anti-clumping agent, the amount of aggregation of HOS TE85 cells could be regulated. Results indicate that further research and experimental work would be needed, tailored to cell type, for the successful single cell suspension of cells paused at ambient temperature.

5.5 CONCLUSION AND OVERALL SUMMARY

The need to transport cells from manufacturing site to medical facility has paved the way for the commercialisation of specialised biopreservation media. While there are no media available specifically tailored for ambient temperature storage, it was important to test options ready available alongside standard media with additives.

Results demonstrated that for ambient temperature pausing, rather than the commercial media available such as HTS-FRS, the addition of a buffer to standard MEM is sufficient to obtain higher viability post-pause and for the cells to adhere to a surface successfully and continue to grow. This signifies the importance of pH stability, which might not be appreciated due the logarithmic scale used to measure changes. The logarithmic scale can give false indications over what might seem a minor deviation, for example, a change of pH 1 translates to a 10-fold change in H^+ concentration (Swain, 2010). The addition of the buffer HEPES counteracts these changes and will therefore be used in the composition of pausing medium for hMSCs in chapter 6. Since there was no significant impact of the addition of the anti-oxidant Trolox, this will not be used in further experiments. The use of different concentrations of Trolox during pausing will be an important avenue to investigate for future studies. It was interesting that the commercial media tested in pausing experiments were unsuccessful for improving cell survival post-pause, especially HTS-FRS at 4°C. As explained in section 5.2.6, there is a high possibility that the complete medium change from MEM to HEPES and back to MEM may have severely impacted cell osmolality.

In terms of a single cell suspension for HOS TE85 cells, there was too much variability between experiments to draw a conclusion. However, what has been highlighted is that it may not be the amount of aggregates that is most important but the size and level of compactness of the aggregates. There are currently no guidelines, in terms of cell therapy, for what size aggregate is safe to deliver to a patient. Whilst the delivery of cell aggregates has been known to be a health risk, there is conflicting evidence that

suggests aggregation may improve cell survival and therapeutic action once *in vivo*. Clearly, there are many routes for examination concerning cell aggregation, and size and prevalence is also dependent on cell type. Whilst only touched upon here, it was important to acknowledge cell clumping during pausing and to highlight that when applied to a therapeutically relevant cell type such as hMSCs, the level of aggregation must be monitored throughout storage, recovery and administration.

CHAPTER 6: APPLICATION OF AMBIENT TEMPERATURE PAUSING TO HMSCS

6.1 INTRODUCTION

This chapter is focussed on the application of what has been learnt from the osteoblasts to the more therapeutically relevant hMSCs. Although the HOS TE85 cells have been a good model to test if ambient temperature pausing was a viable option for cells, they are no doubt extremely different in phenotype and function compared with hMSCs. As HOS TE85 cells have a cancerous make-up, they divide indefinitely and this may give them an element of robustness in response to cold stress. On the other hand, hMSCs are complex cells and their genetic stability is crucial for their clinical use as stem cells (Estrada *et al.* 2013), which together with their limited availability renders them more precious than cancerous cell lines. This reiterates why experiments so far have used the osteoblasts as a model, to gain a further understanding of cell pausing. Human bone marrow derived MSCs are the most commonly used stem cell in cell therapy studies and clinical trials today (Culme-Seymour *et al.* 2012; Heathman *et al.* 2015). Therefore, short-term pausing of hMSCs will be thoroughly examined throughout this chapter to assess the feasibility of this process for cell therapy applications.

Throughout experiments so far, we have learnt that the osteosarcoma derived cell line, HOS TE85, can remain viable following up to 96 hours pausing in ambient conditions. The key points from the osteoblast pausing experiments were the importance of a stable pH, maintained by an additional buffer system, and the sensitivity of cells to osmotic stress during uncontrolled conditions. These findings have led to the use of

standard DMEM medium plus additives, including HEPES, for subsequent hMSC pausing experiments, rather than commercial media.

There has been some degree of research, which examines the chilled temperature storage of stem cells, using temperatures between 2–8 °C (Heng, *et al.* 2006; Ginis, *et al.* 2012). Only a handful of studies have focussed on the ambient temperature storage of stem cells (Heng, *et al.* 2006; Chen, *et al.* 2013; Swioklo, *et al.* 2016), and even fewer of these have looked past initial viability to study the longer term effects of low temperature preservation on cellular health.

The closest research to what is being explored here is work that was published by Swioklo and colleagues, early in 2016; their research demonstrated the successful preservation of human adipose derived stem cells via alginate-encapsulation for 3 days in hypothermic (0–32°C) conditions (Swioklo *et al.* 2016). Whilst this option has great potential for the industry, as explained in the literature review, alginate encased cells will require retrieval at the clinical site, which may complicate the end processing of cells for therapy. Warmer temperatures (15–32°C) for the preservation of cells in liquid form have been touched upon in the literature but research is mainly focussed on the end point of viability and fails to investigate the post-pause effects of cell growth and proliferation. What is also lacking from the literature is an appreciation of the donor variability concerning hMSCs and how differently cells from people of varying ethnicity, age, gender and health can respond to preservation procedures.

The aim of this chapter is firstly to test whether factors shown to be important for HOS TE85 are also important for hMSC pausing, for example, stable pH. Considering that hMSCs may behave extremely differently under pausing conditions compared with the

osteoblasts, it may be that additional elements of control will be required for their successful preservation. Secondly, this chapter will examine the effect of ambient pausing on three different donor-derived cell lines, to demonstrate the variation that can occur between patients in a clinical setting. The donor cell characteristics of the cell lines used in these experiments are provided in table 5. Briefly, the age of donor's ranged between 19–24, each donor was a different ethnicity (Black, Caucasian, and Hispanic) and the ratio of female to male was 2:1. In terms of characteristic variation encountered within the samples and the feasibility of testing multiple cell lines in a panel of experiments, three cell lines was considered an acceptable number for the purpose of this proof of concept investigation.

For all following experiments, hMSCs were paused in ambient conditions for up to 72 hours. Ultimately, a timeframe of 72 hours provides enough flexibility in terms of logistics (transport time and delays) and extending this time length is not essential for this purpose. Demonstrating that hMSCs can remain viable and are able to efficiently grow and proliferate after ambient temperature pausing for 3 days will add substance to the limited literature available and hopefully strengthen the rationale for further research in this area.

6.2 HMSC SURVIVAL POST-PAUSE

Initial survival, in terms of cell membrane integrity and live cell number before and after pausing, were methods used following the pausing of HOS TE85 cells. The same assays were applied to stem cells for consistency, with some additional experiments specific for hMSCs. Immediate cell viability was an important factor to measure, as if most cells were considered non-viable by membrane integrity tests, it would be

unlikely that the cells would survive for further post-pause testing. Other experiments performed in this section include those based on the ISCT guidelines, which state that an MSC must be plastic-adherent in standard culture conditions, characteristically express certain surface markers, and must be able to differentiate into osteoblasts, adipocytes and chondrocytes *in vitro*. In this section, immediate parameters such as cell attachment and immunophenotype are explored, with cell differentiation being covered in section 6.3. Cell attachment is a standard quantitative measure of cell viability for adherent cell types (Humphries, 2001). Specifically for the hMSCs, a multi-parameter flow cytometry assay was employed to examine the expression of several bone marrow-derived hMSC markers, simultaneously at the single cell level. This assay, developed by Chan and colleagues in 2014, provides a more in depth analysis compared with conventional single or dual staining methods as it includes analysis of the co-expression of three positive and two negative extracellular surface markers typical of hMSCs (Chan *et al.* 2014),. Alongside testing the cells, pH measurements were also taken to ensure the medium was maintained at a neutral pH throughout pausing.

6.2.1 OVERVIEW OF EXPERIMENTAL PROCEDURES

Human bone marrow-derived MSCs from three healthy donors (M2, M3 and M4) were used in all pausing experiments throughout this chapter. For donor characteristics please refer to table 5. These cells were originally cryopreserved and banked at passage 1. One vial of MSCs from each donor containing $1–2x10^6$ cells was retrieved from the banks, thawed and cultured for at least one passage before all experimental procedures. All experiments were performed at passage 4 and 5 due to the finite

lifespan of MSCs. Cells were prepared for experiments by being cultured and passaged as described in section 3.2.2. At P4, cells were quenched in standard DMEM medium with 10% (v/v) FBS, 2% (v/v) ultra-glutamine and 25mM HEPES, and seeded into ULA plates (5×10^4 cells/cm^2). The plates were transferred to a plastic, sealed container and kept at ambient temperature in the dark (no atmospheric control) for up to 72 hours. Cell morphology was monitored throughout pausing by collecting images every 24 hours. At 24, 48 and 72 hours pausing, a cell sample was taken for viability and live cell number measurements. DAPI and AO staining were used to decipher between live and dead cells; this was performed on the NC-3000. Immediately after pausing at the three time points (24, 48 and 72 hours), hMSCs were seeded into normal 6-well plates at 5×10^4 cells/cm^2 and cultured in normal culture conditions for 24 hour attachment efficiency tests. Cells were also tested for immunophenotype changes before and immediately after pausing at 72 hours, as described in section 3.10.5. A 1mL sample of medium was also taken at each time point to monitor pH throughout the whole length of pausing.

6.2.2 HMSC VIABILITY POST PAUSE

Figure 49A shows that for all three hMSC lines (M2, M3 and M4), there was a significant drop in membrane integrity following pausing at all three time points ($p \leq 0.001$). Whilst some data points fell below the 70% threshold, indicated by the error bars, the mean viability for all lines remained $\geq 70\%$. There was no significant difference in viability whether hMSCs were paused for 24, 48 or 72 hours ($p > 0.05$). In terms of live cell number before and after pausing (figure 49B), similar to viability results, there was a significant decrease in the number of live cells following ambient

pausing for 24, 48 and 72 hours (p<0.001). The extent of the decrease in live cell number at all three time points was similar and unlike HOS TE85 results, no decrease in viability and live cell number was observed with increased pausing time. No difference was found between hMSC lines (M2, M3 and M4) by multiple comparison tests for viability and live cell number.

6.2.3 HMSC ATTACHMENT POST PAUSE

Immediately after ambient temperature pausing (24–72 hours), cells were re-seeded onto well-plates and cultured in standard DMEM medium in normal culture conditions for 24 hours. Subsequently, the medium was aspirated away and cells were detached, quenched and counted using the NC-3000. The number of live cells retrieved after 24 hours was divided by the average number of live cells that was measured immediately after pausing, to give the percentage of cells attached after returning to normal culture conditions (see equation 2, section 3.9.7). Multiple comparison tests resulted in no significant difference between the levels of attachment at each time point and also between hMSC lines (figure 50). There was a high degree of variability between data points, which may have impacted on the significance of the results.

(A)

(B)
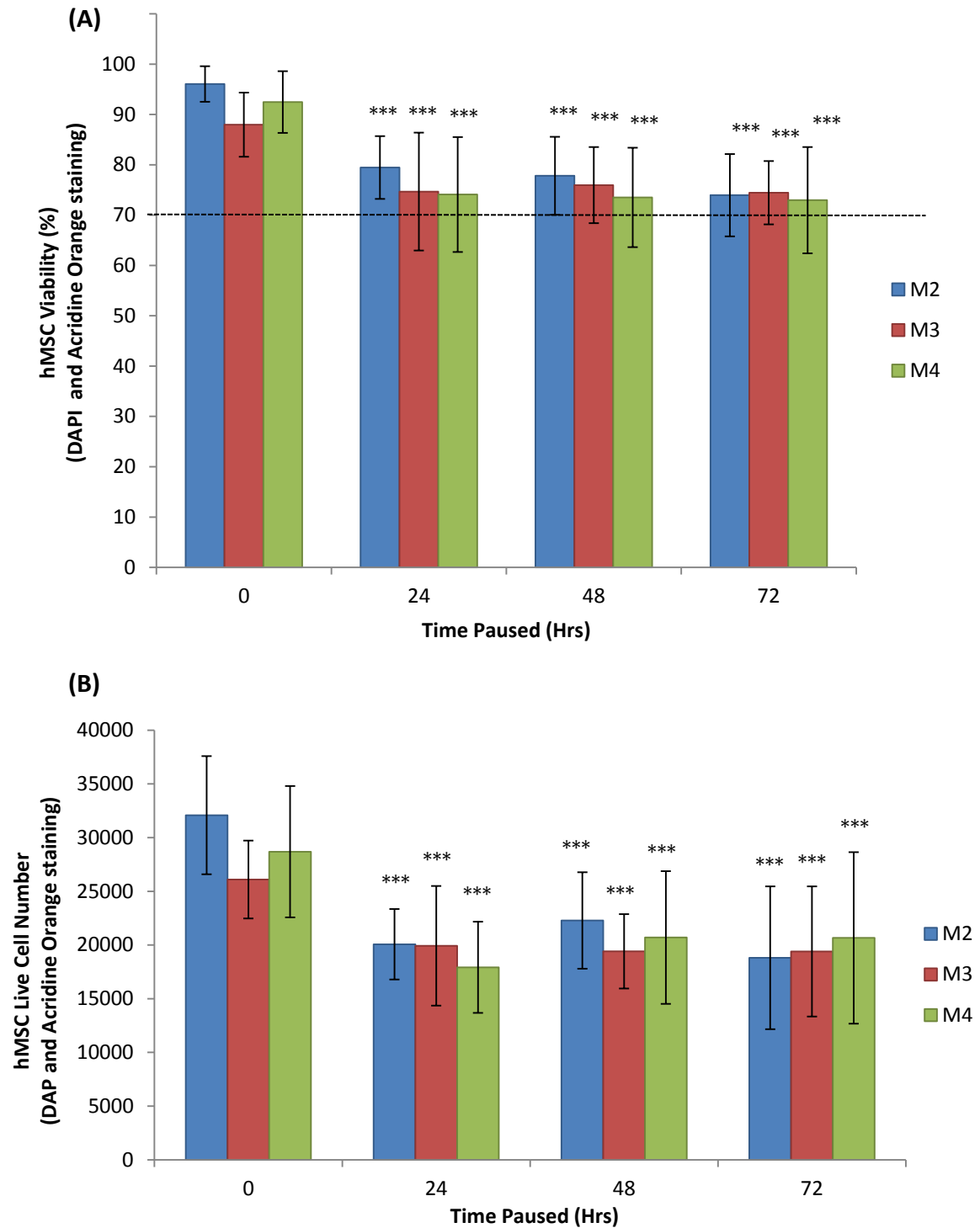
Figure 49: hMSC viability and live cell number, measured by DAPI/AO staining, when paused at ambient temperature for 24–72 hours (n≥4). (A) hMSC percentage viability **(B)** hMSC live cell number. A two-way ANOVA was used to test for significance alongside Tukey's range test to determine which data points were significant. (*** indicates $p < 0.001$). Stars show significance compared with the control (MEM). Error bars show the standard deviation between independent experimental repeats.

6.2.4 HMSC IMMUNOPHENOTYPE POST PAUSE

Immunophenotype investigations, utilising the multi-parameter flow cytometry assay were performed on hMSCs before (0 hours) and following 72 hours pausing. The assay quantified the number of cells that positively expressed the surface markers CD73, CD90 and CD105 and those that were negative for CD34 and HLA-DR, as a single percentage. Figure 51A shows an example of the gating strategy used for the extracellular markers conjugated to fluorescent antibodies. Overall, there was no significant difference between the co-expression of the five surface markers at 0 and 72 hours (figure 51B). There was also no difference in immunophenotype between the three cell lines. Although no significance was found between non-paused cells and those paused for 72 hours, there was a substantial amount of variance between the levels of co-expression across all samples including control cells and the average values were much lower than expected (M2 = 85.8; M3 = 83.2; M4 = 84.4). For example, according to the ISCT criterion, ideally ≥95% of hMSCs should be positive for CD73, CD90 and CD105 and ≤2% should be positive for CD34 and HLA-DR.
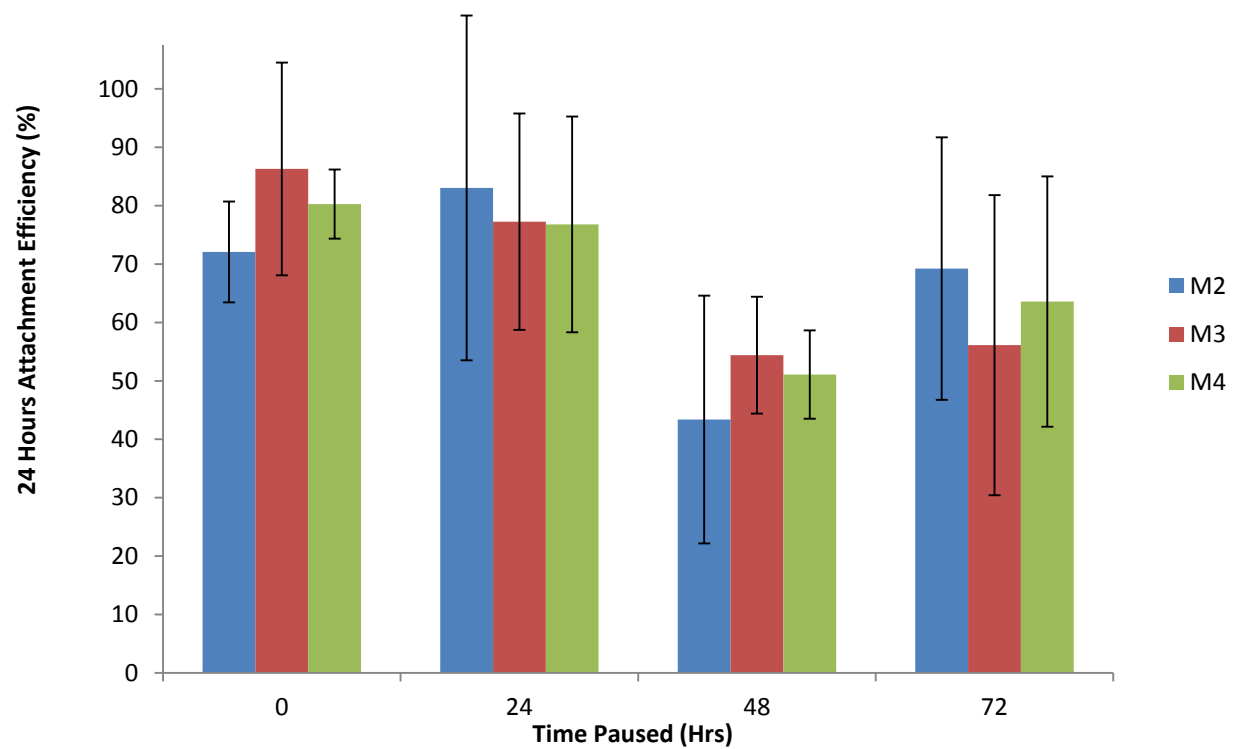
Figure 50: hMSC 24 hour attachment efficiency, measured by DAPI/AO staining, following pausing at ambient temperature for 24–72 hours (n=4). Data are expressed as the percentage of the live cell population recovered immediately after pausing. A two-way ANOVA was used to test for significance alongside Tukey's range test to determine which data points were significant ($p > 0.05$). Error bars show the standard deviation between independent experimental repeats.
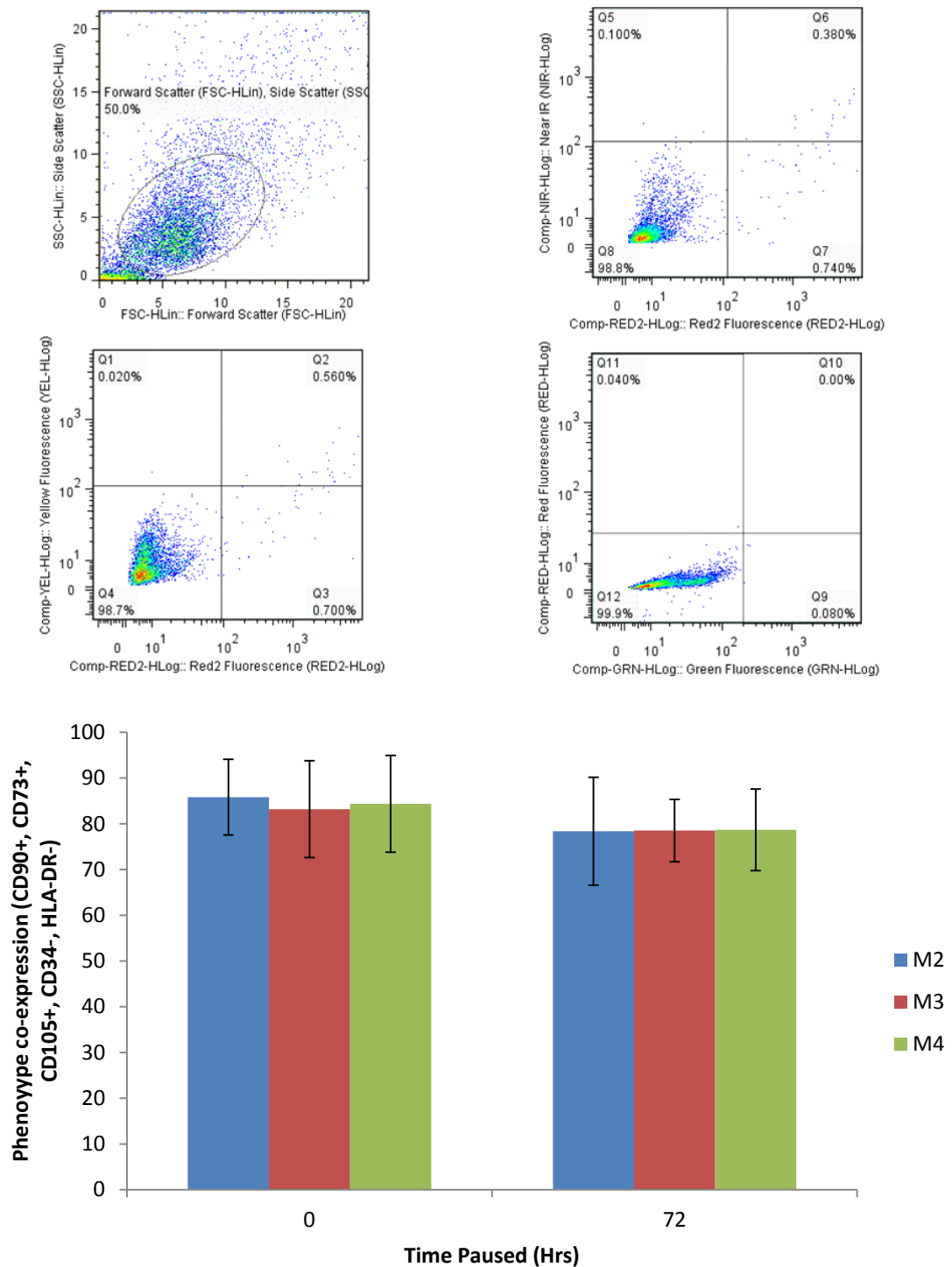
Figure 51: hMSC immunophenotype before and after 72 hours ambient temperature pausing (n=3).
(A) Flow cytometry, multi-parameter gating strategy, for the 5 individual extracellular markers. This sample represents unstained cells, which show negative expression for all extracellular markers by being in the bottom left quadrant (forward versus side scatter / Near IR versus Red2 fluorescence (CD73 versus CD90) / Yellow versus Red2 fluorescence (CD105 versus CD90) / Red1 versus Green fluorescence (CD34 versus HLA-DR)) **(B)** Percentage of cells positive for the markers CD73, CD90 and CD105 and negative for CD34 and HLA-DR; a selection of markers typically found on MSCs using flow cytometry. Results are based on >10,000 events for each individual sample. No significance between time or cell line was found following a two-way ANOVA test. Error bars show the standard deviation between independent experimental repeats.

Measurements of medium pH were taken before pausing (37°C) and every 24 hours throughout cell pausing for up to 72 hours. Standard DMEM medium without any additional components except 10% (v/v) FBS and 2% (v/v) ultra-glutamine had an average pH of 7.64. As indicated on figure 52, following 24 hours in ambient conditions, pH significantly increased to 9.13 (p<0.001). At 48 and 72 hours pausing, pH plateaued at ~9.23. When 25mM HEPES was added to DMEM with supplements, pH was measured at a slightly more acidic 7.01. Following pausing at 24, 48 and 72 hours, pH significantly increased to 7.81, 7.83 and 7.81, respectively.
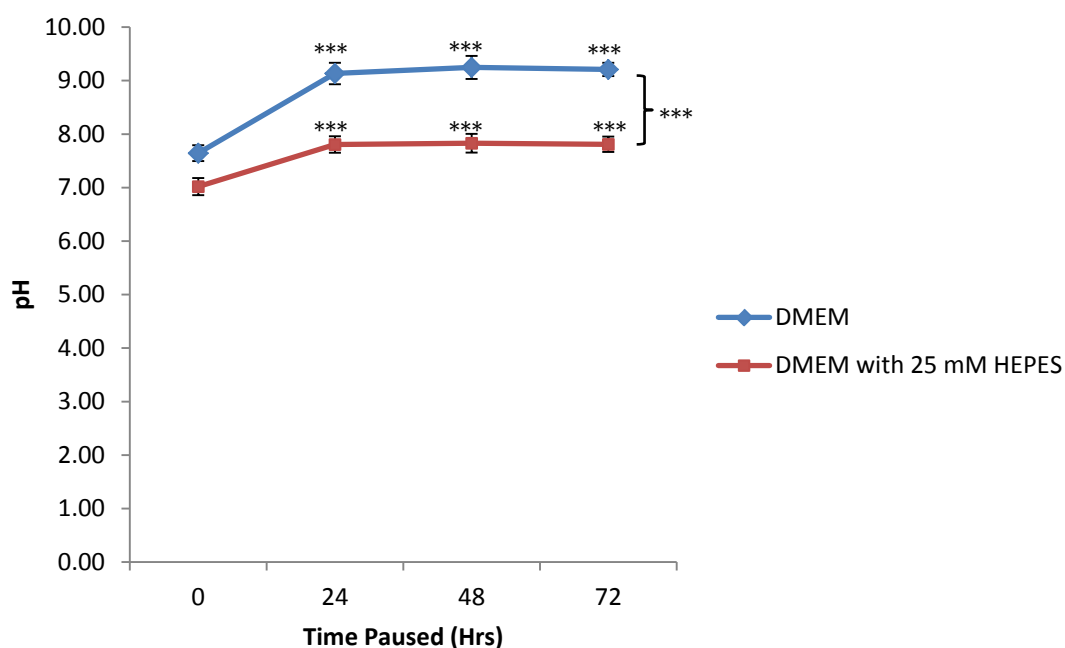
Figure 52: Medium pH throughout the ambient temperature pausing of hMSCs (n=4). 1mL samples of standard DMEM medium and DMEM medium plus 25mM HEPES, were taken for pH measurements, before and after ambient temperature pausing for 24, 48 and 72 hours. A two-way ANOVA was used to test for significance between time points, alongside Tukey's range test, to determine which data points were significant. Stars on top of data points show significance compared with the control (MEM) (*** indicates p<0.001) and the bracket indicate significance between the two media. Error bars show the standard deviation between independent experimental repeats.

While pausing with the robust, cancer-derived HOS TE85 cells was successful for up to 144 hours, it was important to test if this would also be the case for the more complex hMSCs. Instead of pausing the hMSCs for as long as they could survive, it was decided to initially focus on a 72 hour window as this would allow enough time for the storage and transport of cells for allogeneic or autologous therapies. This time frame has also been used in other stem cell preservation research (Swioklo *et al.* 2016).

Overall, the pausing of hMSCs was successful as the average viability remained above 70% after 24, 48 and 72 hours, which is considered safe by US FDA standards. While there was a significant drop in viability for all three hMSC lines (M2, M3 and M4) at all-time points ($p < 0.001$), there was no statistical difference between the viability values at 24, 48 and 72 hours. The fact that the average viability was sustained at >70% and did not decrease with increased pausing time, was likely attributed to the additional HEPES buffer in the medium, helping to stabilise pH at ~7.8. This phenomenon was also observed with the osteoblasts in MEM with 25mM HEPES, which helped retain viability above 90%. What was of interest regarding the pH of the medium was that the experiments only worked when 1 x DMEM was made up from 10 x medium and not when the medium had been previously formulated at 1 x concentration by the manufacturer. The main difference was the starting pH of both media, with the 1 x DMEM made from 10 x DMEM starting off much more acidic ~7.0 and 1 x DMEM at 7.6. After 24 hours pausing, pH, even with the support of 25mM HEPES, increased to ~7.8. With an average rise in 0.8, this pushes the pH of pre-formulated 1 x DMEM to ~8.4. As discussed in the literature review, media with a pH>8.0 can be toxic to cells and lead to

cell death. This may explain why experiments did not work when using pre-formulated 1 x DMEM.

The difference in viability between the osteoblasts and the hMSCs highlights the difference in response to low temperature pausing by various cell types and the need for tailored preservation regimens. Results for live cell number before and after pausing were similar to those for viability, with a significant decrease in viable cell number following 24 hours in ambient conditions. This implies that cells are always lost during the pausing of hMSCs, even if measures are taken to maintain pH. The fact that the decrease in viability and live cell number is only significant between the control (before pausing) and following 24 hours ambient temperature pausing and not between 24 hours pausing and further time points suggests that the most cell damage and death occurs within the first 24 hours of low temperature pausing. However, this reduction in viability may not be an artefact of hypothermia alone; a percentage of the cell population may not have adjusted to suspension culture. Cells that are able to survive the first 24 hours are likely to enter a form of 'stasis' (Muller-Cohn *et al.* 2015), shutting down metabolic and signalling pathways to conserve energy and nutrients. The damage that cells encounter during hypothermia is largely from 'osmostress', which can jeopardise the cells structural integrity as well as internal systems (Mager *et al.* 2000). Cell volume stability is constantly challenged by hydrostatic pressure gradients and the movement of substances across the cell membrane (Lang *et al.* 1998); the magnitude of this challenge is increased during pausing. When cells shrink as a result of water loss, ion uptake and release mechanisms are activated, which temporarily increases cell volume (Lang *et al.* 1998; Lang *et al.* 2007). Whilst this

regulatory mechanism helps return cell volume to normal, the increased ion concentration can lead to impaired cell function. Further to this, a high concentration of Na^+ ions can cause sodium toxicity (Trump and Berezesky, 1995) and increased levels of electrolytes can interfere with cell structure and function (Chakraborty, 2008). As the cells enter the recovery period in normal culture conditions, they will yet again face osmotic challenges. Inevitably, if the regulatory volume response and repair mechanisms fail during osmotic stress, apoptosis pathways may be activated.

The ability of a cell to attach to a surface is a well-known measure of cellular health and is specified as one of the minimum ISCT criteria for determining hMSC identity. Cell attachment assays were performed at 24 hours post-pause as a measure of cell viability and function. Cell attachment assays can be performed as soon as 1–3 hours post-storage or treatment; however, as ambient temperature pausing is a particular harsh stress and it may take extra time for cells to recover and be able to attach to a surface, the assays were performed after a day of culture in normal conditions. Whilst there was some level of variability between independent experimental repeats, no significant difference in attachment ability was found between the control cells and cells paused for 24, 48 and 72 hours. This is a positive result, as it suggests the cells that can survive the stress of pausing in terms of membrane integrity can also attach to a surface and remain viable. It may have been possible that some cell replication occurred within the 24 hour recovery period, increasing the number of supposedly attached cells. However, due to the slow growth rate of cells and the knowledge that a 6 day culture period is generally needed to obtain confluence, the effect of this on cell attachment measurements is considered minimal.

A multi-parameter flow cytometry assay was performed on all three cell lines to investigate if the immunophenotype of the cells changed before and after ambient temperature pausing. As there is no single extracellular marker that can define an hMSC, combinations of a selection of markers are normally used to characterise a population (Chan *et al.* 2014). The expression of specific surface antigen markers is part of the minimum criteria for defining an hMSC in the ISCT guidelines. The guidelines state that hMSCs should positively express CD73, CD90, CD105, CD10, CD146 and GD2 (Dominici *et al.* 2006; Rasini *et al.* 2013) and must lack the expression of the non-stromal markers CD14, CD34, CD45, CD79α and HLA-DR (Dominici *et al.* 2006). The five colour multi-parameter flow cytometry assay measures the co-expression of the three positive markers CD73, CD90 and CD105 and checks for the absence of CD34 and HLA-DR. Overall, the immunophenotype of non-paused cells and cells paused for 72 hours was not significantly different. Proving that the extracellular surface marker expression of cells can be maintained after hypothermic stress, adds to the potential for pausing stem cells for therapy. Although the co-expression percentages were lower than expected, this was most probably attributable to human error regarding the gating strategy utilised and the quantification of cells in each quadrant rather than the cells themselves. All three cell lines have been thoroughly characterised, including immunophenotype and were all considered to meet the criteria set out by the ISCT (Heathman *et al.* 2016).

What is of great importance from a regulatory perspective is cell product consistency whereby the cells that are manufactured, preserved and delivered are unchanged and maintain their therapeutic function. A risk of pausing cells and using the cells that have

managed to survive the stresses of low temperature storage is the development of a resistant cell population that is somehow changed. Experiments so far have been successful in demonstrating the potential for hMSC ambient temperature pausing in terms of cell viability and also that the parameters tested before and after pausing are similar. The next section will investigate the longer-term effects of pausing to examine if cell damage or death is encountered upon further culture and if cell potency if affected.

6.3 LONGER TERM EFFECTS OF PAUSING HMSCS

One of the main messages throughout this research so far has been not only to test cell viability immediately after pausing, but to examine post-pausing effects after a cells return to normal conditions and following further passages. This is due to the phenomenon of delayed-onset cell death, which can ensue after preservation during the cell warming procedure or further culture. Results so far have demonstrated that hMSCs can remain ~70% viable immediately after 72 hours pausing and can maintain an immunophenotype similar to non-paused cells. It will be important to demonstrate that hMSCs are not only viable after pausing but maintain their viability and therapeutic attributes long-term. The mechanism of action behind how and when hMSCs elicit their effects once administered to a patient is currently not understood. There is research to support the idea that hMSCs therapeutic effects are attributable to their ability to differentiate into tissue-specific cells, which help resident cells survive and function due to secreted paracrine factors, and also by their immunomodulatory properties (Li and Fu, 2012). What is not known is when these effects take place after cell administration. A stem cell being able to retain its

therapeutic function long after preservation provides more flexibility when considering the logistics of cell therapy manufacture, delivery and preparation for surgery.

Whilst in an *in vitro* setting is difficult to prove cell potency, there are a number of assays that can help verify hMSC viability and function, including fold growth expansion tests, CFU assays and of course, differentiation into the osteocyte, adipocyte and chondrocyte lineages, specified in the ISCT guidelines. The ability of a cell to grow and replicate is a good assessment of longer-term cell viability following a preservation procedure. The fold growth expansion assays used for the HOS TE85 cells provided a wealth of understanding regarding medium composition and its effects on cell attachment proficiency and proliferation; hence, the assay was used here for the hMSCs.

Originally, hMSCs were referred to as fibroblastoid CFU cells, attributed to their ability to generate colonies when plated at low density (Pochampally, 2008). This led to the development of the CFU assay, used as a measure of cell quality. Here, we test the CFU efficiency of non-paused and paused cells, to further examine if they maintain this attribute following cold stress.

Tri-lineage differentiation assays were performed on all cell lines before and after pausing, which took advantage of the hMSCs multipotent phenotype. This was used as a qualitative assessment of the cells ability to form osteocytes, adipocytes and chondrocytes after pausing.

6.3.1 OVERVIEW OF EXPERIMENTAL PROCEDURES

Fold growth expansion assessments were performed over 3 passages rather than 5 due to the slower growth of hMSCs compared with the cancer derived osteoblasts. Briefly, following pausing at 24, 48 and 72 hours, cells were immediately re-seeded (5.4×10^4 cell/cm^2) onto tissue culture plastic (3 x T25) and cultured in normal conditions (37°C, 5% CO_2, 95% humidity) for 6 days. After this period, cells were counted using the NC-3000 and re-passaged twice more.

For CFU efficiency assays, ~250 cells from each line were seeded into T25 flasks and cultured for up to 14 days, with a complete medium change every 3–4 days. At the end of culture, the cells were fixed and stained with 1% (v/v) crystal violet in methanol to visualise and manually count how many CFUs had been produced from the original starting population of 250. Please refer to section 3.10.7 for more details.

For the differentiation assays, cells after pausing were seeded into 12-well plates and cultured in the presence of specific medium specialised for the differentiation of osteocytes, adipocytes or chondrocytes. Cells were cultured for 21 days with complete medium changes every 3–4 days. After the culture period, cells were fixed and stained as per section 3.10.1. Images were taken using a phase-contrast microscope and were examined to provide a qualitative assessment of the hMSCs differentiation capacity.

6.3.2 HMSC FOLD GROWTH EXPANSION

All hMSC cell lines (M2, M3 and M4) were able to successfully proliferate over 3 x 6 day passages after 24, 48 and 72 hours pausing at ambient temperature. After 24 hours pausing, cells from all lines maintained a similar level of fold growth expansion to non-paused cells (figure 53A). Additionally, M3 generally had a lower fold growth

expansion rate compared with the other two cell lines (M2 = p<0.001; M4 = p<0.05). When cells were paused for 48 or 72 hours hours (figure 53B and 53C, respectively), fold growth expansion on P1 was significantly lower than that achieved on P2 (p<0.001) and P3 (p<0.05). The level of fold growth between P2 and P3 however, was not significantly different.

6.3.3 COLONY FORMING UNIT ASSAY

As a quantitative assessment of cell function, the number of CFUs produced by non-paused cells and cells paused for 72 hours were counted and expressed as CFU efficiency (%). Overall, CFU efficiency was <20% for both the control cells and cells paused for 72 hours at ambient temperature (figure 54). For M2 and M4, there was a significant reduction in the number of colonies formed after pausing compared with the control (p<0.01). Although there was a decrease in CFU efficiency for M3 (0 hr = 4.6%; 72 hr = 3.1%), this was not a significant result (p>0.05). Between cell lines, the only significant difference was found between M2 and M3 at 72 hours pausing (M2 = 4.4%; M3 = 3.1%). The error bars on the graph, which represent standard deviation, show that the variation between independent experimental repeats was minimal for paused cells; however the data were widespread for the control cells.
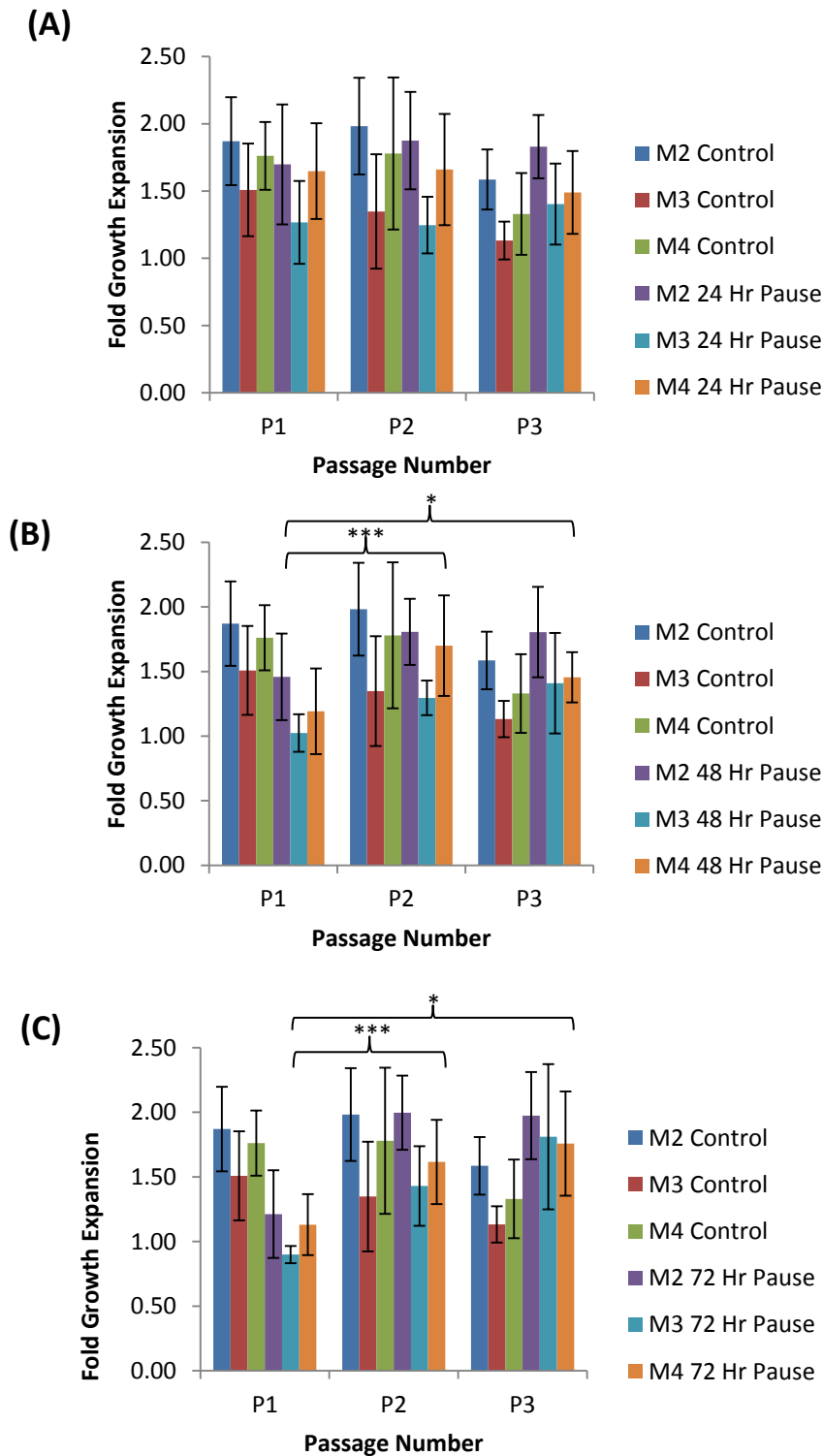
Figure 53: hMSC fold growth expansion, measured by DAPI/AO staining, following pausing at ambient temperature for 24–72 hours (n≥3). (A) Fold growth expansion after 24 hours pausing **(B)** Fold growth expansion after 48 hours pausing **(C)** Fold growth expansion after 72 hours pausing. A three way ANOVA was used, alongside the Bonferroni post-hoc test to determine which data points were significant. Error bars represent the standard deviation between experimental repeats (*indicates $p < 0.05$ and ***indicates $p < 0.001$ between paused samples only).
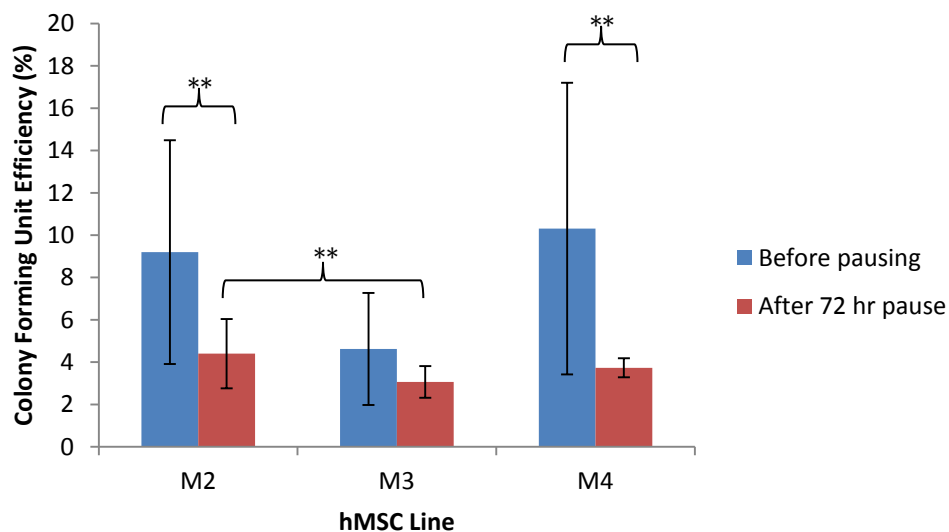
Figure 54: hMSC colony forming unit efficiency (%) before and after 72 hours ambient temperature pausing (n=3). A one-way ANOVA was used, alongside Tukey's range test to determine which data points were significant. Error bars represent the standard deviation between experimental repeats (*indicates p<0.01).

6.3.4 HMSC DIFFERENTIATION

All three lines of hMSCs were tested for the retention of their tri-lineage differentiation potential after pausing at ambient temperature for 72 hours. All cell lines (M2, M3 and M4) whether non-paused or paused for 72 hours stained positively for alkaline phosphatase and showed calcium deposition (figures 55–57). Similarly, cells cultured in chondrogenic medium stained positively for Alcian blue and demonstrated chondrocytic differentiation whether paused or non-paused. All cells formed visible lipid vacuoles stained in red when cultured in adipogenic medium.

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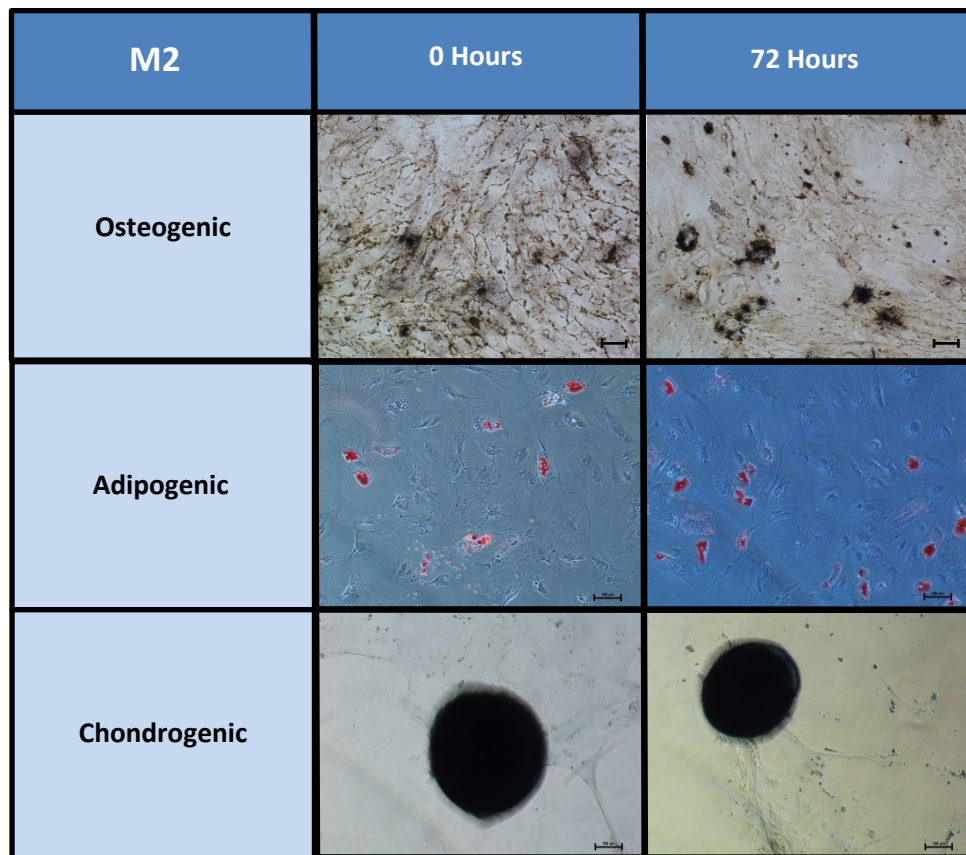
Figure 55: Tri-lineage differentiation potential of the hMSC line M2 (n=3). The differentiation capacity of the M2 cell line was examined before pausing (0 hours) and following 72 hours ambient temperature pausing. Cells were stained for alkaline phosphatase and calcium deposition to test for osteogenic differentiation, Oil Red O for adipogenic differentiation and Alcian Blue for chondrogenic differentiation (scale bar = 100 microns).

M3	0 Hours	72 Hours
Osteogenic		
Adipogenic		
Chondrogenic		
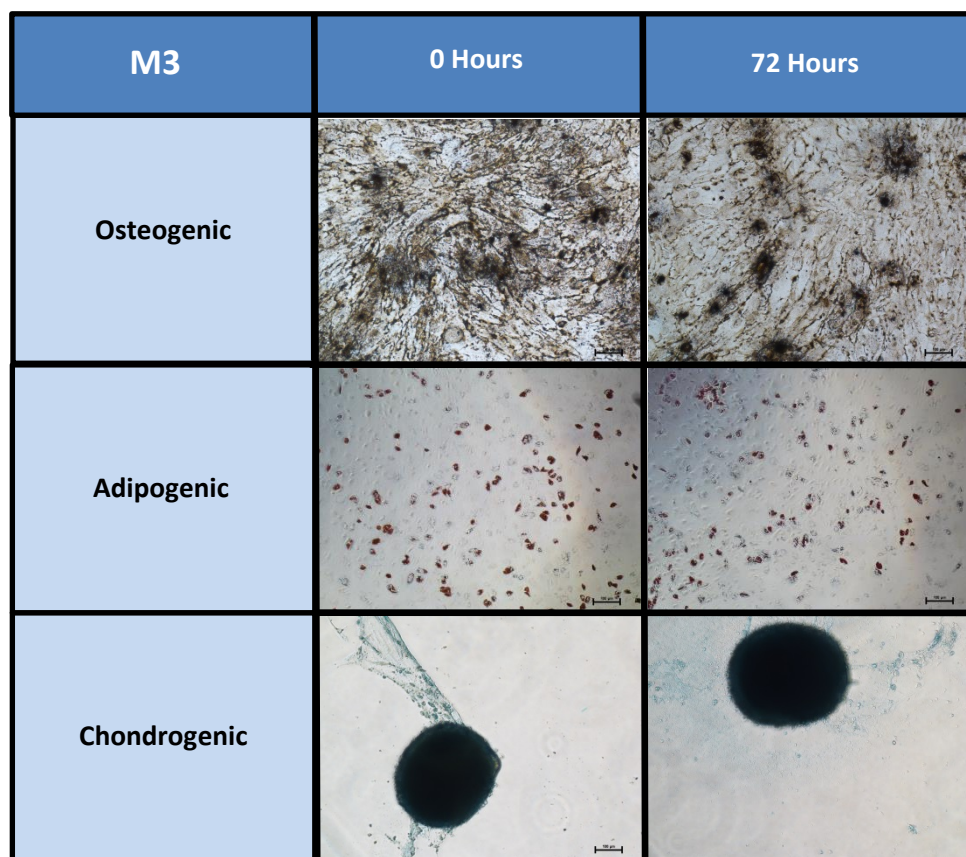
Figure 56: Tri-lineage differentiation potential of the hMSC line M3 (n=3). The differentiation capacity of the M2 cell line was examined before pausing (0 hours) and following 72 hours ambient temperature pausing. Cells were stained for alkaline phosphatase and calcium deposition to test for osteogenic differentiation, Oil Red O for adipogenic differentiation and Alcian Blue for chondrogenic differentiation (scale bar = 100 microns).

M4	0 Hours	72 Hours
Osteogenic		
Adipogenic		
Chondrogenic		
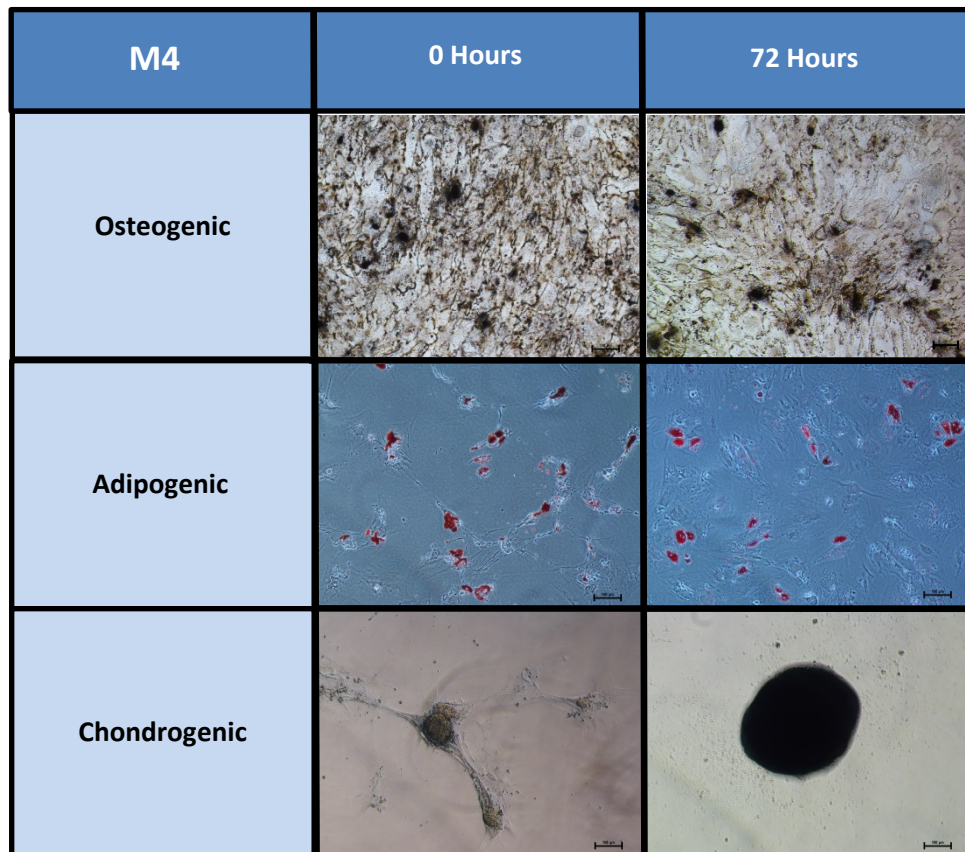
Figure 57: Tri-lineage differentiation potential of the hMSC line M4 (n=3). The differentiation capacity of the M2 cell line was examined before pausing (0 hours) and following 72 hours ambient temperature pausing. Cells were stained for alkaline phosphatase and calcium deposition to test for osteogenic differentiation, Oil Red O for adipogenic differentiation and Alcian Blue for chondrogenic differentiation (scale bar = 100 microns).

It was important to examine the fold growth potential and potency of the three lines of hMSCs after pausing, as this not only demonstrates survivability after preservation but also provides further confidence in the cells therapeutic potential.

For consistency throughout the project, fold growth expansion tests were performed on hMSCs, as this demonstrates that the cells can successfully attach to a surface and proliferate following low temperature exposure. With the osteoblasts, after 96 hours, cells were unable to reattach and proliferate when paused in ambient conditions (without CO_2 control). With a stable neutral pH, the osteoblasts could be successfully passaged five times with a similar pattern to the control cells. Although it was uncertain that the more delicate hMSCs would be able to retain their ability to grow and proliferate after low temperature storage, apart from a short lag phase on P1 after 48 and 72 hours pausing, we see that fold growth expansion was successful at all-time points over 3 x 6-day passages. As already mentioned, the number of passages was reduced to three because of the slower growth rate of hMSCs compared with osteoblasts. Similar to viability results, there does not seem to be a decrease in fold growth ability with increased pausing time. These fold growth experiments were particularly important as they support the survivability of hMSCs after pausing for up to 18 days and considering the finite life span of MSCs, this is highly promising.

Studies have shown that the intravenous infusion of hMSCs generally leads to their entrapment in the lungs; although only temporary, this can delay or negatively impact targeted engraftment (Kean et al. 2013). As it is unknown how long cells can remain viable once administered to a patient, the administered population must be healthy

and able to maintain their potency for as long as possible. This highlights the importance of post-recovery tests, not just hours, but days after pausing.

CFU efficiency for all lines, whether paused or kept in normal culture conditions, was under 20%; whilst these numbers seem low, CFU efficiency was still consistent with values quoted in the literature for similar passage numbers (DiGirolamo *et al.* 1999; Kuznetsov *et al.* 2008). The CFU efficiency of paused cells was significantly reduced when compared with non-paused M2 and M4 lines, whilst M3 retained a similar CFU efficiency to its non-paused counterpart. There was some variability in CFU efficiency and also in the size and morphology of colonies formed in the control samples; the reason for this remains uncertain and may require further experimentation with a larger sample size to reduce the variability encountered. Whilst some of this variability could be from differences in CFU efficiency between donors, another explanation for the variability could be due to a variation in the number of cells originally seeded (~250 cells); a low seeding density is required for CFU assays and the recommended cell concentration of a cell suspension to be read accurately by the NC-3000 is between 5×10^4–5×10^6 cells.

The tri-lineage potential of hMSCs was assessed before and after 72 hours pausing as a quantitative measure of cell function. From the images, it was clear that all lines of MSCs retained their multi-lineage potential. Whilst all three cell lines were successful in differentiating into the typical morphology of osteogenic, adipogenic and chondrogenic lineages, this is only a qualitative assay, which has its limitations. Although the cells might look similar to osteocytes, adipocytes and chondrocytes, the appearance of cells does not confirm their function. Whilst the cell therapy community

is aware of this limitation, tri-lineage differentiation is still part of the ISCT criteria. Experiments focussing on the development of functional assays, specifically immunomodulatory effects, are now an active area of research (Abdi *et al.* 2008; Noort *et al.* 2012; Lee *et al*. 2015) and will provide more information on cell potency than images of stained cells.

Of course, it is difficult to say for certain if cells will be able to perform effectively, once administered into a patient. The *in vivo* environment is considerably different to *in vitro* conditions. However, the ambient temperature storage of cells for therapeutic applications is a fairly new concept, and this early work supports the successful process of ambient temperature cell pausing with retention of proliferative capacity and potency.

6.4 EXAMINING CELL RECOVERY UPON REWARMING

Up until this point, the time for cells to recover from pausing in normal culture conditions has been between 24–48 hours. This time frame is long enough to ensure viable HOS TE85 cell attachment and for metabolic activity to resume to normal (shown by PrestoBlue experiments in figure 20). For the purpose of cell therapy, it would be useful to understand exactly how long hMSCs need for a full recovery, specifically in terms of metabolism.

Timing of cell administration is critical to achieve the best therapeutic action, however this is a severely under researched topic. Administering cells that are recovering and are still 'paused' in terms of metabolic activity, may delay the cells therapeutic effects. Additionally, the stress of the delivery itself, in terms of shear force during the movement through a needle, may hinder the recovery process and lead to cells unable

to perform their function, whether that be paracrine activity or immunomodulation. Delivering cells that have recovered by at least resuming energy dependent processes may mean that they can handle the stress of delivery through a needle and enter an *in vivo* environment better than cells that are still 'in recovery'. On the other hand it might be more detrimental to cells to deliver them after a full recovery and a better approach may be to deliver cells that are paused so that they can recover and acclimatise *in vivo*. Apoptosis is a known mechanism that occurs frequently during cellular stress and its activation during patient delivery may affect the dose and number of cells that need to be administered to a patient. Although this is very early research, an idea of the amount of time required for cells to be actively metabolising and proliferating would be useful for future studies. Efficacy tests, such as those examining a cells immunomodulatory properties would generate more relevant results if performed at the time when cells have regained their original level of metabolism, growth and proliferation.

6.4.1 OVERVIEW OF EXPERIMENTAL PROCEDURE

Following ambient temperature pausing of all three cell lines for up to 72 hours, cells were immediately seeded into 24-well plates (5.25×10^4 cells/cm^2) and PrestoBlue was added to the cell medium to obtain a final concentration of 10% (v/v). The plates were immediately transferred to an incubator set at normal culture conditions. After 1, 3, 6, 9 and 24 hours, samples of medium were taken and fluorescence (relative fluorescence units) was measured on a FLUOstar Omega plate reader, which was equivalent to the reduction of PrestoBlue (non-fluorescent blue resazurin to pink fluorescent resorufin).

Additionally, the average cell size throughout pausing (0, 24, 48 and 72 hours) and after recovery was measured using data from the NC-3000. This analysis was performed for all three cell lines and presented as average cell diameter (microns).

6.4.2 CELL SIZE THROUGHOUT PAUSING AND RECOVERY

Overall, cell size was reduced when paused in ambient conditions (figure 58). For the M2 cell line, the decrease in cell diameter was only significant after 72 hours pausing ($p=0.001$). For M3, the decrease in cell size compared to the control was significant following 48 ($p<0.05$) and 72 hours ($p=0.001$) pausing. For M4, a significant decrease in cell diameter was observed after 72 hours pausing ($p<0.01$) and also between 24 hours and 72 hours ($p<0.05$). Cell size remained significantly smaller upon warming to normothermic temperatures for 24 hours ($p<0.01$).
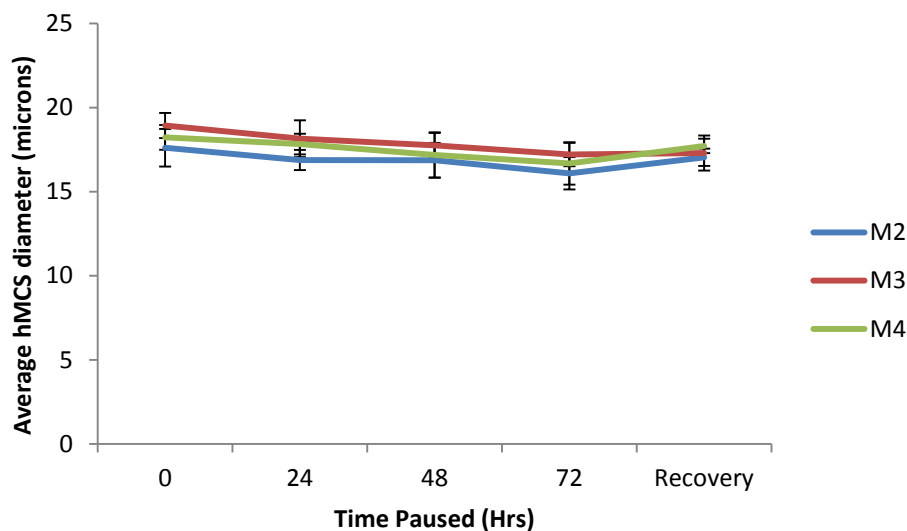
Figure 58: hMSC cell diameter (microns) throughout pausing and recovery following 24, 48 and 72 hours ambient temperature pausing (n=3). A two-way ANOVA was used as a measure of significance between paused and non-paused cells alongside the Bonferroni post-hoc test to determine which data points were significant. Significance results are described in text for clarity. Error bars represent standard deviation between independent experimental repeats.

6.4.3 METABOLIC ACTIVITY THROUGHOUT PAUSING AND RECOVERY

The metabolic activity, determined by PrestoBlue reduction, was measured for all three hMSCs lines upon their gradual return to 37°C (figure 59A, M2; figure 59B, M3; figure 59C, M4). Regardless of cell line, a steady increase in the amount of PrestoBlue reduction is observed with time, with a steep incline between 9 and 24 hours. The difference in the amount of fluorescence at 24 hours between the control cells and cells kept at ambient temperature was significant ($p \leq 0.01$), irrespective of time paused (24, 48 or 72 hours).
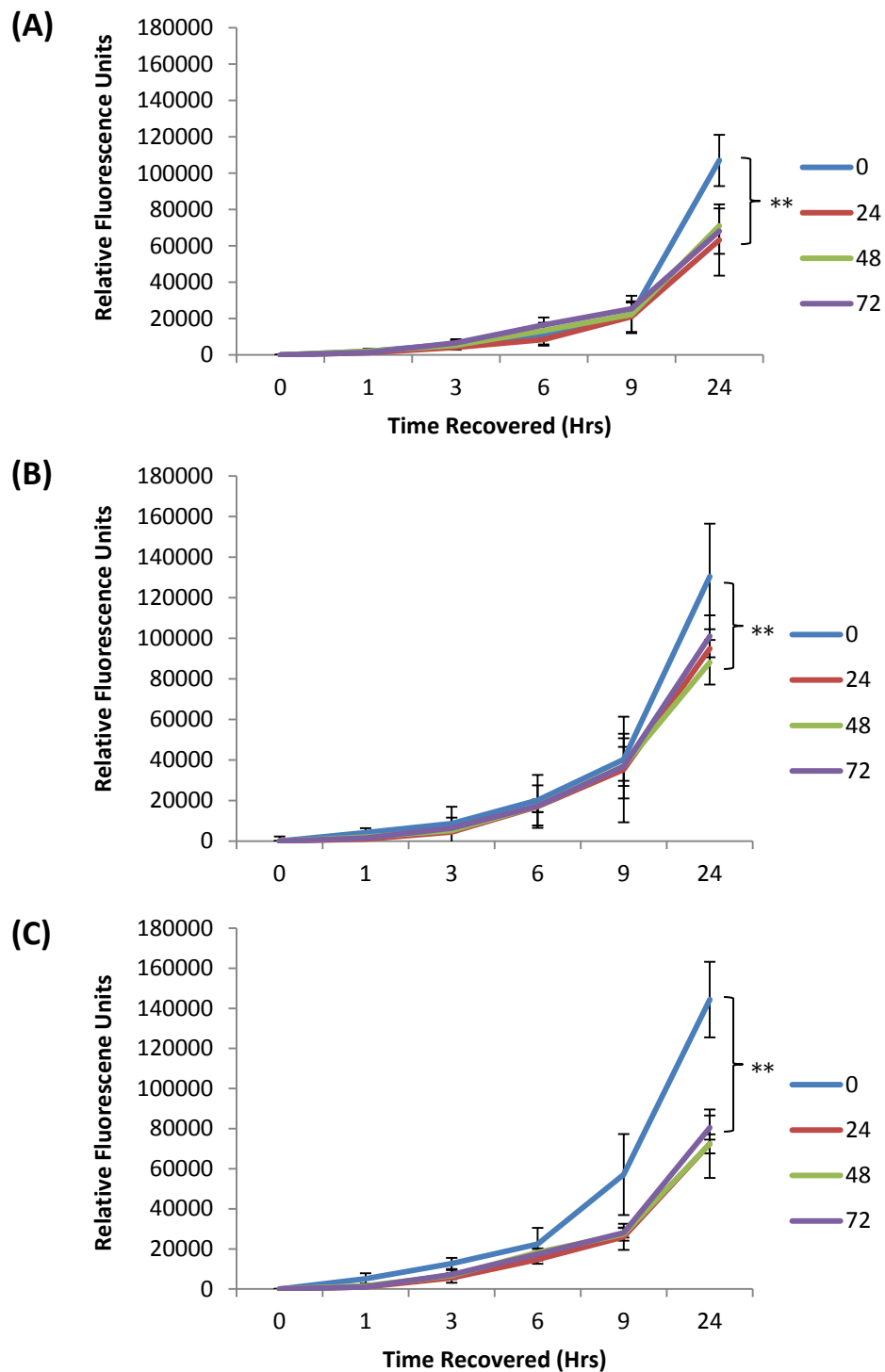
Figure 59: PrestoBlue fluorescence at various time points throughout hMSC recovery to normal culture conditions, following pausing at ambient temperature (n=3). (A) M2 **(B)** M3 **(C)** M4. A two-way ANOVA was used as a measure of significance between paused and non-paused cells alongside the Bonferroni post-hoc test to determine which data points were significant (**indicates $p \leq 0.01$).Error bars represent standard deviation between independent experimental repeats.

6.4.4 DISCUSSION

Cells that are preserved, either frozen, chilled or kept at ambient temperature will undoubtedly have a recovery period during their return to normal temperature, where the cells energy dependent systems will begin to resume and metabolic and proliferative rates return to normal levels. There is no research to date which looks in depth at this 'recovery time', which is essential for the development of successful cell preservation protocols.

In this section, the recovery of cells in terms of cell size and metabolic activity was assessed after ambient temperature pausing for up to 72 hours. Overall, cell diameter decreased during low temperature storage and remained significantly smaller compared to non-paused cells, even following a 24 hour recovery period. As there is a 'pausing' of cell membrane transport systems with lowered temperature, water is likely drawn out of the cell via osmosis, reducing the overall volume of the cell. Furthermore, in cooler temperatures membrane fatty acids and sterols undergo a phase transition from a fluid to a gel like state; this can cause the membrane to become leaky (Walker, 1998). The reason why cell size is still smaller than control cells after their return to normal temperature is unknown but maybe attributable to unrecoverable membrane damage which affects ion uptake.

PrestoBlue experiments showed that fluorescence increased at a similar level and time frame to control cells up until 9 hours into recovery. At 24 hours recovery however, paused cells had a significantly higher reduction capacity compared to control cells. An explanation for this is possibly due to a higher number of cells being able to reduce the resazurin to resorufin, as the control cells were able to complete the cell cycle and

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propagate. Considering the therapeutic action of stem cells, be it the cells themselves, paracrine activity or immunomodulation, efficient cell proliferation may not be as important as resuming other cellular systems such as protein synthesis and cell signalling. Another positive finding from this experiment is that cells recover at a similar rate to the control whether paused for 24, 48 or 72 hours. This suggests that increased pausing time does not necessarily increase time to recovery. Importantly, this will help simplify pausing protocols, as the recovery time required will remain the same regardless of time paused. To summarise, these initial experiments are promising as they show that paused hMSCs from three different donors can increase in metabolic activity at a similar rate to non-paused cells when recovered in normal conditions for up to 9 hours. Further research is required to understand when, in terms of hours, cells should be delivered to patients after pausing so that they can effectively elicit their therapeutic effects.

It is the general consensus amongst cell biologists, that if a cell population has been cryopreserved, they need to undergo at least one passage before any experimental procedures are performed. This ensures the cells have survived the suboptimal conditions of freezing and reduces the risk of 'cryostress' affecting a subsequent procedure. In a clinical setting it is not feasible to passage the cells before use as this would require laboratory facilities and trained staff. Instead, quality control checks are implemented backed up by a wealth of research based evidence. More work demonstrating cells can fully recover after a certain time paused is needed to optimise cell preservation and administration protocols.

6.5 CONCLUDING DISCUSSION

With the preservation of stem cells being critical for cell therapy research and clinical application (Hanna and Hubel, 2009), it was important to test if the methods used for the successful pausing of HOS TE85s were also suitable for the therapeutically relevant cell type, hMSCs.

Whilst all three cell lines were able to remain above the 70% viability threshold following pausing for 24, 48 and 72 hours, percentages were expressed as the arithmetic mean, with some values falling below 70%. From a regulatory point of view, it would be unethical to use a cell for therapy from a population that was >30% non-viable, as this may affect the therapeutic quality of the dose. In order to save cells undergoing apoptosis in response to suboptimal preservation conditions, the use of an apoptotic inhibitor might be required so that the average viability of hMSCs is kept way above the 70% mark (Hanna and Hubel, 2009).

From the literature, it is understood that membrane integrity assays frequently do not correlate with functional measures of viability (Pegg, 1989), which led to the investigation of immunophenotype post-pause as well as CFU efficiency tests and differentiation assays. Although early in its development, this research has demonstrated that hMSCs are robust enough to recover after a 3-day period at suboptimal conditions and show similar levels of fold growth expansion, immunophenotype and multi-lineage potential to non-paused cells. Whilst there is no way of proving that these cells will perform exactly the same following patient administration, results have been encouraging. Preclinical testing and clinical trials in

human subjects would be the only sure way of confirming cell potency following pausing and administration.

Various cell types may perform very differently during chilled, ambient or cryopreservation procedures and the number of hours required for transport or hold time may vary considerably depending on the treatment. It is therefore of great importance that there are more cell preservation options available, other than the standard cryopreservation method, with proven efficacy post-recovery so that the most suitable protocol can be implemented. Additionally, cost is a major consideration in the development of a medical treatment. The more simple a regimen or transport procedure, if proven to retain cell viability and potency, the easier it will be to obtain approval from the regulator.

Few studies to date have examined the role of cell storage conditions, such as temperatures and duration, and the influence of warming conditions upon recovery. This research has been successful in providing further understanding and evidence to support the ambient temperature pausing of cells for therapy applications.

CHAPTER 7: CONCLUSION AND FUTURE WORK

7.1 OVERALL SUMMARY

With the advances taking place in cell therapy clinical trials, it is anticipated that treatments may become closer to approval in the next 10–20 years. Whilst the manufacture of cells for therapy is well understood and highly regulated, less focus has been applied to the logistics of getting these therapies to the patient. Although cryopreservation is a standard protocol for the storage of cells in research and medicine, there are concerns with the use of cryoprotectants, which may cause adverse events in patients. Moreover, for short-term transport the freeze-thaw procedure is a particularly risky process for cells to endure and more often than not sees a decline in cell viability, which also varies with cell type. Alternative options including chilled and ambient cell storage need to be explored and the current literature is substantially lacking in this area. This project aimed to examine the potential of ambient temperature pausing for short time periods (24–144 hours) and hoped to add to this severely limited area of research. Overall, results for both the osteoblasts and the hMSCs have been encouraging and deserve further exploration. Whilst some differences have been encountered between the two cell types, the main messages are as follows:

- Mammalian cells can survive ambient temperature pausing for up to 72–144 hours, if pH is neutral and stable.

- An element of cell death (10–30% for HOS TE85 and hMSCs) is unavoidable during the stresses of pausing and recovery, most likely driven by osmostress and changes to the cellular membrane.

- Cells will encounter a lag phase in fold growth expansion following pausing and the length of this delay is dependent on cell type.

- Time to recovery in terms of hMSC metabolic activity is similar regardless of time paused.

Initially, the cancer-derived osteoblastic cell line, HOS TE85, was used as a test system to examine the impact of keeping cells in ambient conditions for short-time periods. This provided a wealth of understanding surrounding the cell pausing process and biological mechanism. These early experiments demonstrated that HOS TE85 cells could survive ambient pausing conditions for up to 48 hours with a similar level of metabolic activity (PrestoBlue), proliferative capacity and ALP expression to non-paused cells. Although cell morphology changed during pausing as well as a higher level of suspended cells, the cells that were capable of attachment after their return to normal culture conditions regained their fibroblastic morphology. These findings suggest that even with no additional components to standard MEM medium, this cell type is somewhat resilient to the stresses endured during 48 hours ambient pausing. After this time however (>48 hours), there was a decline in membrane integrity and the cells were unable to re-attach to tissue culture plastic and proliferate when CO_2 was not controlled. With a stable atmospheric environment of 5% CO_2 or with the addition of 25mM HEPES to standard MEM medium, the pausing time frame was extended to 144 hours and cell viability was successfully maintained above 90%. Cells

were also capable of fold growth expansion over 5 passages. This led to the finding that a stable pH during pausing is perhaps more important than a stable temperature. The reasoning behind the non-significant improvement in cell viability with the anti-oxidant Trolox is uncertain; however, as only one concentration was used (1μM) it is fair to say that higher concentrations need to be investigated. Whilst cells kept in HTS-FRS at both 4°C and ambient temperature produced the highest membrane integrity results, cells were unsuccessful in their ability to ensure cell attachment and further growth. As this was an unexpected finding, it is possible that the complete medium change back to standard MEM medium for both HTS-FRS and PLA was detrimental to cells in terms of osmotic stress and membranous damage. Due to this, media additives may be a more successful method for short-term storage rather than a complete medium change and more thought into step wise cell recovery protocols is required.

As a result of the commercial media not performing as well as expected and the lack of improvement in viability observed with Trolox, for hMSC investigations DMEM was only supplemented with FBS, ultra-glutamine and 25mM HEPES. Results for the ambient temperature storage of hMSCs over 72 hours were extremely positive. Whilst an initial drop in viability was observed over the first 24 hours, viability was maintained ~70%, which is defined as safe by the US FDA. As some data points fell below 70%, work to counteract this using an apoptosis inhibitor may be the next step in ensuring as high viability as possible. As the cell is the product, regulatory bodies require proof that cells can remain as genetically and phenotypically unchanged as possible following a preservation procedure. Whilst there is no guarantee that cells for therapy will act with a certain therapeutic efficacy once *in vivo,* a panel of tests

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examining various cell parameters can be used to provide information on any cellular changes that may arise after the preservation procedure. Therefore, for the hMSCs, 24 hour attachment, fold growth expansion, CFU efficiency, immunophenotype and differentiation tests were performed to provide an overall picture of cells before and after ambient temperature pausing. To summarise, there was no difference was found between attachment efficiency, immunophenotye and tri-lineage differentiation potential. An extended lag phase upon the first passage after pausing with no atmospheric control was identified for the hMSCs. This phenomenon also occurred with the HOS TE85 upon the first two passages. For the effect of pausing stress to delay growth and proliferation for between 4–6 days after their return to normal conditions highlights the importance of these longer term tests. A significant drop in CFU efficiency was also encountered with lines M2 and M4 compared with the control, however the non-paused cells produced high levels of variation between experiments. More experimental repeats would be required to confirm this difference.

Overall, all the three donors performed similar in terms of morphology, viability, cell surface marker expression and differentiation. The only differences were the lower fold growth expansion rate of M3 compared with the other two cell lines and a non-significant reduction in CFU efficiency after pausing. In previous characterisation experiments however, M3 was found to be a slow growing cell line overall in terms of cumulative population doublings, specific growth rate and fold increase (Heathman *et al.* 2016). The fact that M3 is a slow grower in comparison to M2 and M4 may have been the reasoning behind the lack of significance in CFU efficiency; it may be that more time would be required to see the effect.

With regards to maintaining cells in a single cell suspension, it seems that the size of cellular aggregates may be more important than prevalence, as the larger the aggregate the more central cells will be more likely to encounter damage from hypoxia and lack of nutrients. Additionally, larger sized aggregates may increase the risk of promoting a reaction upon administration or causing a pulmonary embolism. Attempts to use a range of anti-clumping agents to curb cell-cell attachment were unsuccessful here, however it seems that cell 'stickiness' is cell type dependent as aggregation levels were considerably lower for hMSCs compared with HOS TE85s. As no guidance exists from regulatory bodies on cell aggregate size and what is safe to deliver to a patient, research in this area is required to provide information on how to best measure aggregation levels and size and what this means for patient administration.

As highlighted in literature review, methods employed to measure viability and cell quality vary considerably across research institutes and scientists, making the comparison of results difficult. As most papers investigating the effects of cell preservation describe the use of membrane integrity tests and colorimetric assays for measuring metabolic activity, these methods were focussed on throughout this project. What is apparent is that methods looking into cell preservation for the purpose of cell therapy need to be standardised; this will enable accurate comparisons of data and may lead to more efficient ways of progressing in this already small field.

Whilst this work has provided a further understanding of cell pausing, it has produced many questions including:

- How can optimum viability (>70%) be obtained during pausing?

- How large is the range of ambient temperature cells can be paused in to remain viable and functional?

- What is happening to cells during pausing at the genetic level?

This research has added to the limited work available on the ambient temperature pausing of mammalian cells, by looking at two cell types (HOS TE85 and bone-marrow derived hMSC) in depth and not only documenting initial viability but examining effects post pause.

7.2 FUTURE DIRECTION

Although an average viability of 70% was obtained when hMSCs were paused at ambient temperature, with some variation in the data and some results falling in the 60–70% range, work to ensure the range remains above 70% for the lowest data point is required from a regulatory standpoint. Inevitably, some cell death will occur during pausing, however the addition of an apoptosis inhibitor that helps prevent the mediators of apoptosis, c-Myc, Bax, p53, tBid, and BCL may limit cell death (<30%). Research including a range of different apoptotic inhibitors at various concentrations would be the next logical step for the hMSC work. Any additives however, would require regulatory approval.

With a wealth of literature on CSPs and their upregulation during low temperature storage, this would be an interesting area of research to follow on from investigations so far. Cold shock immediately before hypothermic exposure may improve a cells capacity for coping with subsequent cold induced stress. During low temperature exposure, the expression of certain proteins such as CSPs are elevated and are suggested to offer protection by slowing cellular metabolism, proliferation and other

energy-dependent systems (Nishiyama et al. 1997; Matijasevic et al. 1998; Ohnishi et al. 1998; Sonna et al. 2002). Whilst a cold-shock period was introduced in section 4.4 when examining temperature fluctuations during pausing, the timing of the cold-shock was after 24 hours at ambient temperature. This negatively impacted cell viability and fold growth capacity, possibly due to the sharp change in temperature soon after cells had acclimatised to ambient conditions. It will be valuable to investigate whether cold shock immediately before pausing may help cells survive and recover. Instead of adding warmed fresh medium to cells immediately before pausing, the addition of medium cooled to room temperature or 4°C may be enough to initiate the expression of CSPs and offer a protective mechanism. Whilst work using real time-PCR was initiated at the end of hMSC investigations, results that were achieved were too variable and of insufficient power to be included in this thesis. It will also be useful to identify if the upregulation of CSPs is ubiquitous across different cell types.

Donor cell variability is expected when considering the heterogeneity of different ages, health, ethnicity and gender. Three donors of varying characteristics were used for hMSC experiments in this project and testing more donors would strengthen the potential of pausing cells for therapy applications. It may even provide information on the characteristics of cells that cope better under pausing conditions than other methods of preservation.

With ambient temperature varying greatly around the world, it would be useful to know the cut off points at each end of the scale regarding temperature. The difficulty is developing a protocol that is capable of testing the spread of temperatures that cells may encounter during transport, in the most efficient way. This presents the question

of how large should the range of temperatures be to still guarantee a cell product of acceptable quality. Cells that have been stored outside of these ranges, measured by temperature monitors, would then be considered unusable for therapy. Knowing the upper and lower limits of temperatures exposure to ensure cellular health is important for the safety and regulation of cell therapy protocols.

One major limitation of this work is the use of FBS in standard medium, required for the successful culture of cells. Apart from the obvious xeno safety concerns, lot-to-lot variation in terms of signalling molecules, apoptotic factors and nutrients also creates problems for hMSC research. The use of hMSCs in clinical trials that have been previously expanded in FBS has led to some patients having anaphylactic reactions, and the risk of this is not ideal. Substituting FBS for a chemically defined medium which supports the growth of hMSCs is required for future research. With a wide variety of serum-free medium to choose from including Prime-XV® (Irvine Scientific®) and Stemulate™ (Cook Regentec), this would be a project in itself. The movement away from using animal derived components for medical research will ensure that work does not have to be repeated at later stages and will of course aid the progression of cell therapies towards regulatory approval.

7.3 THE BIGGER PICTURE

The innovative field of hypothermic preservation has advanced over the years with regards to mammalian tissues and organs; however, its application in stem cells is relatively new and opportunistic. Not only does cell preservation mean cells can be banked or stored until needed for use, it also permits time for transport to their clinical destination, whether for allogeneic or autologous therapy, and allows time for

the completion of quality release and safety testing. The focus of this project has been ambient cell storage, which is a simpler, cost effective method compared with chilled storage and cryopreservation. Following manufacture and distribution, short-term ambient cell preservation can facilitate the coordination of therapy with patient care regimens, which ultimately reduces the number of staff needed to deal with deliveries of cells and the expensive equipment needed to keep vials of cells fit for use. By comparison, if cells were cryopreserved and shipped to their destination, the complex thawing procedure would have to be performed at the medical site and will no doubt encounter differences in standards across hospitals.

A system has been designed to enable the examination of pausing mechanisms and critical factors which are required for effective short-term storage of cells for therapy. This positive work has demonstrated that hMSCs from three individual donors are capable of surviving the stresses of ambient temperature pausing for three days, which is a suitable time frame regarding the logistics of cell therapy. Encouraging results bode well for future investigations. This work not only supports the use of cell pausing for medical applications but also for laboratory research and within biotechnology. With many active clinical trials utilising hMSCs to treat a wide range of diseases, cell pausing offers another potential option for their storage and transport alongside chilled methods and cryopreservation. With more strategies to choose from, protocols can be tailored for the individual cell type and therapy procedure. Regenerative medicine is a rapidly emerging field and the preservation and transport of the cells used in these therapies is as important as the therapy itself. The effective

low temperature pausing of therapeutically relevant cells will assist with their application within the cell therapy arena.

APPENDIX A

PSEUDO LYTE A FORMULATION

As the US Federal Law restricts the use of Plasma-Lyte A to licensed physicians or veterinarians, an in-house formulation (Pseudo-Lyte A) was used instead for experiments throughout chapter 5. The formulation was based on the composition of Plasma-Lyte A listed by manufacturers (Baxter International Inc.).

Per 1000mL

- Sodium chloride (NaCL) – 5260mg

- Sodium gluconate ($C_6H_{11}NaO$) – 5020mg

- Sodium acetate Trihydrate ($C_2H_3NaO_2 \bullet 3H_2O$) – 3680mg

- Potassium choride (KCl) – 370mg

- Magnesium chloride ($MgCl_2 \bullet 6H_2O$) – 300mg

- pH was adjusted to ~7.4 with sodium hydroxide (NaOH)

- Osmolarity ranged between 280–310 mOsmol/L

HOS TE85 CELL FOLD GROWTH EXPANSION FOLLOWING CHILLED STORAGE

In section 5.2.4, alongside fold growth expansion experiments for cells paused in ambient conditions, experiments were also performed for cells chilled at 2–8°C for 96 and 144 hours. Few cells in MHT, HTS and PLA were able to attach to tissue culture plastic and survive in terms of membrane integrity following pausing; however, all cells regardless of media, were unable to attach and proliferate past P1 (figure B1). Data is not shown for 144 hours as cells were unable to demonstrate attachment ability immediately after pausing in chilled conditions.
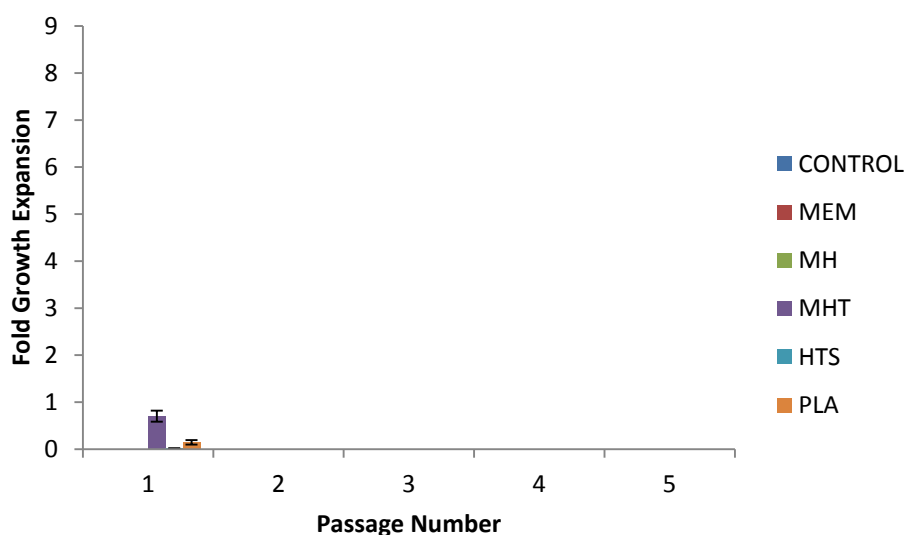

Figure B1: HOS TE85 cell fold growth expansion when paused at 4°C for 96 hours in various media (n=2). Error bars show the standard deviation between independent experimental repeats.

ASSESSMENT OF ANTI-CLUMPING AGENTS BY MORPHOLOGY

For single cell suspension experiments (section 5.4), a high and low concentration of each anti-clumping agent was tested, as shown in table 6. To help decide what concentrations would be best to use, HOS TE85 cells were cultured for 24 hours in the presence of various different concentrations of each anti-clumping agent, typical for use in cell culture. The agents were assessed for their toxicity effects on the cell membrane via morphology and membrane integrity. Unfortunately, due to the high amount of cell clumping and increased viscosity of the cell medium with certain anti-clumping agents and concentrations, many of the samples could not be effectively ran on the NC-3000. Therefore, the concentrations chosen were based on the images acquired (figures C1–C6) with an optical light microscope after 24 hours culture and were compared with control cell morphology. Briefly, cells cultured in various concentrations of EDTA (0.01–1mM) had no clear change in morphology (figure C1). The only difference to the control was that cell size marginally increased and cells were more spread out on the surface as concentration increased. Due to this, 0.1mM was chosen as the low concentration and 1mM was chosen as the highest concentration. Interestingly, increasing concentrations of P68 seemed to encourage cell aggregation (figure C2); therefore, the highest concentration used was 1% (v/v). Cells cultured in Tween-80 looked more triangular and granular in appearance than control cells at all concentrations used and a higher proportion of these cells were detached from the surface (figure C3). Due to this abnormal HOS TE85 morphology, it was decided that Tween-80 would not be carried through to single cell suspension experiments. The manufacturers of Agent A (anti-clumping agent) suggested the use

of between 0.01–1% (v/v) to use in cell culture (Gibco®). As expected, cell morphology appeared similar to the control at all three concentrations (figure C4). Therefore, 0.01% and 1% (v/v) were used in experiments. For PPG and PEG, 0.05% and 2% (v/v) were chosen for the low and high concentrations. Cell morphology remained similar to the control with all concentrations of PEG tested (figure C5). For PPG, attached cells had a typical fibroblastic appearance; however, at 2% the cells were more dispersed and some cells were suspended, possibly attributable to the surfactant properties of PPG (figure C6).

This experiment (n=1), was performed in order to help justify the high and low concentrations used in following single cell suspension experiments with cells in suspension.

Figure C1: HOS TE85 cells incubated for 24 Hours at 37°C in complete MEM media with various concentrations of EDTA (scale bar = 100 microns).

Figure C2: HOS TE85 cells incubated for 24 Hours at 37°C in complete MEM media with various concentrations of Pluronic F-68 (scale bar = 100 microns).
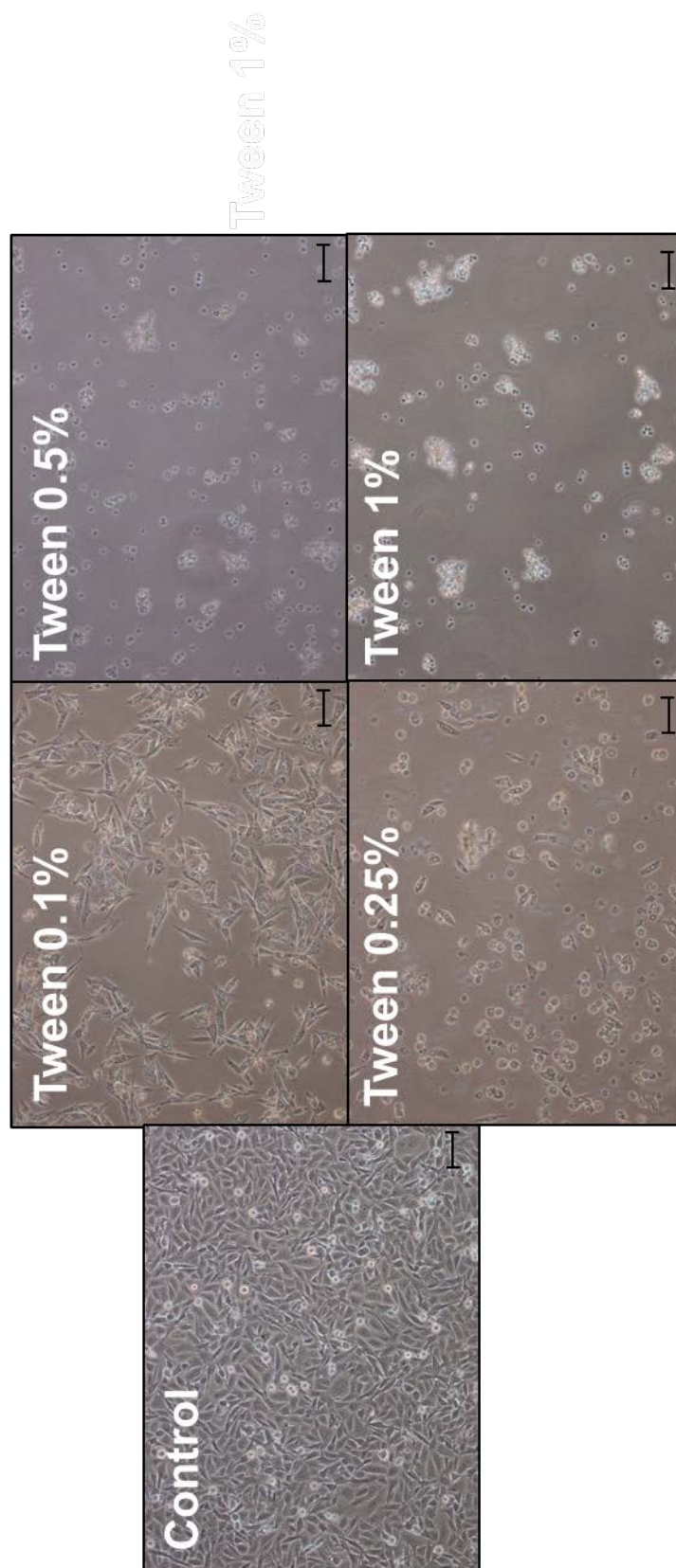
Figure C3: HOS TE85 cells incubated for 24 Hours at 37°C in complete MEM media with various concentrations of Tween 80 (scale bar = 100 microns).

Figure C4: HOS TE85 cells incubated for 24 Hours at 37°C in complete MEM media with various concentrations of Agent A (scale bar = 100 microns).

Figure C5: HOS TE85 cells incubated for 24 Hours at 37°C in complete MEM media with various concentrations of polyethylene glycol (scale bar = 100 microns).
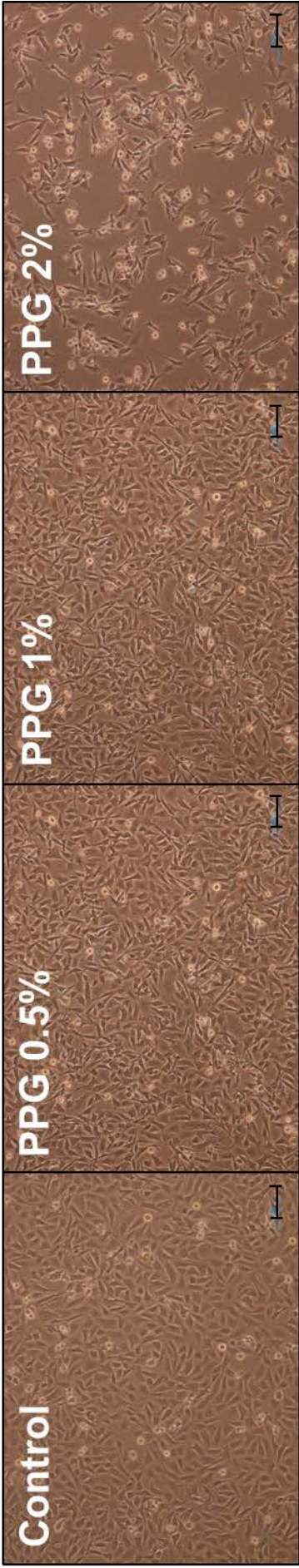
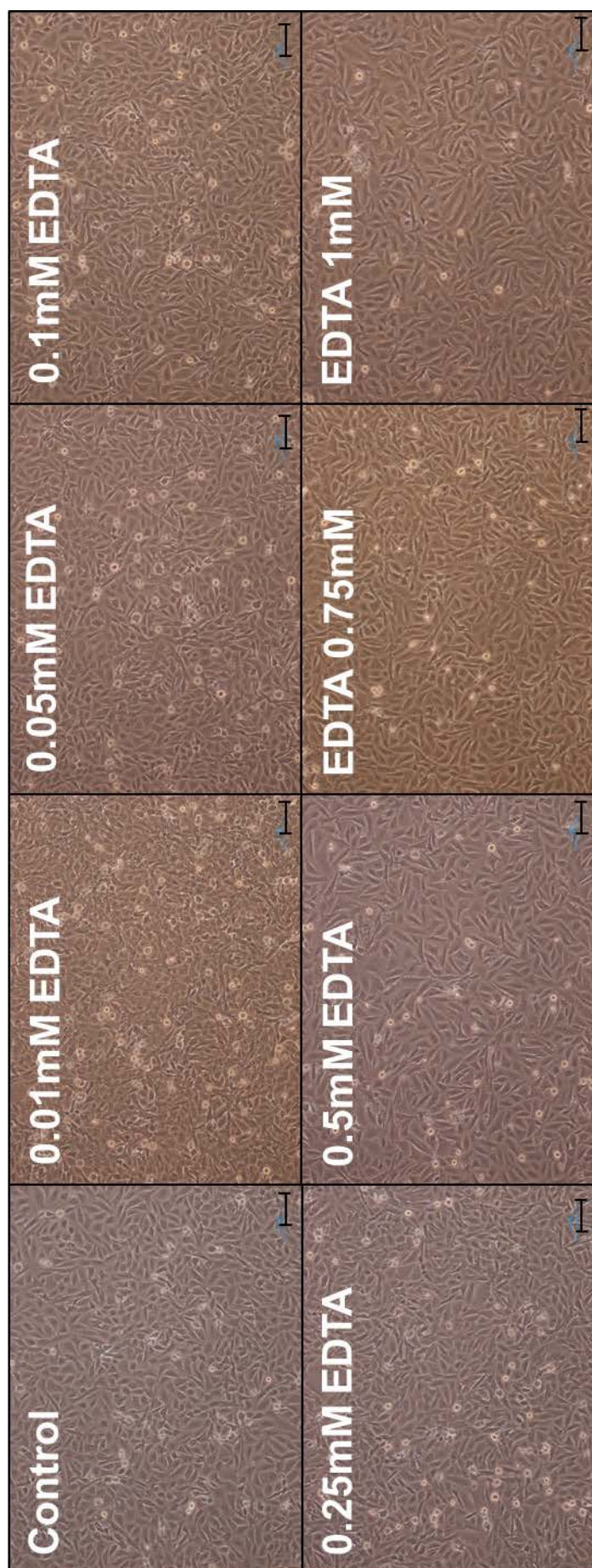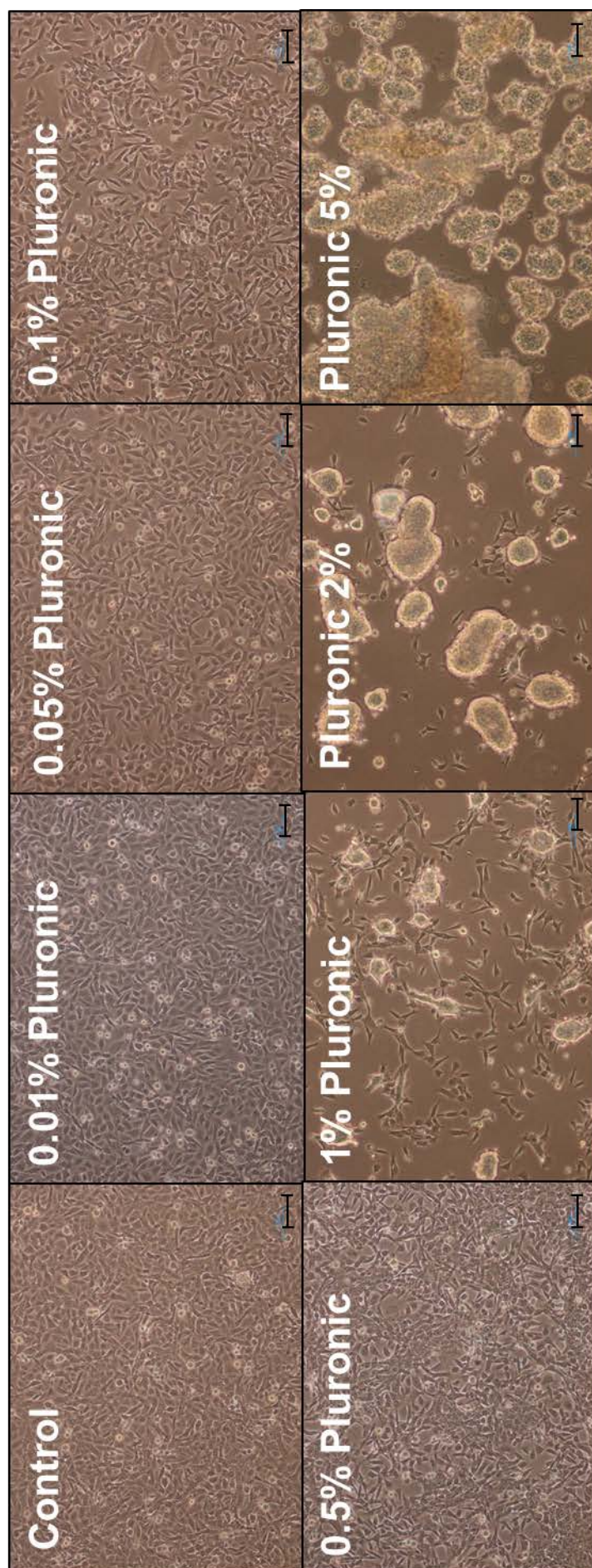**Figure C6: HOS TE85 cells incubated for 24 Hours at 37°C in complete MEM media with various concentrations of polypropylene glycol** (scale bar = 100 microns).
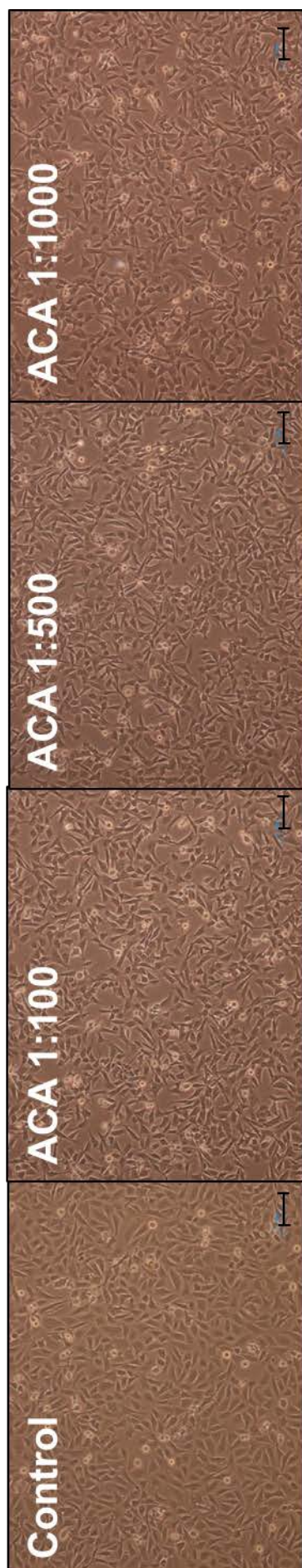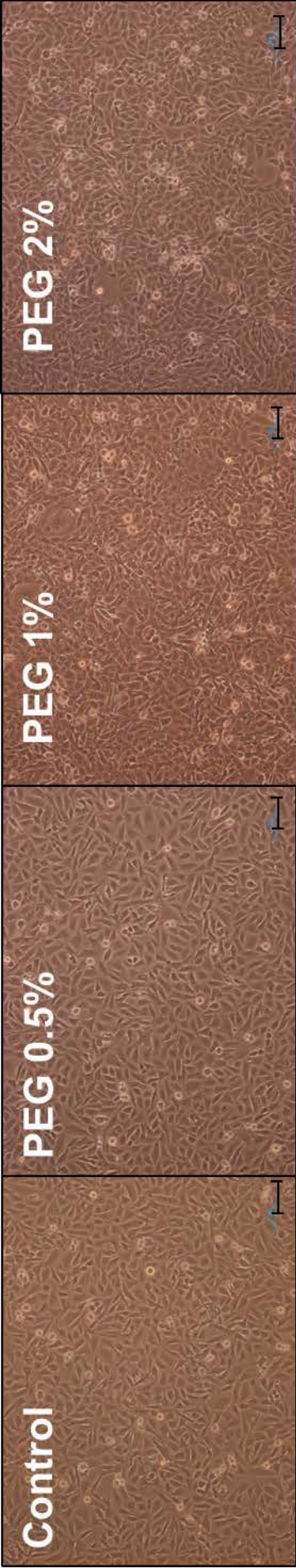
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