Improving Transport and Storage of Mesenchymal Stem Cells through Investigations into Their Energy Metabolism

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Abstract

Mesenchymal stem cells (MSCs) are plastic adherent spindle shaped cells that express stem cell markers CD73, CD90 and CD105, while lacking leukocyte, endothelial and haematopoietic markers such as CD34 and CD45. They can differentiate into a range of different cell types. Compared to embryonic stem cells, they are easier to purify and can be used for allogenic therapy. As of 2021, over 900 trials have taken place, primarily involving tissue repair, treatment of immune disorders and more recently, COVID-19. Cryopreservation damages cells, while current non-freezing methods require cold temperature, expensive special materials or serum, and have not been tested beyond 7 days. The project aims to improve on current work by using a simple and chemically defined medium to maintain cell survival, consisting of a DMEM base to provide the necessary substrates, vitamins, salts and amino acids, insulin supplement (ITSE) to keep cells viable in the absence of serum and a low bicarbonate buffer to maintain physiological pH at atmospheric CO_2.

The medium was developed at 37°C using hTERT MSC monolayers. When hTERT human MSCs were cultured in the insulin based preservation medium, cells proliferated between day 6 and 12, while in DMEM basal medium cell death occurred. Cell monolayers in ITSE based medium displayed higher levels of oxidative stress, but no significant difference in autophagy compared to cells in expansion media (DMEM/FBS). hTERT MSCs in ITSE based media displayed lower levels of glucose consumption, lactate production and proliferation, and displayed a wire like morphology. When seeded in DMEM/FBS after 12 days in ITSE based media, metabolic and morphological changes were reversed, and cells retained proliferation and differentiation potential.

The ITSE based medium was then adapted to work at room temperature by lowering the bicarbonate concentration from 44 mM to 0.9 mM. The majority of hTERT human and primary bovine MSCs remained viable as monolayers by day 12. hTERT MSCs retained proliferation potential, but primary bovine MSCs remained permanently anchored to the flask. When preserved as 3D structures in alginate beads, at 12k cells/bead and 360k cells/mL of ITSE based media, both hTERT and primary MSCs remained viable and functional for 8 days. At this point the limiting factor was lack of serum and pH acidity. In alginate beads at atmospheric conditions, hTERT MSCs still underwent low levels of glycolysis over 6 days. For primary MSCs, metabolite changes over 6 days were too low to measure.

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Statement of Originality

I declare that the work described in this thesis is entirely my own, and to the best of my knowledge it contains no content from previously published work or any materials submitted for a degree, except where due acknowledgement is made. Any input made by others is explicitly acknowledged in this thesis.

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List of Abbreviations

2-DG	2-deoxy-D-glucose
6-OHDA	6-hydroxydopamine
α-MEM	Alpha minimal essential medium
AGEs	Advanced glycation end products
AMI	Acute myocardial infarction
AMP	Adenosine monophosphate
APC	Allophycocyanin
APP	Amyloid precursor proteins
ARDS	Acute respiratory distress syndrome
ARG-1	Arginase-1
ATP	Adenosine triphosphate
AUC	Area under curve
BAC	Beta antigen c
bFGF	Basic fibroblast growth factor
CoA	Coenzyme-A
COVID-19	Coronavirus disease 2019
DAPI	4’,6-diamidino-2-phenylindole
DCs	Dendritic cells
DCFDA	2',7'-dichlorodihydrofluorescein diacetate
DMEM	Dulbecco’s Modified Eagle’s Medium
DMEM/FBS	DMEM expansion media + 10% FBS
DMSO	Dimethyl sulfoxide

E4P	Erythrose 4-phosphate
ECAR	Extracellular acidification rate
EGF	Epidermal growth factor
ESCs	Embryonic Stem Cells
FAD/ $FADH_2$	Flavin adenine dinucleotide
FBS	Foetal bovine serum
FCCP	Carbonyl cyanide-p-trifluoromethoxyphenylhydrazone
FEV1	Forced expiration volume
FITC	Fluorescein isothiocyanate
FMN	Flavin mononucleotide
G6P	Glucose-6-phosphate
GC-MS	Gas chromatography and mass spectroscopy
GFP	Green fluorescent protein
GMP	Guanine monophosphate
HBSS	Hank's balanced salt solution
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
HPA	Hydroxyapatite
HSA	Human serum albumin
hTERT	Human telomerase reverse transcriptase
ICMP	Ischaemic cardiomyopathy
IDO	Indolamine-2,3-dioxygenase
IL- (1β, 2-10)	Interleukin 1β, 2-10
iNOS	Inducible nitric oxide synthetase

ITSE	Insulin-transferrin-selenium-ethanolamine supplement
mAb	Monoclonal antibody
MCL-1	Induced myeloid leukaemia cell differentiation protein Mcl-1
M-CSF	Macrophage colony stimulating factor
MCT	Monocarboxylate
MHC	Major histocompatibility complex
MI	Myocardial infarction
MND	Motor neurone disease
MOPS	3-(N-morpholino) propanesulfonic acid
MPC	Mesenchymal precursor cells
MSCs	Mesenchymal Stem Cells
NAC	N-Acetyl cysteine
NK cells	Natural killer cells
NO	Nitric oxide
OCR	Oxygen consumption rate
OD	Optical density
OXPHOS	Oxidative phosphorylation
PBMCs	Peripheral blood mononuclear cells
PE	Phycoerythrin
PBS	Phosphate buffered saline
PGE2	Prostaglandin E2
PI	Propidium iodide
qPCR	Real time polymerase chain reaction

ROS	Reactive oxygen species
SBS	sucrose based solution
SCDF-1α	Stromal cell derived factor-1 alpha
SG	Sulphated glycosaminoglycans
SO	Safranin-O
Stat-3	Signal transducer and activator of transcription 3
TCA	Citric acid cycle
TGF-β1	Transforming growth factor beta-1
TNF-α	Tissue necrosis factor-alpha
UW	University of Wisconsin solution
VEGF	Vascular endothelial growth factor
VPA	Valproic acid

Chapter 1

Introduction and Project Aims

1.1 – What are Mesenchymal Stem Cells?

1.1.1 Characterisation of Mesenchymal Stem Cells

Freidenstein *et al.* (1970) reported that the guinea pig bone marrow consisted of both haematopoietic stem cells and plastic adherent cells, the latter of which rapidly proliferated *in vitro* and displayed osteogenic potential (1). Over the next few decades the multipotency of these plastic adherent cells was demonstrated: they were shown to differentiate into osteoblasts, adipocytes, chondrocytes in the 1980s (2), myocytes (3) and astrocytes (4) in the 1990s and hepatocytes (5) and cardiomyocytes (6) in the 21st century. The term "mesenchymal stem cells" was coined by Caplan (1991), after reporting their involvement in the development of embryonic mesenchymal tissues (7). Human bone marrow mesenchymal stem cells (BMMSCs) have also been shown to reversibly inhibit T-lymphocytes (8). Their differentiation and immunosuppressive properties prompted their investigation for the treatment of tissue damage and inflammatory diseases.

Da Silva Meirelles *et al.* (2006) reported that MSCs can be isolated from the bone marrow of mice,alongside the liver, spleen, pancreas, lung, kidney, aorta, vena cava, brain and muscle (9). They concluded that MSCs can be isolated from all post-natal organs and tissue (9).

The criteria defined by the international society for cell therapy (ISCT) for MSCs is as follows: adherence to plastic during culture, the expression (≥95%) of cell surface markers CD73, CD90, CD105, the absence (≤2%) of haematopoietic and endothelial markers such as CD34, CD45, HLA-DR, CD14, CD11b, CD79a or CD19, and the ability to differentiate into osteoblasts, adipocytes and chondrocytes *in vitro* (10). Differentiation lineages for MSCs are shown in **Figure 1.1.**

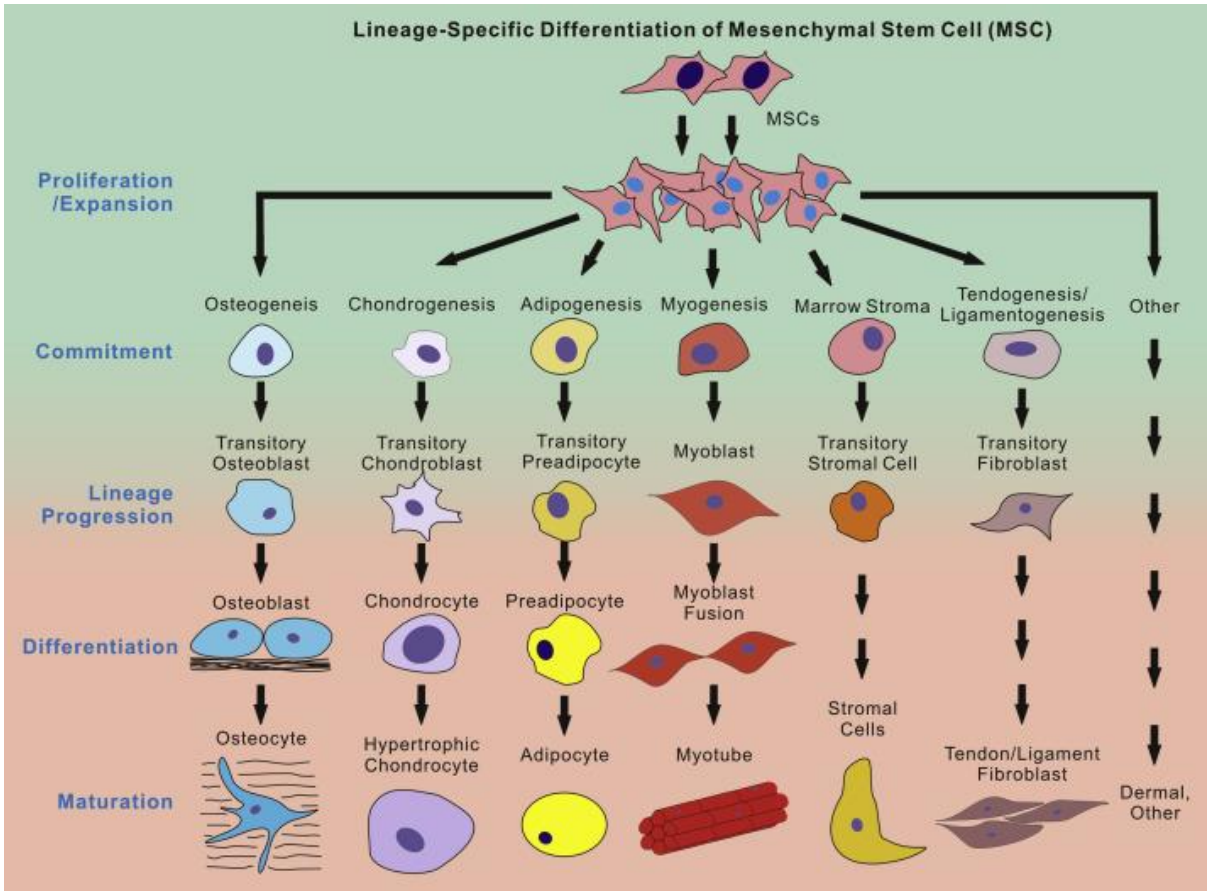

Figure 1.1: Multipotency of MSCs. MSCs can differentiate into a range of cell types, such as osteocytes, chondrocytes and adipocytes. From (11) with permission from Elsevier Copyright © via CC BY-NC-ND 4.0 license (https://creativecommons.org/licenses/by-nc-nd/4.0/)

1.1.2 Advantages and Disadvantages Compared to Embryonic Stem Cells

MSCs have a lower risk of tumour formation compared to embryonic stem cells (ESCs) due to replicative senescence. MSCs undergo asymmetric cell division, where they yield a mixture of rapidly proliferating spindle shaped cells, rapidly proliferating small and round cells, and slowly proliferating large and flat cells (12). During the expansion of human adipose MSCs, Schellenberg *et al.* (2012) reported that <5% of cells retain colony forming potential at passage 10 and <1% at passage 20 (13), which means that MSCs lose proliferation potential with increasing passage number. In ESCs, telomerase activity is maintained, which allows indefinite cell division (14). This makes it easier to establish a cell bank of ESCs. Also research involving ESCs may generate controversy due to their potential to become humans, and therefore may face opposition from religious groups (15). MSCs can release various growth factors required for cell proliferation, cell migration, angiogenesis and blood vessel maturation, making them useful in tissue repair. Secreted growth factors and cytokines are summarised in **Table 1.1** (16).

Table 1.1: Growth factors and other signaling molecules released by MSCs. Table reproduced from Nie *et al.* (16).

Type of Secreted Factors	Active Molecules
Hematopoietic growth factors (17) (18) (19)	SCF, FLT3LG, Thrombopoietin, IL-3, IL-6, GM-CSF, M-CSF
Angiogenic growth factors (17) (18) (20) (21)	HGF, VEGF, Angiopoietin, PDGF, IGF-1, FGF-2, FGF-4, FGF-7
Trophic molecules (17) (18) (22)	Adiponectin, Adrenomedullin, Osteoprotegerin, MMP10, MMP13, TIMP-1, TIMP-2, TIMP-3, TIMP-4, Leptin, IGFBP-1, IGFBP-2, IGFBP-3, IGFBP-4, BDNF, GDNF, NGF, PIGF
Immunomodulatory cytokines (18) (19) (23)	IL-1α, IL-1β, IL-2, TSG-6, OSM, IL-7, IL-10, IL-11, IL-12, IL-13, IL-16, IFN-γ, TNF-α, LIF, TGF-β, MIF
Chemokines (17) (18) (24) (25)	CCL1, CCL2, CCL5, CCL8, CCL11, CCL16, CCL18, CCL22, CCL23, CCL24, CCL26, CXCL1, CXCL2, CXCL3, CXCL5, CXCL6, CXCL8, CXCL11, CXCL12, CXCL13, CX3CL1, XCL1

Abbreviations: SCF, stem cell factor; FLT3LG, Fms-related tyrosine kinase 3 ligand; IL, interleukin; GM-CSF, granulocyte macrophage colony-stimulating factor; M-CSF, macrophage colony-stimulating factor; HGF, hepatocyte growth factor; VEGF, vascular endothelial growth factors; PDGF, platelet-derived growth factor; IGF, insulin-like growth factor; FGF, fibroblast growth factor; MMP, matrix metalloproteinase; TIMP, tissue inhibitor of metalloproteinase; IGFBP, insulin-like growth factor-binding protein; BDNF, brain-derived neurotrophic factor; GDNF, glial cell-derived neurotrophic factor; NGF, nerve growth factor; PIGF, placenta growth factor; TSG, tumor necrosis factor-stimulated gene; OSM, oncostatin; IFN, interferon; TNF, tumor necrosis factor; LIF, leukemia inhibitory factor; TGF, transforming growth factor; MIF, macrophage migration inhibitory factor; CCL, C-C motif chemokine ligand; CXCL, C-X-C motif chemokine ligand; CX3CL, C-X3-C motif chemokine ligand; XCL, X-C motif chemokine ligand.

MSCs can be harvested from any tissue, peripheral blood (26), umbilical cord blood (27), amniotic fluid and placenta, and are easier to isolate than ESCs and induced pluripotent stem cells (iPSCs). For example, plating bovine bone marrow directly into culture media and subsequent 48 hour media changes have been shown to yield a high purity of MSCs, where qPCR demonstrated high expression of CD73, CD90 and CD105, and no or minimal expression of CD34 and CD45 (28).

Abdullah *et al.* (2018) reported that mice BMMSCs can be obtained direct plating, where media changes were performed after 24 hours (29). Immunohistochemical staining of mice BMMSCs has shown that all cells stained for MSC markers CD44, CD90 and CD105, while none stained positive for CD34, a haematopoietic and endothelial cell marker. However, Baddoo *et al.* (2003) reported that media changes 72 hours after plating of mice bone marrow leads to a low purity of MSCs, where 46.8% of cells were cd11b positive (macrophages) and 36.69% test positive for CD45 (haematopoietic cells), where marker expression was studied using flow cytometry (30). Kannan *et al.* (2019) reported that direct plating of porcine bone marrow in α-MEM and subsequent 24 hour media change yields only cells that express CD90 and CD105, where immunostaining with CD90, CD105 and 4',6-diamidino-2-phenylindole (DAPI) was used (31). These studies demonstrate that media changes must be performed within 24-48 hours after direct plating of marrow to guarantee a pure MSC sample.

Extraction of ESCs is more complex, as it requires a fertilised embryo (32).

Furthermore, MSC efficacy falls with age: the % of attached MSCs in 1 year old mice did not exceed 40% at 24 hours after plating, compared to 80% attachment for 6 week and 6 day old mice (33). Proliferation rate over an 8 day period decreased with age: 500% increase in cell count for the 6 day group, 400% for the 6 week group and 300% for the 1 year group (33). Older mice experienced more rapid losses of trilineage differentiation potential with increasing passage number (34). Duscher *et al.* (2014) reported weaker angiogenic, differentiation and wound healing potential in the adipocyte mesenchymal stem cell (ASCs) of 21 month old mice over 3 month old mice, but no change in proliferation capacity (35). BMMSCs from human patients with ischaemic cardiomyopathy (ICMP) demonstrated roughly a 30% loss of angiogenic potential and half the production of VEGF compared to healthy patients (36). Advanced glycation end products (AGEs), present in higher levels in elderly and diabetic patients, have been shown to reduce human MSC survival by half; in addition they reported a 2-3x increase in ROS production when MSCs were cultured with AGE concentrations ranging 10-100 μg/mL (34). This may suggest that MSCs derived from elderly or diabetic patients may be less potent.

Overall, the research suggests that MSCs are damaged in elderly and diabetic patients, or patients with cardiovascular disease. As a result, autologous therapy may not be possible for these patient groups. Allogenic MSCs may be required for these patient groups.

When placed in culture medium and stored at atmospheric conditions (room temperature and 0.04% CO_2) as monolayers, BMMSCs do not remain viable for long periods of time following extraction and must therefore be preserved during transit. The current method is cryopreservation in 90% foetal bovine serum (FBS) and 10% dimethyl sulfoxide (DMSO). DMSO has been shown to disrupt mitochondrial integrity and membrane potential in cultured astrocytes, disrupt the structure of the actin cytoskeleton in MSCs and cause inflammatory responses in patients (37). It is impossible to avoid the use of DMSO completely; cell damage has been reported with concentrations below 2%. (38). MSCs are less potent in elderly and diabetic patients, so autologous therapy may not always be suitable (39) (34).

Current non-freezing methods involve the use of isotonic solutions (40-42), gels (43, 44) or spheroid culture to keep MSCs in a quiescent state (48), enabling them to withstand colder temperatures and atmospheric CO_2. More recently, MSCs have been encapsulated in alginate beads, enabling them to withstand a range of temperatures (4-37°C) and shock (43, 44). Current

preservation methods will be discussed in further detail in **Chapter 2.** However, preservation times have not been tested beyond 7 days for any solution thus far. Additionally, current methods require cold temperature or serum.

The project aimed to investigate if a combination of Dulbecco's Modified Eagle's Medium (DMEM) and insulin based supplement (ITSE) could be used to keep bone marrow mesenchymal stem cells (BMMSCs) viable and functional for long periods of time in the absence of cryopreservation, refrigeration and serum to aid their clinical applications.

1.2 – Proposed Goals for the Thesis

The first objective was to expand and characterise both primary bovine and immortal human MSCs, and study proliferation, glucose consumption, lactate production, and morphology under standard culture conditions (monolayer, 37°C, 5% CO_2). With the hTERT MSCs, the effect of low glucose on proliferation and metabolism was studied; which confirms whether culture can be carried out at lower starting glucose concentrations, and create a Lineweaver-Burke plot, which would yield the Michaelis constant (K_m). Proliferation, metabolism and morphology of hTERT MSC monolayers under 25°C and 4°C were also studied to determine if cells survive and use glucose under room temperature and cold storage. With primary bovine MSCs, the effect of 1 year cryopreservation on proliferation and metabolism was investigated.

The second goal was to develop the insulin-transferrin-selenium-ethanolamine (ITSE) based medium using hTERT MSC monolayers at 37°C and 5% CO_2, and to study the differences between cells cultured in DMEM/FBS and ITSE based preservation media. Serum cannot be used in a clinical setting, so the use of ITSE an alternative was investigated. Firstly, cells were cultured for 12 days in blank DMEM and DMEM with 1% ITSE supplement. The number of attached cells were measured throughout, to determine whether the ITSE supplement was required to prevent cell death in the absence of serum. Since cells synthesize pyruvate as a result of glycolysis, the effect of media pyruvate removal on cell growth and metabolism in ITSE based medium was studied. The effect of ITSE based media culture on cell metabolism, proliferation, and morphology and trilineage differentiation potential was measured. Unregulated levels of reactive oxygen species (ROS) may damage cells, by oxidation and nitration of lipids, proteins, RNA and DNA (45). However, regulated ROS production is required for cell proliferation, differentiation, survival and apoptosis (45). The project also aimed to investigate whether ROS production rises during preservation in ITSE based medium, and whether autophagy increases as an indication of damaged cell components.

To eliminate the need for incubators and CO_2 cylinders, the final goal was to preserve both primary bovine and human hTERT MSCs in 3D at atmospheric conditions (20°C, 0.04% CO_2), maintaining >70% survival, proliferation and differentiation after 12 days. Culture media typically uses 44 mM bicarbonate, which rapidly turns alkaline at atmospheric CO_2. Therefore a range of alternative buffers were tested, where the formulation was deemed suitable if complete cell attachment and elongation occurred by day 2 of culture. Cells were first preserved as monolayers for 12 days, measuring survival and pH at regular intervals. The preservation experiments were repeated in 3D, both in suspension culture and in alginate beads. Specific glucose consumption and lactate production in alginate beads were measured to estimate the number of cells that can be packed per mL of media. Previous authors have not tested cell survival and function beyond 7 days in the published literature. Most did not test longer times than their original target, where cell survival rates were approx. 70% or higher, so it is not known at which point cell survival rates become low (<40%). Longer times of 12 days have been tested in this project to determine when significant cell death occurs, which in turn will allow the determination of limiting factors that prevent long term survival. Additionally, extending preservation times to 12 days may allow greater treatment flexibility, as the patient can reschedule treatments at later times. Usually, the cell processing site is separate from the clinic. Achievement of longer preservation times will also reduce the amount of cells wasted should a delay occur during transit, particularly during international transport. It will allow more accurate study of metabolic requirements, since metabolite changes will be larger and therefore more easily measured.

The following challenges below in media formulation and cell preservation, along with proposed resolutions is summarised in **Table 1.2**.

Table 1.2: Current Challenges in MSC Preservation

Drawbacks of current media and preservation methods	Proposed solution
MSCs cannot survive without serum, yet serum cannot be used in a clinical setting	Used a proprietary insulin supplement containing insulin, selenium and transferrin to maintain cell survival without serum.
44 mM bicarbonate is not suitable for atmospheric CO_2	Tested combinations of phosphate, MOPS, HEPES and reduced bicarbonate buffers and ensured that they maintain pH 7-8 over 12 days without compromising cell survival
MSC glucose consumption, lactate production and amino acid consumption during preservation has not been studied.	Measured glucose and lactate concentrations of spent novel preservation media to determine the optimal number of cells per mL to use. Employed GC-MS to determine which amino acids and vitamins are needed for MSCs in ITSE based medium.

1.2.1 – Thesis Outline

The overall aim of this project was to determine if an insulin based supplement can keep MSCs viable and functional for up to 12 days in the absence of serum, cryopreservation and refrigeration, using DMEM as a base medium along with a buffer to maintain physiological pH at atmospheric conditions.

Chapter 2 consisted of a literature review of current research regarding MSC metabolism and preservation, with additional information on trials involving the use of MSCs to treat various diseases. The drawbacks of current preservation methods in this chapter have been discussed.

In **Chapter 3**, the experimental protocols were described in detail. This involved primary cell extraction, cell expansion, and characterisation. It also involved the study of glucose consumption, lactate production, amino acid consumption and assessment of differentiation potential. The methods used to study intracellular ROS production, autophagy were described.

The purpose of **Chapter 4** was to characterise both the primary bovine and human hTERT MSCs, and study monolayer proliferation and metabolic activity under standard culture conditions. For hTERT MSCs gas chromatography and mass spectroscopy (GC-MS) was carried out to determine

amino acid and vitamin requirements during expansion, and how proliferation, metabolism and morphology changed at 25°C and at 4°C. For the primary MSCs, the effect of prolonged cryopreservation on growth and metabolism was studied.

The ITSE based medium was developed using hTERT MSCs in **Chapter 5.** DMEM basal medium was supplemented with ITSE to determine if it could prevent cell death over a 12 day period in the absence of serum. The proliferation, metabolism, oxidative stress, autophagy and morphology was compared for cells in DMEM/FBS and in ITSE based media. Since cells required extracellular pyruvate, the effect of low and zero pyruvate for cells cultured in ITSE was studied. GC-MS was employed to determine amino acid and vitamin requirements during preservation. The recovery of cell proliferation, differentiation potential, metabolic activity and morphology was assessed following 12 days in ITSE preservation as monolayers at 37°C.

In **Chapter 6**, the ITSE based medium was adapted to work at atmospheric conditions (20°C and 0.04% CO_2) by using a low bicarbonate buffer. hTERT and primary MSC survival in monolayer over a 12 day period was tested. Experiments were then repeated in 3D in suspension, 4 mm beads with 49k cells/bead and 2-2.7 mm beads with 12k cells per bead. For the 12k cells/bead group, proliferation and differentiation potential after 8 days preservation was assessed, and the lack of serum components as limiting factor for cell survival beyond 8 days was investigated. Glucose consumption and lactate production during alginate preservation was measured to determine the number of cells that can be stored per mL of ITSE based media. Bead size distribution for both 4 mm beads made by the syringe pump and 2-2.7 mm beads made by hand have been characterised.

Contributions to the field of MSC preservation were highlighted in **Chapter 7**, and discussed limitations to the thesis and proposed future work to address them.

1.2.2 – Central Hypothesis

In the absence of serum, supplementation of DMEM basal media with ITSE will improve cell survival compared to basal DMEM alone, and keep MSCs functional in the absence of refrigeration, cryopreservation or serum. Previous authors have demonstrated that at 37°C and 5% CO_2 cells can survive in basal media and ITSE supplement in the absence of serum (46) (47), and that MSCs can survive for 7 days in culture media at room temperature and atmospheric CO_2 (48), providing they are cultured as spheroids in alpha minimum essential medium and serum. However no author has measured pH during preservation or developed a suitable buffer for 0.04% CO_2. Preservation times can be further extended to 12 days by using an alternative buffer to maintain media pH at physiological levels during preservation.

Chapter 2

Literature Review

2.1 – MSC Immunology

Administration of MSCs in a clinical setting requires 1-10 million cells per kg of body weight (49), yet MSCs are found in very low concentrations in the bone marrow, where Pittenger *et al.* (1999) reported that 0.01 – 0.001% of cells from isolated human marrow adhered and proliferated as MSCs (50). Therefore it is important to establish a MSC cell bank in advance for allogeneic therapy, where there may be an immediate need for cells. MSCs have immunomodulatory properties (51), due to low expression of MHC class I, CD40, CD80, and CD86 and lack of expression of MHC class II leading to the absence of T-cell activation (52). However, loss of function and survival in MSCs have been reported after infusion *in vivo* due to activation of the complement system of the innate immunity upon contact with serum (53). Complement mediated cytotoxicity in MSCs can be reduced by CD55 overexpression in MSCs, MSC treatment with complement inhibitors such as anti-C5 IgGs or autologous therapy (53).

2.1.1 – MSC Interactions with T-cells

Tse *et al.* (2003) reported that human bone marrow mesenchymal stem cells (BMMSCs) suppressed T-cell proliferation, where the T-cells were stimulated by CD3, CD28 or allogenic peripheral blood mononuclear cells (PBMCs)(54). No apoptosis of T-cells was observed. (54). Separation of MSCs and T-cells using a semi permeable membrane showed a partial loss in T-cell suppression, which may suggest that T-cell suppression is partly due to cell contact (54). An increase in media prostaglandin E2 (PGE2) was observed when MSCs were co-cultured with T cells, in comparison to MSCs alone or T cells alone. T-cell suppression by MSCs was abolished upon supplementation with PGE2 inhibitor indomethacin. Overall this study indicates that MSCs reversibly suppress T-cell proliferation and activation by cell contact and the production of PGE2 (54).

Sato *et al.* (2005) reported that mice MSCs produce nitric oxide (NO) when co cultured with concanavalin A activated splenocytes, or with activated $CD4^+$ and/or $CD8^+$ T-cells (5). Increasing the number of MSCs lead to a dose dependent increase in NO production (5).NO production did not occur in MSCs when cultured with inactive splenocytes or B-cells (5). NO production was partially abolished when MSCs were separated from splenocytes using a partially permeable membrane, and T-cell inhibition was absent in inducible nitric oxide synthetase (iNOS) knockout mice (5). This demonstrates that MSC produced NO is a mediator of immune cell suppression.

2.1.2 – MSC Interaction with Natural Killer (NK) Cells

Krampera *et al.* (2006) reported that human BMMSCs inhibit natural killer (NK) cell proliferation but only if the MSCs: T-cells ratio does not fall below 1:10. Upon the addition of MSCs to NK cells, a reduction in media interferon gamma (IFN-γ) was reported (55). The inhibitory effect of MSCs was abolished by an anti IFN-γ monoclonal antibody (mAb) (55). The results show that lymphocyte suppression by MSCs is dependent on IFN-γ production by the target lymphocyte. IFN-γ upregulates Indolamine-2,3-dioxygenase (IDO) in MSCs, which converts tryptophan to kynurenine, exerting an inhibitory effect on lymphocytes by tryptophan depletion (55).

2.1.3 – MSC Interaction with Neutrophils

Human MSCs have been shown to prevent spontaneous apoptosis of both inactive and IL-8 activated neutrophils, where the greatest anti-apoptotic effect was observed at MSC: neutrophil ratios between 1:1 and 1:100 (56). The anti-apoptotic effect was diminished at ratios exceeding 1:500, and was abolished at a ratio of 1:2000 (56). When neutrophils were co-cultured with MSCs, the authors observed a fall in the expression of apoptotic protein beta antigen C (BAC) and an upregulation of anti-apoptotic protein MCL-1, alongside a shift away from oxidative metabolism in neutrophils (56). An increase in Stat-3 expression in neutrophils was also observed (56). Separation of MSCs and neutrophils using a semi permeable membrane did not abolish the anti-apoptotic effect, which may indicate that the effect of MSCs is solely due to its secreted molecules. The anti-apoptotic effect was abolished upon treatment of neutrophils with an anti-interleukin-6 (Il-6) monoclonal antibody, which blocks Il-6 receptors (56). This may suggest that MSCs exert their effect on neutrophils via IL-6 release, which subsequently activates the stat-3 pathway (56).

2.1.4 – MSC Interaction with Dendritic Cells

Maturation of CD14a+ monocytes into and dendritic cells (DCs) was abolished upon co culture with MSCs, where the MSC:DC ratio for maximal inhibition was 1:20 or higher. When MSCs and DCs were separated by a semipermeable membrane, the maximal inhibition ratio increased to 1:10, indicating that the inhibitory effect of MSCs was partially abolished (57). DC maturation was restored upon supplementation of anti-IL6 and anti-macrophage colony stimulating factor (m-CSF). This may suggest that the effect of MSCs on dendritic cells may be mediated by IL-6 and M-CSF (57).

In summary, MSCs interact with a range of leukocytes by secreting various growth factors and through cell-cell contact. There interactions prevent immune responses which allows allogeneic MSC therapy, and makes them useful in the treatment of inflammatory diseases. (7).

2.1.5 – Effect of Allogeneic MSC Injection on Tissue Transplants

Unlike metabolic studies in this project, where millions of cells per experiment are used, allogeneic cell therapy may require up to billions of MSCs depending on the indication (58). For low dose therapies for diseases such as back pain where tens of millions of cells are required, cell banks are generated using multilayer flasks (58). For therapies that require hundreds of millions of cells per dose or more, bioreactors may be used, where cells are cultured on microcarriers or hollowfibres for a greater surface area (58). Current approved MSC therapies are summarised in **Table 2.1.**

Allogeneic BMMSCs have been shown to alleviate skin graft rejection in baboons. Injection of 10-30 million MSCs at day 0 prolonged the skin graft survival from 7 days to 11 days (59). However, additional MSC injections at day 3 failed to extend the survival time beyond 11 days (59). This may indicate that MSC injection may suppress the initial T-cell response, but fails to prevent the recruitment of other additional lymphocytes beyond day 3 (59). The results were comparable to immunosuppressant drugs such treatment with an anti-CD80 antibody and cyclosporine in combination, or treatment with fludarabine alone (59). At day 3, an increase in interleukin-2 (IL-2) production was observed, and addition of exogenous IL-2 to the MSC/lymphocyte co culture abolished the inhibitory effect of MSCs (59). This indicates that that tissue rejection beyond day 3 is mediated by Il-2 production from lymphocytes (59).

However, challenges remain in MSC therapy, which may delay the approval of novel treatments. Substantial variation in proliferation potential (60), differentiation potential (61) , metabolic requirements (60) and immunomodulatory function (60) have been reported between donors, which may make it difficult to combine cells from different donors to create a universal cell bank. Manufacture of medication usually involves the mass production of cheap products that can be used universally. MSC production, on the other hand, requires expensive components (serum and/or growth factors), special protocols, and have a smaller market size, making them less profitable (62). At present it is also important to determine how therapy is affected by dosing regimens (single, repeat or escalating doses), the method of delivery (direct, local or systemic) or the dosage size (number of MSCs delivered/kg of body weight) (63).

Table 2.1: Indications, dose, cost and country of origin for government approved MSC therapies available on the market. AL = allogeneic. AT = autologous.

Product name	Company	Therapy area	Type	Dose	Price	Country	Approval
Aloficell® (64)	TiGenix (USA) and Takeda (UK)	Complex perianal fistulas in Crohn's	AL	5 M cells/mL dose	$47,485 per dose	USA and UK	EMA 2018 (64)
Cartistem®	Medipost	Osteoarthritis	AL	2.5M cells/cm2	$20-40k per round of treatment	South Korea	MFDS 2012 (65)
Cupistem®	Anterogen	Crohn's fistula	AT	30M cells/cm	$3000-$5000 per treatment	South Korea	MFDS 2012 (64)
Heartcelligram-AMI®	Pharmicell	Post-acute myocardial infarction	AT	Not known	$19,000 per round of treatment	South Korea	MFDS 2011 (65)
Mesestrocell®	Cell Tech Pharmed	Osteo- and knee joint arthritis	AT	20 million cells/knee	Not known	Iran	Iran FDA 2018 (64)
Neuronata-R®	Corestem	ALS	AT	1 M cells biweekly	$55,000 per year	South Korea	MFDS 2014 (64)
Prochymal®	Osiris	Transplantation rejection	AL	2 M cells/kg body weight	$20,000 per dose	Canada & New Zealand	2012 Canada + NZ (65), US FDA 2015 (64)
Queencell®	Anterogen	Subcutaneous tissue defects	AT	1 M cells/mL, depend on defect size	Not known	South Korea	MFDS 2010 (64)
Stempeucel®	Stempeutics Research	Burger's disease	AL	1-2 M cells/kg body weight	$2,200 per treatment	India	India DCGI 2016 (64)
Temcell®	Mesoblast	Transplantation rejection	AL	1.2-1.7 bil cells/dose	$7079/72M cells	Japan	Japan, 2015 (62)

2.2 – Current Uses of MSCs in Therapy

As of 2021, over 900 clinical trials involving MSCs have taken place (66), primarily involving tissue injury and immune disorders. One example is the NEPTUNE trial where MSCs are being used to attenuate the immune response in kidney transplant patients.(67) Other recent trials include the treatment of bladder tuberculosis (68) and the use of MSC spheroids in fibrin hydrogels to aid wound healing (69). More recently, MSCs have been employed to treat COVID-19 patients (see **section 2.3.3**).

2.2.1 – MSC Treatment of Neurodegenerative Diseases

MSC therapy has been attempted in neuronal diseases with limited success. Autologous BMMSCs suspended in cerebrospinal fluid have been injected in 7 patients with motor neurone disease (MND) via the spinal cord. No severe adverse events, such as death, respiratory failure or post-surgery neurological deficits were observed at 6 months following treatment, while minor side effects included reversible intercostal pain and leg dysesthesia (70). A third of patients exhibited an increase in muscle strength 3 months after treatment. For the remainder, the decline in muscle strength was slowed. This demonstrated that MSCs can be safely injected into the spinal cord (70). The mechanism behind MND treatment was not investigated by the authors.

Green fluorescent protein (GFP) labelled MSCs have been investigated as a potential treatment for Parkinson's disease (71). Intravenous administration of 10 million MSCs into 6-hydroxydopamine (6-OHDA) mice models lead to the restoration in forelimb function and a >50% reduction in amphetamine induced rotations (71). No alleviation of symptoms occurred for the fibroblast treated group or the phosphate buffered saline (PBS) control (71). *In vitro*, PC-12 cancer cells of the rat adrenal medulla were treated *in vitro* with 6-OHDA, followed by stromal cell derived factor-1 alpha (SCDF-1α), a signalling molecule released by MSCs (71). Maximal dopamine secretion was achieved with of 1 ng/mL SCDF-1α, and PC-12 survival was improved. The effect was abolished by addition of SCDF-1α antibodies, which indicates that MSCs exert their anti-Parkinsonian effects by the secretion of SCDF-1α (71). However, DAPI staining showed that the majority of MSCs migrated to pulmonary tissue at day 2 of MSC injection and at 4 weeks no MSCs were detected in the brain (71). Therefore, MSCs may not exert their therapeutic effect by engraftment.

Injection of adipose MSCs in hank's balanced salt solution (HBSS) reduced the escape latency in APP/PS1 mice models of Parkinson's disease, alongside a reduction in the area of amyloid precursor proteins (APPs) in brain slices (72). The authors also observed an increase in the area covered by microglia, which migrated to amyloid plaques (72). Further real time polymerase chain reaction (qPCR) studies on the microglia revealed the up regulation of anti-inflammatory mediators interleukin 4 (IL-4) and arginase-1 (ARG-1), alongside the down regulation of tissue necrosis factor-alpha (TNF-α) and interleukin-1 beta (IL-1β) (72).

Overall, the results suggest that MSCs treat Parkinson's disease via 2 mechanisms: stimulation of anti-inflammatory mediator production in macrophages to clear amyloid plaques, and SCDF-1α production to stimulate dopamine production.

2.2.2 – MSC Treatment of Cardiovascular Diseases

Silva *et al.* (2005) reported the restoration of cardiac output following allogeneic MSC injection (100 million in 10 mL saline) in canines with acute myocardial infarction (AMI). For the MSC treatment group, the authors observed a 20% reduction in the area of fibrotic tissue and two fold increase in vascular density (73). Immunofluorescence studies showed that MSCs localised and then differentiated into smooth muscle cells and endothelial cells (73). Different doses of MSCs have been tested in AMI swine models: 24 million, 240 million and 480 million, yet there was no significant difference in efficacy or safety profile between the 3 doses (74).

Additional studies were performed on sheep using STRO-3 positive mesenchymal precursor cells (MPCs), which are thought to exert increased proliferative and differentiation potential compared to its STRO-3 negative counterpart (75). Animals were treated with either 25 million, 75 million, 225 million or 450 million MPCs following coronary artery occlusion. The authors observed the maximal reduction of end diastolic volume for sheep treated with 75 million MPCs. The duration of myocardial infarction (MI) was significantly reduced in all doses but the 450 million group (76). This may suggest that injection of tens of millions of MSCs is sufficient for the treatment of cardiovascular disease.

Tang *et al.* (2005) reported that MSCs modified to express heme oxygenase-1 (HO-1) exhibited a 5 fold increase in survival rate upon implantation for myocardial infarction models of mice(77). The modification enabled MSCs to resist ischemia/reperfusion damage (77). At day 14 of MSC infusion mice treated with HO-1 MSCs exhibited higher cardiac output, a reduction in fibrotic tissue and a reduction in production of inflammatory mediators compared to the wild type MSCs (77). The data so far may suggest that MSC treatment of cardiovascular disease may be enhanced by expression of HO-1.

MSCs fail to remain for adequate periods of time in the heart following injection. When female swine were injected male swine MSCs in the heart, no Y-chromosome positive MSCs were observed at 8 weeks (76). This may indicate that MSCs exert their therapeutic effect by releasing signalling molecules rather than engraftment.

Autologous injection of MSCs has been carried out in humans with myocardial infarction, at doses of 0.5, 1.6 and 5 million cells per kg of body mass. Data from all 3 doses were combined in the study, where 39 patients received MSCs while 21 patients received the placebo (78). At 6 months after injection, the treatment group saw a reduction in the arrhythmia event rate (36.8% for the placebo group vs. 8.8% for the treated group) and a reduction in the incidence of ventricular

tachycardia (26.3% for placebo group vs. 2.9% for the treated group), alongside a 16% increase in the forced expiration volume (FEV1) (78). No difference adverse events were observed in either group (78).

A total of 69 Chinese acute myocardial infarction patients were injected with autologous BMMSCs or 6 mL saline. At 3 months, the treatment group experienced a reduction in functional defects (28% for the placebo group vs. 11% for the treated group), a 2 fold increase in wall movement velocity for the treated group (vs. none for the placebo) and a reduction in the incidence of perfusion defects (36% for the placebo group to vs. 20% for the treated group) (7).

In summary, MSC therapy can alleviate symptoms of cardiac disease by promoting tissue repair. Efficacy can be further enhanced by genetic modification.

2.2.3 – Treatment of COVID-19 with MSC Therapy

MSC therapy has been used to treat coronavirus disease 2019 (COVID-19), a virus spread that across the world in 2020, infecting millions. COVID-19 has been shown to cause hyper inflammation (cytokine storm) in severely infected patients, who end up producing excessive amounts of inflammatory mediators such as interleukin (IL)-2, IL-7, granulocyte-colony stimulating factor, interferon-γ inducible protein 10, monocyte chemoattractant protein 1, macrophage inflammatory protein 1-α, and tumour necrosis factor-α (79). This may lead to multiple organ damage and subsequent mortality from respiratory failure (79). The immunomodulatory and tissue repair properties of MSCs may therefore be useful in preventing the cytokine storm and reversing organ damage from the disease.

Allogenic menstrual-blood-derived MSCs for the treatment of 61 H7N9 avian flu patients with acute respiratory distress syndrome (ARDS) have been tested, where 44 of the 61 served as the control, and the remaining 17 were injected with MSCs. The patients were collected between March 2013 to Feb 2014, and were infused with 1 million cells/kg of body weight. After 5 years of follow up, the authors observed no significant change C-reactive protein levels (a marker of systemic inflammation) or lung function tests (as forced expiration volume), but anatomical damage to the lungs was reversed. The overall mortality was significantly lower in the treatment group (17.4%) vs. the control (54.5%) (80). As the symptoms of H7N9 were similar to COVID-19, this may suggest that MSC therapy can be employed to repair pulmonary damage caused by COVID-19.

Intravenous allogenic umbilical cord MSCs at a dose of 1 million cells/kg have been trialled in 7 COVID-19 patients over a 7 week period, where MSC infusion occurred after week 1. Within the treatment timeframe, the following improvements were observed: the reversal of pulmonary

damage, normalisation of blood test results, reversal of fever and shortness of breath, and a 1.5 – 2 fold increase in anti-inflammatory mediators and signalling molecules associated with tissue repair, such as IL-10 and VEGF and decrease in the number T cells and Natural Killer cells (81).

2.2.4 - Limitations of MSC Therapy

Rosland *et al.* (2009) reported malignant transformations in cells from 11 of 24 human MSC healthy donors within 5-106 weeks of culture (82), yet Bernardo *et al.* (2007) reported no chromosomal abnormalities among 10 donors human BMMSCS that were cultured until passage 25 (P25) or senescence (83). Rosland *et al.* (2009) had used patient samples ranging from 18 to 53 years old, and cultured cells in Dulbecco's Modified Eagle's Medium (DMEM) or alpha minimal essential medium (α-MEM), while Bernardo et al. (2007) used patients with a median age of 18 years, and expanded cells using Mesencult medium (82) (83). It is important to investigate whether patient age and culture media can raise the risk of tumour formation in MSCs.

In the literature, MSC have demonstrated both pro and anti-oncogenic effects. Interactions between MSCs and breast cancer cells have been demonstrated via the collagen receptor discoidin domain receptor 2 (DDR-2) (84). In 17 out of 21 human samples of breast cancer cell metastases, the expression of DDR-2 was reported in both tumour cells and adjacent MSCs (84). When the adjacent MSCs were extracted from the metastatic environment (Met-MSCs) and injected into the mammary fat pads of immunocompromised mice, no tumours were formed (84). Upon co-culture of Met-MSCs with breast cancer cells (BCCs), BCC proliferation was enhanced, and had gathered around a central core of Met-MSCs (84). A 5 fold increase in DDR-2 expression in BCCs was observed when cultured with MSCs, though MSC DDR-2 expression was not altered when co-cultured with non-malignant breast epithelial cells (MCF-10A) (84). The proliferative effect of MSCs on BCCs were abolished when DDR-2 knockout MSCs were used. Overall, the results may suggest that MSCs do not form tumours alone *in vivo*, and do not promote tumour formation in healthy cells but may enhance proliferation of existing tumours via DDR-2 mediated interactions.

In other trials, MSCs have also demonstrated anti-oncogenic effects. Hoechst 33258 staining demonstrated that human U251 glioma cells co-cultured with primary human BMMSCs exhibited nuclear fragmentation and condensation, along with increased expression of activated caspase-3 (7x increase) and caspase-9 (2x increase) compared to U251 cells that were cultured alone, indicating higher levels of U215 cell death. The nuclei of control U251 cells that were not cultured with MSCs remained round and intact (85). At day 7, the rate of apoptosis of control cells was reported as 3.48%, compared to 22.36% for BMMSC treated cancer cells. Through the downregulation of cyclin

D1 and upregulation of p21, a higher percentage of BMMSC treated U251 cells were held in the G1 phase of the cell cycle (67.39% of BMMSC treated U251 cells vs. 41.55% of untreated cells) (85). Using a matrigel invasion assay, an 8 fold reduction in invasion cell number, and migration cell number for BMMSC treated U251 cells was reported compared to control cells (85). Proteomics revealed reduced expression of phosphoinositide-3-kinase (PI3K) and protein kinase B (Akt) for MSC treated U251 cells, which may suggest that BMMSCs inhibit U251 proliferation via modulation of the PI3K/Akt pathway (85).

Human BMMSCs have been investigated as a delivery vehicle for anti-cancer agents in mice that had undergone intracranial engrafting of U87, U251, and LN229 glioma cells (86). When harvested brain slices were placed in serum free medium, BMMSCs (labelled by Sp-Dil) had migrated to the site of the glioma (GFP labelled) (86). However, migration was dependent on the presence of platelet derived growth factor, EGF and SDF-1α in the surrounding serum free medium, and was abolished upon the addition of anti-PDGF, anti-EGF and anti-SDF-1α antibodies (86). In the same study, mice survival rates were prolonged when injected with IFN-β^+ BMMSCs, compared to those treated with wild type BMMSCs (0% survival reached at day 26 for the wild type BMMSC group, compared to day 60 for the INF-β^+ MSC group) (86). For both groups, the total dosage was 250,000 cells. Overall, this demonstrates that MSCs can be used as a vehicle for the delivery of anti-cancer treatments, as they are able to migrate to the tumour in the presence of growth factors.

In carbon tetrachloride induced cirrhosis models of mice, injection of BMMSCs contributed little to the recovery of parenchymal cells, but lead to the formation hepatic stellate cells and myofibroblasts, which constitutes liver fibrosis (87).

MSC doubling time, metabolic activity and immunomodulatory function varies greatly from patient to patient; making it difficult to mass produce MSCs to treat multiple patients (60). This will also cause batch to batch variation in their efficacy (60). In addition, human BMMSCs from patients varied in their Hayflick limit, where cells could be cultured for as little as 3 passages or as many as 25 passages before senescence (83).

Alterations in cell morphology, alongside loss in proliferation and differentiation potential (88) occurs with increasing population doublings when cultured at normoxic conditions *in vitro*, due to a fall in telomerase activity (89) and increases in oxidative stress production (90). This may make it difficult to produce billions of cells required for autologous therapy.

2.3 – Current Methods of Cell Preservation

2.3.1 - Novel Cryopreservation Media for MSCs to Circumvent Damage by DMSO

When temperatures fall below 0°C, water molecules adopt a crystal arrangement maintained by hydrogen bonding. Ice crystal formation may deform or compress cells, and damage intracellular structures (91). Diffusible or intracellular active cryoprotectants such as DMSO may exert the following protective effects: prevent water crystallisation, protect cell proteins from denaturisation and increase water viscosity to prevent ice crystal aggregation (91). DMSO was first used in 1959 (92) for the cryopreservation of human and bovine erythrocytes and bull spermatozoa. However, DMSO exerts cytotoxic effects, and therefore must be thoroughly washed out prior to cell therapy (94). Following cryopreservation of $CD4^+$ T cells in 10% DMSO, reductions in cell survival, proliferation potential and cytotoxic response to immunologically relevant stimuli have been reported (93), yet removal of DMSO from thawed cells is time consuming, expensive and may decrease cell yield (94). It is therefore important to find non-toxic alternatives to DMSO.

To address the problems with DMSO, alternative cryopreservation mediums have been tested to determine if the structural and epigenetic changes caused by DMSO can be avoided. MSCs derived from mice H9 embryonic stem cells were cultured and frozen in the following osmolyte solutions (see **Table 2.2**), with standard DMSO, or in culture media to serve as the control (95).

Table 2.2: Constituents of osmolyte solutions to be tested as an alternative to DMSO. The differential evolution algorithm on MATLAB was used to determine suitable candidates for the experiment. Reproduced from Pollock *et al.* (95).

Osmolyte Solution	Constituents
SGC	1.25% glycerol, 2 mM creatine
SGI	30 mM sucrose, 1.25% glycerol and 7.5 mM isoleucine
SMC	150 mM sucrose, 62.5 mM mannitol and 6.25 mM creatine

Cells were incubated in the SGC and SGI solutions at room temperature for 1 hour and the SMC solution for 2 hours prior to a multistep freezing procedure and subsequent transport to liquid nitrogen. Cells were then defrosted within 2 minutes and analysed for survival, proliferation rate, marker expression and stemness. (95)

For all groups, cells were positive for CD73, CD90 and CD105 markers (>99%) while lacking CD45 (<1%). This shows that freezing with DMSO or osmolyte solution does not alter the marker

expression. Staining with Calcein AM dye shows that proliferation capacity was maintained only in the SGI and DMSO (0h) group.

Measurement of senescence using Beta-glo assay shows no difference in any of solutions (95). Disrupted actin alignment was reported in the DMSO group (peak strength of 200), while the actin alignment in all 3 osmolyte groups resembled fresh cells (peak strength of 1400). (95) Significant increases in DNA hydroxymethylation, which is thought to be responsible for a reduction in cell functionality, was observed only in the DMSO and SMC group. (95)

The results suggest that the SGI group is the most suitable cryopreservation media, as it maintained MSC survival and marker expression, yet does not disrupt the actin alignment. An MSC preservation media that works at room temperature must be developed to ensure cell survival if the fridge/freezer breakdown were to occur.

Preservation media consisting of 7.5% carboxylated-poly-l-lysine (CPLL) has been investigated as an alternative to 10% DMSO in DMEM media for human BMMSCs over a 24 month period at -80^{o}C, where cells were frozen at 1 million cells/mL (96). For thawed cells that were transferred to DMEM expansion media, both the DMSO and CPLL group demonstrated no loss in proliferation potential compared to fresh cells, where there was a 5x change in cell count at day 5 of culture. Post thaw survival rates, measured by Trypan Blue, were near 90% for all groups (96). Both DMSO and CPLL preserved cells maintained stem cell marker expression (CD13 and CD29), and maintained differentiation potential, where osteogenesis, adipogenesis and chondrogenesis were assessed by alkaline phosphatase, GDPH and glycosaminoglycan formation respectively (96). It is important to compare the extent of actin alignment and DNA hydroxymethylation between the two groups, to determine if MSCs preserved in CPLLS evade cell damage that occurs with DMSO.

Pentaisomaltose (PIM) has been studied as an alternative to DMSO for the cryopreservation of human hematopoietic progenitor cells (HPCs) from apheresis products (97). Cells were frozen for up to 9 months at 95 million cells/mL in a base containing 4% human albumin and 2 IE/mL heparin, with either 32% PIM or 20% DMSO as a cryoprotectant (97). For the PIM group, the authors reported a significant improvement in post thaw cell viability for granulocytes (81% for the PIM group vs. 27% for the DMSO group), but no significant differences in the percentage viability for mononuclear cells (MNCs), total nucleated cells (TNCs) and $CD34^{+}$ cells, where the % cell survival ranged from the high 60% to mid-80% (97). Similar engraftment rates in the bone marrow of immunodeficient mice were reported for both PIM and DMSO cryopreserved $CD34^{+}$ cells, along with similar colony-forming potential (97). The results demonstrate that PIM may be used as an alternative to DMSO for HPC cryopreservation, though additional studies may be required for MSCs.

Trehalose, a non-toxic disaccharide, has been found in a range of organisms such as bacteria, yeast, fungi, insects, invertebrates and plants, where it is thought to protect the organism from a range of abnormal environmental conditions, including desiccation, dehydration, heat, cold, and oxidation (98). In *E.Coli*, trehalose levels were shown to increase 8 fold when the temperature was reduced from 37°C to 16°C (99). After 5 days of storage at 4°C, *E.Coli* overexpressing enzymes required for trehalose synthesis (otsA/B) exhibited the highest rates of survival (80%), compared to wild type (10%) and otsA knockout strains (0%) (99). It has been proposed that at cold temperatures trehalose may prevent protein denaturation and aggregation, along with cell membrane stabilisation (99).

The cryopreservation of primary human adipose and umbilical cord MSCs have been tested in trehalose as an alternative to DMSO (100). Cells were frozen in liquid nitrogen (-196°C) for 1 week at a density of 1 million cells/100 µL in either 90% FBS/10% DMSO or trehalose media (10 mM K_2HPO4, 10 mM KH_2PO4, 1 mM $MgCl_2$ with either 0, 10, 50, 100 or 250 mM trehalose) (100). For the trehalose group, electroporated cells were included to determine whether trehalose must enter the cell to exert its cryoprotectant effect (100). For both cell types, cell survival (assessed by trypan blue) after 1 week of cryopreservation increased with higher trehalose content in the media, with a concentration of 250 mM required to match the survival of the DMSO group (80%) (100). Electroporation did not improve cell survival compared to unmodified cells, which indicated that intercellular trehalose was not required to exert its cryoprotectant effect (100). Following preservation, a similar trend was observed for the post proliferation potential in adipose derived MSCs, with 250 mM trehalose exhibiting similar recovery to the DMSO group irrespective of electroporation (100). For umbilical cord derived MSCs preserved in 250 mM trehalose, MSCs that underwent electroporation exhibited superior recovery of proliferation compared to the DMSO and the unmodified cell group (100).

Indolamine-2,3-dioxygenase 1 (IDO1) production by human MSCs deplete tryptophan, generating kynurenines, which are further degraded downstream to form kynurenic acid and 3-hydroxy-anthranilic acid, which suppress leukocyte activity by binding PDK1 or aryl hydrocarbon receptors (101). In the 250 mM trehalose group, both unmodified and EP MSCs exhibited a 2.7-3 fold increase in IDO1 production compared to the DMSO group (100). Umbilical cord MSCs followed a similar pattern with a 4 and 5.6 fold increase in IDO1 expression in trehalose for unmodified and EP MSCs respectively compared to DMSO. MSCs also secrete TNF-α-stimulated gene 6 protein (TSG6), which suppresses the TLR2/ NF-κB signalling pathway to attenuate the immune response (102). TSG6 expression was highest in unmodified cells preserved in trehalose for both umbilical cord (4.5 fold

higher than DMSO, 1.3x higher than electroporated cells in the same group) and adipose derived MSCs (37.2 fold higher than DMSO, 4.3x higher than electroporated cells in the same group)(100).

Overall, the results demonstrate that 250 mM trehalose can be used as an alternative cryoprotectant to DMSO, with little or no further improvement in cell survival and recovery for electroporated cells.

Although previous authors have developed alternative cryopreservation methods to DMSO, it is advantageous to avoid the cold chain requirements for short term preservation. Once thawed, cells cannot immediately be refrozen should the patient cancel their appointment. Additionally, cell loss via fridge or freezer breakdown can be avoided if the cold chain requirements are no longer present.

2.3.2 - Use of Saline to Preserve MSCs at 4°C

Saline has been investigated as a potential transportation solution for *ex-vivo* expanded and freshly harvested clinical grade BMMSCs for bone regeneration. A total of 20 million cells were transported in 5 mL media and a 3D hydroxyapatite (HPA) scaffold, making the cell density 4 million cells/ml. To mimic the transportation conditions, human BMMSCs were suspended in the following transportation buffers for a total of 18 hours at 4°C: culture medium (MM) to act as control, 0.9% saline (NS) (308 mOsm/L and pH-7.0), and NS with 4% v/v human serum albumin (HSA). HSA was supplied by two different donors, referred to as HSA1 and HSA2 (42).

For BMMSCs transported in MM, the total cell death stained by propidium iodide (PI) was equivalent to 12.13% ± 1.58% of the population. Cells transported in NS or HSA exhibited significantly higher cell death than the control MM group ($p<0.01$), but did not exceed 20%. A total of 19.8% ± 5% of MSCs failed to adhere to a 3D hydroxyapatite scaffold in MM, compared to the 44.5% for the NS group, and >55% for HSA1 and HSA2. The non-adherent cell survival was 90.3% for the MM group. Non-adherent cell survival was significantly higher for the NS transportation buffer (86.4%) compared to HSA1 and HSA2 (<75%). (42) In 2D monolayers, all 4 transportation conditions showed similar proliferation potential after preservation, averaging a 13.5 fold increase in 6 days. (42) In a 3D scaffold, the von Kossa dye, a measure of osteogenesis, stained an average scaffold surface area of 17.6% for maintenance media and the NS group. For the HSA1 group, von Kossa stained <15% of the scaffold, compared to 9.7% for the HSA2 group (42).

The results show that supplementation with serum albumin is not beneficial to cell preservation, as it does not increase survival, does not enhance post preservation proliferation and

lowers osteogenic potential. Furthermore, use of HPA scaffolds leads to 50% of cell attachment. However, this is not suitable for the clinic as the cells need to be readily injectable into the patient.

Others have investigated primary human adipose tissue MSC survival at 4°C in physiological saline (0.9% w/v NaCl) for up to 96 hours (103). Cell survival was reported as 81.78% at 24 hours, 70.20% at 48 hours, 59.94% at 72 hours and 28.80% at 96 hours for the saline group (103). Cell survival in saline was inferior to DMEM at the 72 and 96 hour timeframe, where cell survival was reported as 74.13% and 48.32% respectively for the DMEM group (103). To assess whether proliferation potential was maintained after storage, cells were transferred to DMEM/FBS expansion media and cultured for 4 days at 37°C. Cells preserved in saline exhibited faster doubling times compared to DMEM; 47.05h after 48h in saline vs. 207.81h after 48h in DMEM (103). After 96 hours of preservation, cells preserved in saline maintained slow doubling times when transferred to DMEM expansion media (248.22h), while cells preserved in DMEM failed to proliferate (103). This demonstrates that for short term preservation in cold storage, DMEM is superior to saline in maintaining cell survival, at a cost of proliferation potential.

2.3.3 - Spheropreservation of MSCs in Culture Media

Jiang *et al.* (2017) has reported that human MSCs cultured as spheroids exhibit greater tolerance for room temperature in comparison to 2D monolayer culture. MSC were derived from ESCs, and spheroids were formed using the hanging drop method and transferred to a 1.5 mL sealed tube on a horizontal rocker with α-MEM and FBS at room temperature for 7-9 days. (48) A total of 2 million cells were used per vial, making the density 1.3 million cells/ml.

Survival at day 7 was comparable between spheropreserved cells at room temperature and cryopreserved cells (>90%). For monolayer preserved MSCs, survival fell below 10%. This shows that spheropreservation is equally as effective as cryopreservation, but is more convenient since freezing in liquid nitrogen is not required. At day 9, 61% of cells in the spheroid remained viable. (48) ECAR and OCR were 3 fold lower for spheropreserved MSCs compared to monolayer preservation. For spheropreserved MSCs, the authors also reported the downregulation of genes associated with metabolism (e.g. lactate dehydrogenase, phosphofructokinase) and proliferation (cyclin dependent kinase-1, P53) and upregulation of genes associated with survival (such as PI3K-AKT) (48).

Spheropreserved MSCs remained positive for CD90, CD73 and CD105 and lacked haematopoietic stem cell markers, showing that cells remain as MSCs after preservation (48). Spheroidal MSCs also retained the capability of tri-lineage differentiation into osteocytes, chondrocytes, and adipocytes. (48) These MSCs also retained their lymphocyte

suppression activity and production of anti-inflammatory mediators (48). Recovered cells retained their therapeutic effect in colitis models of mice, shown as the prevention of weight loss over a 5 day period (48).

This study demonstrates that MSCs can be kept viable and functional for 7 days as spheroids in standard culture media. Cells are thought to be quiescent in spheroid form due to downregulation of metabolic and proliferative genes, enabling them to withstand abnormal culture conditions. However, the presence of serum may lead to immune responses in patients and therefore cells will need to be thoroughly washed prior to therapy. In addition, spheroids may need to be dissociated prior to therapy. It may be better to create a product that can be administered directly to patients with no manipulation. A survival rate of 61% at day 9 means that this method cannot keep cells viable for the proposed target of 12 days.

The effect of alginate hydrogels containing Arginylglycylaspartic acid (RGD-alginate) and spheroid culture on cell survival and function has been investigated for primary human bone marrow MSCs (BMMSCs) at 37°C (104). The preservation density was 40 million cells/mL of alginate, where 15,000 cell spheroids were created using the hanging drop method. Hydrogels were supplemented with 0.32 mM RGD to trigger the integrin mediated cell adhesion required for survival and proliferation, before dilution to 2% (w/v) using α-MEM (104). The following groups were tested at 37°C in α-MEM/FBS:

- Dissociated cells in unmodified alginate
- Spheroids in unmodified alginate
- Dissociated cells in RGD-alginate
- Spheroids in RGD-alginate

Among the $CaCl_2$ concentrations (100, 200 and 300 mM) and crosslinking times (10 or 30 minutes) studied, a crosslinking protocol of 200 mM for 10 minutes yielded the lowest levels of caspase 3/7 activity in MSCs (104).

After 5 days of culture in the alginate hydrogel, cell elongation was only maintained for the RGD-alginate group (104). For cells expanded in unmodified alginate, MSC proliferation (DNA content/ng gel) was 2x greater in spheroids compared to dissociated cells (104). For MSC spheroids, supplementation of alginate gel with RGD yielded a further 2x increase in proliferation compared to spheroids in unmodified gel (104). When cultured in unmodified gels, caspase 3/7 activity was 2.8x smaller in MSC spheroids compared to dissociated MSCs, with no further improvement in spheroid survival when the gel was supplemented with RGD (104). In unmodified alginate, MSC spheroids

exhibited 1.4x more VEGF secretion compared to dissociated cells, with a further 3x enhancement of VEGF release in the MSC spheroid group for RGD-alginate compared to unmodified alginate (104). Spheroids in RGD-modified alginate exhibited the highest amounts of pro-angiogenic proteins such as growth-regulated oncogene (GRO), monocyte chemoattractant protein 1 (MCP-1), and interleukin 8 (IL-8) compared to groups with dissociated cells and/or unmodified alginate (104).

Following 14 days treatment with differentiation media, spheroids embedded in unmodified alginate exhibited the greatest extent of osteogenesis *in vitro* (2-4x greater than other groups), quantified as intracellular alkaline phosphatase (ALP) activity, osteocalcin secretion and calcium secretion (104). When MSCs were implanted in diabetic immunodeficient mice following 5 days of culture, explants from MSC spheroids embedded in RGD-alginate exhibited the greatest area of osteocalcin staining (104).

Although current MSC preservation studies at room temperature and cold storage involve dissociated cells inside unmodified alginate (43, 44), these results demonstrate that MSC spheroids in RGD-alginate demonstrate enhanced survival, therapeutic potential and differentiation potential. Therefore, if dissociated MSCs exhibit poor survival and function in unmodified alginate when testing the novel preservation medium, MSC spheroids in RGD-alginate could be tested as an alternative. If bead formation with the current protocol (102 mM $CaCl_2$) yields poor cell survival rates, 200 mM could be used instead for future work.

2.3.4 - Preservation of MSCs in Trehalose

Human primary BMMSCs have been preserved in buffered trehalose solution (BTS) over a 3 day period at 4°C. A total of 10 million cells were stored 1 mL of solution. The BTS consisted of 30 mM KH_2PO_4, 15 mM Na_2HPO_4, 0.5 mM $CaCl_2$, 1 mM $MgSO_4$, 250 mM trehalose (pH = 7.2, osmolarity = 300 − 350 mOsmol). Cell survival and differentiation potential was assessed after preservation and compared to Ringer's solution, Plasma-Lyte, and HypoThermosol (41).

The authors reported that cell survival was highest at 72 hours in the buffered trehalose and hypothermosol group (77-78%), in comparison to 40% in plasma-lyte and 20% in ringer's solution. Post preservation levels of proliferation over a 4 day period were similar to that of fresh cells, and MSC markers (CD73, CD90 and CD105) were maintained in all experimental groups (41). T-cell suppression was the highest for cells preserved BTS and Hypothermosol (65%), followed by Plasma-Lyte (60%) and Ringer's solution (55%). T-cell suppression in fresh cells was 80%. For all groups cells retained their ability to secrete anti-inflammatory mediators programmed death-ligand 1, IDO and

IL-6 (41). No additional studies have investigated the use of trehalose for cold storage of MSCs.

In summary, MSCs can be preserved in BTS for 3 days without loss of survival, proliferation, differentiation potential, and immunomodulatory function, yielding superior results to existing cold storage solutions. As with saline, BTS is unlikely to keep cells viable for the proposed target time of 12 days, as cell survival rates already dropped to 78% by day 3.

2.3.5 – Preservation of MSCs in Wisconsin Solution

Preservation of primary human dermal MSCs in University of Wisconsin solution (UW) have been undertaken, either in suspension or within alginate microspheres for 7 days at 4°C. UW consisted of 105 mM KOH, 4 mM NaOH, 2.6 mM KH_2PO_4, 10 mM $MgSO_4$, 108 mM lactobionic acid, 31 mM raffinose (adjusted to pH 7.4). They compared the results to sucrose based solution (SBS), consisting of 30 mM KH_2PO_4, 1 mM $MgSO_4$, 15 mM Na_2HPO_4, 0.5 mM $CaCl_2$, 250 mM sucrose, 1% polyethylene-glycol 8000 (adjusted to pH 7.4). Prior to survival measurements, alginate was dissolved with 50 mM Sodium Citrate (40). MSCs preserved in α-MEM and FBS served as the control.

At day 7 of preservation in suspension, MSC survival rates, as measured by the MTT cell viability assay, were highest in the UW group (76%) followed by SBS (56%) and the culture media control (5%). Marginal improvements were observed when MSCs were encapsulated in alginate beads. Both UW and SBS preserved MSCs retained their adipogenic and osteogenic potential, as shown by positive staining of oil red O and Alizarin Red respectively (40).

UW appears to be superior to previous methods, as it is able to preserve MSCs for longer periods of time without the use of special materials or serum. However, the disadvantage of UW is that only cold temperatures have been tested. The data also demonstrates that encapsulation in alginate microbeads does not improve cell survival during cold storage. It is important to investigate if MSCs can be stored in UW at room temperature, and whether alginate encapsulation is needed for warmer conditions.

Isolated mice adipose tissue have been stored for 16 hours at 4^oC in either Wisconsin solution or Hank's balanced salt solution (HBSS) prior to adipose derived stem cell (ADSC) extraction to determine the optimal preservation media (105). Cells extracted immediately after adipose tissue harvest served as the control. Following cell extraction, viability (Trypan blue), was significantly higher for the Wisconsin solution group (82%) vs. the HBSS group (74%), where cells from non-

preserved tissues had 84% viability following extraction (105). When isolated cells were expanded, loss in cell proliferation for the HBSS group was more profound compared to the UW group (105). Cells from adipose tissue stored in either UW solution or HBSS exhibited stem cell markers (CD44 and CD90.2) while lacking haematopoietic, endothelial or leukocyte markers (CD34 and CD45) (105). Adipogenesis and osteogenesis was maintained regardless of the preservation technique (105). This demonstrates that for short term overnight preservation, Wisconsin solution is a superior choice compared to HBSS. Ultimately, longer preservation times (3 days and 7 days) must be investigated prior to selection of the appropriate solution.

2.3.6 - Use of Mucin Hydrogel to Preserve hESCs and Embryos

Canton *et al.* (2016) have reported that hESCs and embryos can be kept quiescent by encapsulation in a mucin based poly (glycerol monomethacrylate)-poly(2-hydroxypropyl methacrylate) [$PGMA_{55}$-$PHPMA_{135}$] diblock copolymer worms. HESCs and embryos were kept viable for 8 days and 14 days respectively, and remained functional after preservation. The proportion of ESC colonies that maintained growth was 70% for fresh cells, falling to 60% at day 14 of preservation and 15% at day 21. ESCs retained their pluripotent (Tra-1-60 and SSEA-4) and differentiation markers (SSEA-1) following 21 days of preservation. Although longer preservation times have been achieved with mucin gels, it has not been tested with MSCs. Furthermore, gel synthesis in this publication required expensive materials (2-cyano-2-propyl benzodithoiate at £100 per gram, Sigma) and complex equipment such as nuclear magnetic resonance and freeze dryers (106). Mucin hydrogels have not been investigated for the preservation of MSCs.

2.3.7 - Encapsulation of MSCs in Alginate Gel for Both Cold Storage and Room Temperature Preservation

Human limbus derived MSCs at P3 have been encapsulated in alginate beads and stored the cells at 4°C or room temperature (RT) for 3-5 days in DMEM/F-12 with 2% FBS. For the room temperature (31°C) group, cells were stored in a temperature conditioned container, where the temperature rose from 3-23°C over an 80 hour period (43). They used a total of 2.5 million cells/mL of alginate solution, and beads were created by hand by dripping alginate into $CaCl_2$ solution. Non-encapsulated cells served as the control. At day 5, the survival rates of encapsulated MSCs at RT and 4°C was 76.9% and 64.5% respectively, compared to <10% for non-encapsulated cells. During recovery after preservation, cell survival rates at RT and 4°C were 67.7% and 52.4% respectively. MSCs retained their ocular biomarkers (PAX6), stem cell biomarkers (p63-α and ABCG2),

mesenchymal biomarkers (VIM, Col III, CD105) and surface biomarkers (CD73, CD90 and HLA-DR) after 5 days preservation at both temperatures tested (43).

A total of 1 million human adipose MSCs have been stored in 2 cm diameter x 2 mm thick alginate disks, placed in 1 mL of MesenPro low serum medium (2% FBS) (44). Alginate encapsulation improved survival (65-85% survival rate at 72 hours) over a range of different temperatures (4, 11, 13, 15, 17, 19 and 21°C) compared to unencapsulated cells, where the survival rates were 50-60% at 11-21°C and 20% at 4°C (44). However, the survival rate for encapsulated MSCs at 23°C was 30%, yielding inferior results to unencapsulated cells (50% survival rate). For cells stored at 15°C, there was no significant loss of proliferation and differentiation potential after preservation compared to non-preserved cells (44). At 15°C, the authors reported no difference in survival rate when encapsulated cells were stored in StemPro serum free/xeno free medium in comparison to the MesenPro (44). Repeat experiments at 15°C using 500k and 2 million cells per disk yielded no major changes in survival rate at day 3 compared to 1 million cells per disk, where the survival rate was just above 70% in at all 3 densities (44).

These studies demonstrate that MSC encapsulation improves their survival during preservation, and enables the cells to withstand a range of temperatures. For the final preservation media, it is proposed to investigate if encapsulation improves survival. A summary of current preservation methods are shown in **Table 2.3.**

Table 2.3: A summary of current cell preservation methods. Current methods primarily involve keeping cells quiescent by spheroid culture, gel encapsulation or storage in isotonic solutions.

Author	Cells	Preservation method	Outcome	Drawback
Jiang *et al* (2017)(48)	Human ESC derived MSCs and BMMSCs	Cell spheroids incubated at room temperature in α-MEM/FBS for 7 days	91% viability by day 7, retention of proliferation, differentiation and immunomodulatory function	Batch to batch variation and immune response from FBS.
Petrenko *et al* (2019)(41)	P2-4 Primary Human BMMSCs	250 mM buffered trehalose solution at 4°C for 3 days	78% viability by day 3, retention of proliferation, differentiation and immunomodulatory function	Trehalose is expensive. Preservation time too short. Refrigeration needed.
Canton *et al* (2016)(106)	Human ESCs and embryos	Mucin inspired gel for 21 days	ESCs and embryos remained viable and functional for 14 days and 8 days respectively.	Expensive materials and equipment needed to synthesize gel. No data on MSCs.
Veronesi *et al* (2014)(42)	P1 Primary Human BMMSCs	0.9% saline on calcium phosphate scaffold. 20 million cells in 5 mL saline for 18 hours and 4°C	Proliferation and osteogenic potential maintained. >70% of detaches cells viable after 18 hours.	Preservation time too short. Refrigeration needed.
Tarusin *et al* (2014)(40)	P4-8 Primary Human Dermal MSCs	MSCs were seeded directly or encapsulated in alginate in Wisconsin Solution. 7 days at 4°C	>75% viability at day 7, no benefit to alginate encapsulation. Recovered cells maintained trilineage differentiation.	Refrigeration needed.
Damala *et al* (2019)(43)	Human Limbus MSCs	Encapsulation in alginate and storage in DMEM/F-12 for 5 days, at both 4°C and room temperature	68% viability by day 5 at room temperature. 52% viability at day 4 for cold storage. Cells retained marker expression.	Cells unlikely to survive 12 days using this method.
Swioklo *et al* (2016)(44)	Human Adipose MSCs	Encapsulation in alginate and storage in MesenPro for 3 days at (4-23°C)	Cells remain viable at 21°C and below for 3 days (65-85%). Most cells do not survive at 23°C (30% viable). Recovered cells maintained trilineage differentiation and growth.	Cells unlikely to survive 12 days using this method.

2.4 - Current Understanding of MSC Metabolism

During preservation and expansion, large quantities of MSCs are required *in vitro*. It therefore necessary that the glucose, pyruvate, amino acids and vitamins required to keep cells viable and functional are not depleted, and metabolic waste such as lactate, ammonia and reactive oxygen species (ROS) do not reach toxic levels.

Glucose metabolism provides energy and building materials for the synthesis of proteins, fatty acids and neurotransmitters. In the glycolytic pathway, glucose is converted into pyruvate,

forming adenosine triphosphate (ATP) and reduced nicotinamide adenine dinucleotide (NADH). Pyruvate is converted into Acetyl-CoA by coenzyme A, with NADH as a cofactor (107). Acetyl-CoA enters the citric acid cycle (TCA), where it generates NADH and Flavin adenine dinucleotide ($FADH_2$), both of which participate in oxidative phosphorylation (108). Additional ATP molecules are generated during TCA and oxidative phosphorylation. The pentose phosphate pathway takes place in parallel to glycolysis, where glucose-6-phosphate (G6P) is converted into nucleotides and erythro 4 phosphate (E4P), the latter of which is used for the synthesis of aromatic amino acids (109). The cellular metabolic pathways are illustrated in **Figure 2.1** (110). Glutamine is transported into the cell via a glutamine transporter, where it may serve as a precursor in the synthesis of proteins, amino sugars, nucleic acids and nucleotides, or act as a carbon source for glutamine metabolism (111). Cells require both essential and non-essential amino acids to proliferate during *in vitro* culture (112) (113).

Current studies on MSC metabolism primarily involve glucose consumption and lactate production, with few papers on amino acid consumption. So far only end point measurements have been made, and MSC metabolic activity at room temperature and cold storage has not been investigated. Obtaining the glucose consumption and lactate production per cell per day during preservation will enable us to estimate the number of cells that can be stored per mL of novel medium.

2.4.1 - Effect of Valproic Acid (VPA) on MSC Metabolic and Immunosuppressive Activity

Francois *et al.* (2012) reported a decline in metabolic activity and the immunosuppressive properties in cryopreserved human MSCs (114), which may be alleviated by the addition of VPA (115)

The XF96 Extracellular Flux Analyser was employed to measure the extracellular acidification rate (ECAR) and oxygen consumption rate (OCR). (115) ECAR is an indication of glycolysis. Culture of MSCs with proliferating peripheral blood mononuclear cells (PBMCs) lead to a 2-3 fold increase ECAR (from 200 to 400-650 mpH/min) and oxygen consumption (from 200 to 500-550 pmoles/min), ($P < 0.05$). Addition of DMSO suppressed metabolic activity in a concentration dependent manner. DMSO at 1% reduced ECAR and OCR by 20% while 5% DMSO reduced ECAR by 80% and OCR by 90%. (115) Enhancement of immunosuppressive activity in MSCs was greatest when VPA was added following DMSO treatment (45-60% T-cell suppression) compared to VPA pre-treatment (25-40% T-cell suppression) (115). No additional studies have investigated the effect of

VPA on MSC metabolism, or investigated the use of VPA to prolong cell survival or function during preservation.

When formulating the preservation media for human BMMSCs, it would be worth testing if the addition of VPA to the media will enhance immunomodulatory activity. It is important to test that VPA does not reduce trilineage differentiation capability. If a loss of immunosuppression occurs after MSC preservation, the preservation media could be supplemented with VPA to prevent that loss.

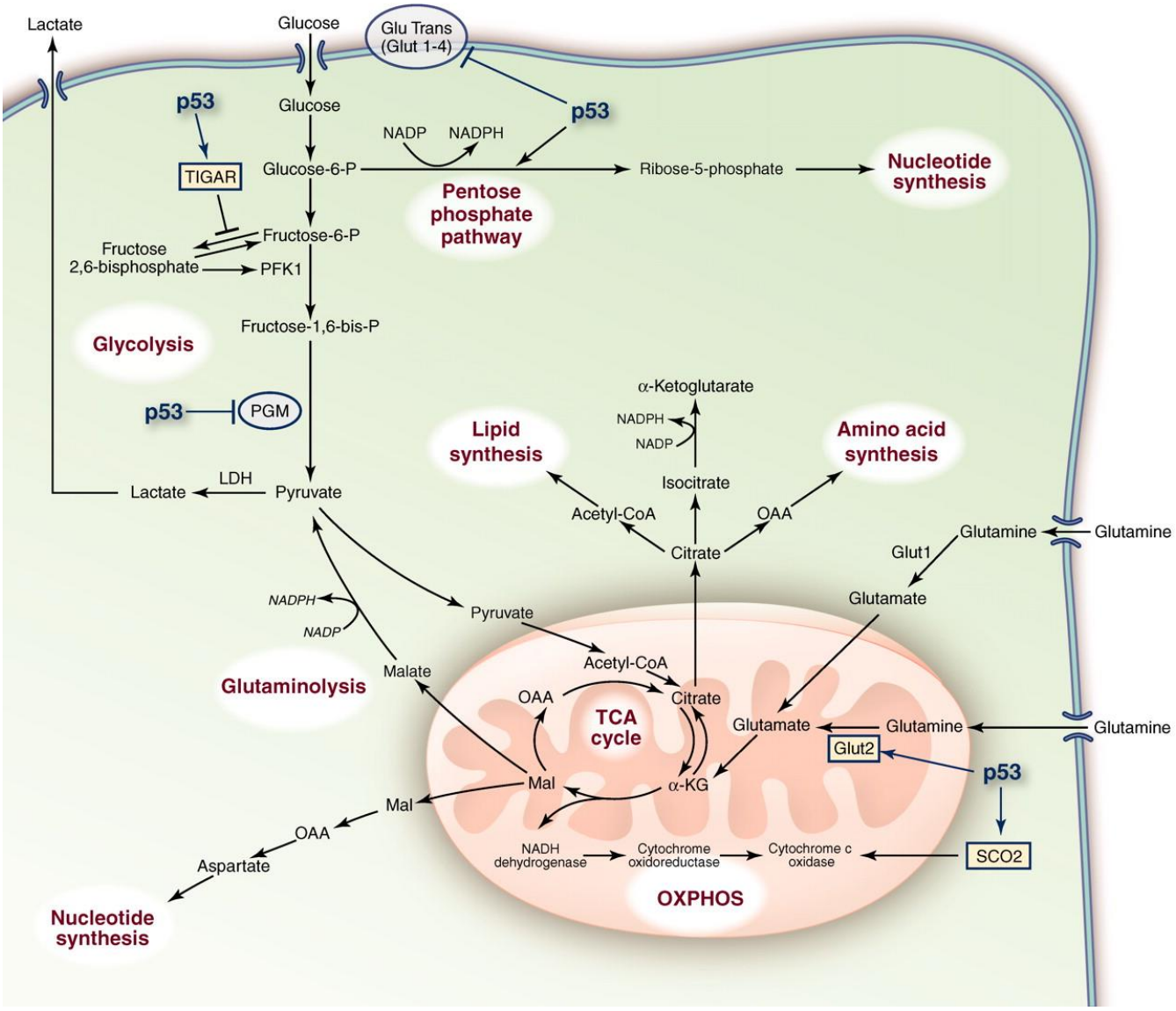

Figure 2.1: Cell metabolic pathways. Glucose is converted into pyruvate, which is converted into Acetyl-CoA; Acetyl-CoA enters the citric acid cycle, which generates ATP molecules and precursors for the synthesis of fatty acids, lipids and amino acids. Glutamine may also act as a carbon source for TCA. The pentose phosphate pathway, where G6P used for nucleotide synthesis, is illustrated in the top half of the figure. From (116) with permission from Science AAAS via Rightslink (order number 5126030907570)

2.4.2 - ATP and Reactive Oxygen Species (ROS) Yield from Various Substrates

MSCs rely primarily on glucose, glutamine and pyruvate as the energy source for *in vitro* culture, but may also rely on other energy sources in an in vivo environment. During the starvation state, plasma ketone levels increase from 0.1 mM to 7 mM (117).

The metabolic activity of human MSCs have been investigated from P2-P5 under both normoxic (20%) and hypoxic (5%) conditions (117). Between P2 and P5, the authors reported a 2 fold increase in glucose oxidation rate (from 0.1 to 0.22 nmol/min/10,000 cells) and a 2.6 fold increase in acetoacetate oxidation rate under normoxic conditions (from 3 to 7.8 nmol/min/10,000 cells). At P2, hypoxia had no clear effect on metabolic activity. At P5, glucose consumption was not altered by hypoxia, but lactate production fell by half. No significant changes between passage numbers were observed for glutamine or pyruvate oxidation. Acetoacetate metabolism was 27-35 fold higher than glucose depending on the passage number (117).

An 8 fold reduction in acetoacetate oxidation was reported in the presence of 5.5 mM glucose, but a 1.5 fold increase when 2 mM pyruvate was added to the culture media. The introduction of pyruvate at 2 mM may have activated the enzyme pyruvate carboxylase, which adds extra oxaloacetate into TCA. Smaller amounts of pyruvate generated from glycolysis may not be sufficient to activate the pyruvate carboxylase enzyme (117).

The greatest amount of ATP was produced when acetoacetate served as the substrate (60 nmol/min/10,000 cells compared to <10 nmol/min/10000 cells for other substrates (117). The preference for acetoacetate by MSCs demonstrated in this study may warrant its inclusion in culture media (117).

The results demonstrate that glucose consumption rate increases with passage number, but only under normoxic conditions, and that acetoacetate yields more energy and exerts less oxidative stress than other substrates. However the authors need to ensure that acetoacetate does not alter the stemness and immunomodulatory activity of BMMSCs. Acetoacetate may be a good candidate as an ingredient for the expansion and differentiation media for human BMMSCs. Oxidation of acetoacetate produces fewer molecules of ROS, and therefore differentiation media with acetoacetate and a lower amount of glucose may have less inhibition on MSC differentiation and proliferation. If 2.5 mM or 5.5 mM glucose is not sufficient for 12 days of preservation, the media could be supplemented with acetoacetate as an additional energy source. Additional studies on the use of ketones for MSC culture or preservation have not been undertaken.

2.4.3 - Effect of Spheroid Size on the Metabolic Activity of MSCs

Less than 1% of MSCs survive 4 days following infusion into the hearts of adult mice (6) - reasons may include the sudden shift to a normoxic *in vitro* environment during culture from an *in vivo* hypoxic environment (118). Murphy *et al.* (2014) reported that culturing MSCs as 15,000 cell, 30,000 cell or 60,000 cell spheroids may improve their survivability post implantation compared to disassociated cells due to decreased metabolic demand (119). It is thought that the lack of oxygen in the core of the spheroid promotes the cell survival and increases the production of trophic factors (119).

There was no significant change in oxygen tension between the surface and core. Glucose consumption rates and lactate production rates decreased with increasing spheroid size. Larger spheroids exhibited greater caspase activity, which indicates the extent of cell death (119). If cells cannot survive in suspension, it is important to determine whether spheroid culture improves survival rates, and which spheroid size is optimal for preservation.

2.4.4 – Species-Species and Donor-Donor variation in Proliferation, Metabolic Activity, Differentiation potential and Immunomodulatory Function

MSC metabolism has been compared between 3 different species: human, goat and rat (113). Glucose, glutamine and lactate measurements were conducted using the VTROS DT60 II chemistry system (Ortho Clinical Diagnostics, USA). Glucose consumption rates were as follows: 2.59 pmol/cell/day for goats, 5.89 pmol/cell/day for rats and 9.19 pmol/cell/day for humans (113). Relative to glucose, lactate concentrations were 0.80 mol/mol for rats, 1.86 mol/mol for goat and 1.96 mol/mol for humans. Ammonia production rates were 1.35 pmol/cell/day for rats, 1.60 pmol/cell/day for goats and 1.63 pmol/cell/day for humans. For each sample, MSCs from 5 donors were obtained (113). The accumulation of ammonia or lactate in culture media has a profound effect on rat MSC morphology after 7 days of exposure: high lactate medium (30 mM) causes MSCs to become more elongated, while cells adopt a cuboid shape in medium with 4 mM ammonia. This may be due to the effect of abnormal pH on the cytoplasm of the cell. In the presence of high lactate levels, adipocyte differentiation was inhibited (113). As metabolic waste accumulation has been shown to alter morphology and gene expression, the novel preservation media must therefore slow down cell metabolism to keep waste levels low during storage and transport. It is proposed to achieve this by keeping cells at cold storage or room temperature.

Differences in the mechanism of immunomodulatory activity have also been reported between primary human and primary mouse BMMSCs at passage 5 (120). The effect on MSC suppression on PBMC proliferation was tested in the presence of various antibodies and inhibitors for anti-inflammatory mediators. For human MSCs, PBMC suppression was maintained in the presence of L-NMMA (nitric oxide synthetase inhibitor), anti-TGFβ, anti-IL-10 and indomethacin (prostaglandin E2 inhibitor), while PMBC suppression was abolished upon the addition of anti-IFNγ and 1-methyltryptophan (1MT) (IDO Inhibitor) (120). For mice BMMSCs, on the other hand, PBMC suppression was mainly due to NO synthesis. Stimulation of BMMSCs using IFNγ lead to an increased production of IDO in human cells and iNOS in mice cells, but not *vice versa* (120). Overall this may suggest that immunosuppression from human BMMSCs is mainly due to IFNγ production while mice BMMSCs exert their immunomodulatory functions via NO production. The study needs to be repeated with multiple samples within the same species to determine if there is donor-donor variation in the mechanism of PBMC suppression.

Primary human BMMSCs have also been cultured in a proprietary medium containing deoxyribonucleotides, ribonucleotides and 10% FCS, along with 15 mM lactate for 1h, 6h, 24h, 72h or 144 h to assess the effect of media lactate on gene expression (121). As controls, MSCs were cultured in parallel without background lactate. After 3 days of lactate culture followed by microarray analysis, the authors reported a 4.11-fold increase in interleukin-6 (IL-6) expression, 2.36-fold increase in heat shock protein 70 (HSP70) expression and a 2.09-fold increase in hypoxia-inducible factor-1α (HIF-1α) expression compared to cells cultured in lactate free media (121). After 7 days of lactate treatment, a downregulation of SOD2 (0.32 fold) and BCL2-associated X protein (BAX) (0.4 fold) was observed (121). There was no significant change in stem cell marker expression (CD 29, 44, 59, 73, 90, 105, 106 and 146) regardless of the duration of lactate exposure (121).

Heathman *et al.* (2016) reported considerable variation in the metabolic activity, growth rate and immunosuppressive activity in BMMSCs from 5 different human donors (labelled M0 to M4). The age ranged between 19 and 27, and a variety of ethnic groups were included. Metabolic activity was measured by the Bioprofile FLEX cell culture chemistry analyser (Nova Biomedical, USA) (60). Growth rate varied from donor to donor, ranging from 7.81 ± 0.32 to 13.74 ± 0.33 cumulative population doublings in 30 days of culture. For all samples, specific growth rate decreased with higher passage numbers (60). The authors reported variations in metabolic flux between the 5 samples. Glucose consumption (averaging 26.98 pmol $cell^{-1}$ day^{-1}) and lactate production rates (averaging 29.45 pmol $cell^{-1}$ day^{-1}) remained stable from P4-P8, with the exception of M3 where both parameters increased with passage number. Ammonium production averaged 1.35 pmol $cell^{-1}$ day^{-1} (60). For the M3 and M4 group, osteogenic potential decreased significantly

from P3 to P10, while it remained low for the M2 group at all passages. (60) Immunomodulatory activity varied between 36.3 ng/mL ± 3.65 of Il-6 production to 80.8 ng/mL ± 8.4 from passage 3-5 depending on the donor.

The metabolic activity, morphology and proliferation potential of porcine BMMSCs from 3 different samples were assessed from passages 1-3. Cells were cultured for a total of 5 days at each passage in a-MEM/FBS (61). Proliferation potential in flask culture at passage 3, assessed by the alamar blue assay, was reported as 25% for Pig 2 and approx. 40% for Pig 1 and Pig 3 (61). When cultured in flasks, cells from Pig 1 and Pig 2 developed a long spindle morphology, though the nuclei was larger in Pig 2. Pig 3, however, adopted a cobblestone like morphology (61). Due to poorer proliferation rates in Pig 2, only P1 cells from Pig 1 and Pig 3 were expanded for an additional 2 passages in a Quantum hollow fibre bioreactor, where culture lasted for 8 days per passage (61). To assess metabolic activity, glucose and lactate media concentrations were assessed daily using handheld glucose/lactate devices (61). At both passages, glucose and lactate media levels followed a similar pattern of change for both Pig 1 and Pig 3, indicating no difference in cell metabolism between the 2 groups (61). Oil Red Staining showed that Pig 3 exhibited a greater number of lipid droplets compared to Pig 1 following 2 passages of quantum expansion, while alizarin red staining showed more rapid osteogenesis in Pig 1 relative to Pig 3 (61). By Alcian blue staining, it was demonstrated that Pig 1 yielded larger chondrocyte spheroids than Pig 3 (61). Regardless of flask or hollowfibre expansion, all 3 samples expressed stem cell markers (CD44, CD90 and CD105) while lacking haematopoietic/leukocyte markers (CD31, SLA-DR and CD45) (61).

The results show that sample to sample variation occurs in the growth rate, metabolic activity, differentiation potential and the immunomodulatory activity of MSCs therefore, it may be better to use autologous BMMSCs for therapy. For each sample, growth rate falls with increasing passage number. The measure of IL-6 production in MSCs can be used in future experiments to assess the effect of the novel transportation media on immunomodulatory function.

2.4.5 - Effect of 3D Printing Materials on MSC Survival and Metabolism

During preservation, MSCs may need to be encapsulated with special materials to withstand shaking and keep cells in a quiescent state. The material must be non-toxic, and must not raise metabolic activity, which may otherwise lead to glucose depletion and accumulation of metabolic waste. The container must also be safe for preserved cells. Disks consisting of either acrylonitrile butadiene styrene (ABS), a proprietary medically approved thermoplastic for hearing aids and

dentistry (MED), polycarbonate (PC) or polylactic acid (PLA) have been tested as platforms for MSC culture, or as indirect culture where cells and discs were separated by inserts (122). This will determine whether the effect of 3D printing materials is due to cell contact. MSCs cultured in the absence of printing materials served as the control. (122)

At 48 hours, MSC proliferation was enhanced 8 fold compared to control when cultured directly on ABS or PC. No significant changes in proliferation was observed upon direct culture on MED or PLA. During indirect culture, none of the materials tested had a significant effect on MSC proliferation. Lactate dehydrogenase (LDH) activity, an indication of cell damage, was significantly raised in all but indirect culture with ABS or MED. Glucose uptake and lactate production was significantly lowered (>50%) for all materials with the exception of indirect culture on PLA. (10). (122)

When indirectly cultured in 3D printing materials, elevated expression of the gene CTSD was observed in the presence of MED, while elevated levels of CTSD, CASP3 and THY1 occurred in the presence of PLA. These proteins are thought to be associated with MSC migration to areas of inflammation (123), apoptosis (124) and osteogenic differentiation (125) respectively. For direct culture, expression of CTSD, tumour suppressor gene EGR1 and CASP3 was elevated in in all 4 materials. In addition, up regulation of osteogenic differentiation genes THY1 and BGLAP was observed for direct culture on ABS, alongside a fall 25-50% fall in ACAN expression, which is associated with chondrogenesis. Direct culture on MED also caused a 40% fall in MK167, which is thought to be related to cell proliferation. A 25-50% reduction in ACAN expression was also observed for indirect cultured on PC and ABS (122).

The data may suggest that MED may be suitable container or encapsulation material for MSC preservation, as it does not enhance cell proliferation, does not cause cell damage, lowers metabolic activity does not alter genes associated with MSC differentiation. However, longer exposure times of up to 12 days should be tested to assess the suitability of MED as a cell preservation material. Additional studies on the effect of printing materials on MSC metabolic activity has not been carried out.

2.5 – Cell Culture Media Components

To support cell survival and expansion, the basal medium contains the required macronutrients (glucose and pyruvate), B vitamins, amino acids and salts, while the serum provides albumin, fat soluble vitamins and growth factors. It is important to determine which of these components are required for cell preservation so the final preservation medium can be formulated.

2.5.1 – Importance of Serum for Cell Survival

Nuschke *et al.* (2016) reported that human BMMSCs become apoptotic after 4-5 days if FBS is absent, even if key substrates such as glucose, glutamine and pyruvate are present; the same occurs in reverse (127). This indicates that glucose, glutamine and pyruvate, along with a blood free and animal free alternative supplement is required to maintain healthy function of the MSCs during preservation. Exposure of MSCs to blood components such as FBS during transportation may cause an immune response in patients (126). Therefore, spheropreserved MSCs in α-MEM/FBS may not be suitable for therapy.

2.5.2 – Formulation of a Chemically Defined Serum-free Media for MSC Expansion

Insulin is an essential component of cell metabolism, where it is thought to upregulate glucose transporter proteins on the plasma membrane and promote glucose uptake, along with the synthesis of proteins, lipids and glycogen (128). Transferrin is also essential for cell culture, as it binds iron, an essential co factor for many cellular processes (129).

Accumulation of reactive oxygen species (ROS) are thought to cause a loss of differentiation and proliferation potential, possibly by disruption of the membrane structure and damage to the DNA (130). Therefore, to maintain MSC survival and function during transportation, a ROS scavenger was required for the media. Selenium acts as a ROS scavenger by serving as a co factor for the glutathione peroxidase family of enzymes, which converts ROS into H_2O. Park *et al.* (2018) have reported that selenium (5 ng/mL) reduces oxidative stress in human primary amniotic fluid MSCs by 3 fold, without altering its proliferation, marker expression or trilineage differentiation (131).

MSCs culture is typically supplemented with FBS, which includes factors that promote cell proliferation, attachment and survival.(132) However, issues include batch to batch variation, contamination and the risk of an immune response, making FBS unsuitable for use in a clinical setting.

Jung *et al.* (2012) reported that a serum free and chemically defined DMEM/F-12 based expansion medium could be used for MSC culture. The media (PPRF(-)) was supplemented with 4 mM L-glutamine, 0.1% v/v lipid concentrate, 20.5 mM Sodium Bicarbonate, 4.9 mM HEPES, 4.01 µM bovine insulin, 0.318 mM human transferrin, 55.9 µM putrescine dihydrochloride, 28 nM sodium selenite, 0.018 µm progesterone, 0.7 U/mL heparin and 4 g/L human serum albumin. The authors reported that MSCs proliferate more rapidly in PPRF (-) (6x increase in cell number over 10 days) in comparison to DMEM and pre-existing MSC culture media such as MSCBM and MCDB-201 (4 fold increase) (133). By eliminating each compound one by one, the authors determined that selenium and heparin inhibited MSC proliferation by roughly 50%. From this point the authors removed selenium and heparin prior to the testing of growth factors. (132) The authors tested the effect of various growth and attachment factors on cell proliferation (see below). DMEM/F-12 and 10% FBS served as the control.

- basic fibroblast growth factor (bFGF)
- keratinocyte growth factor/fibroblast growth factor-7 (KGF/FGF-7)
- platelet-derived growth factor-BB (PDGF)
- Dickkopf-related protein 1 (Dkk-1)
- acidic fibroblast growth factor (aFGF)
- fibroblast growth factor-4 (FGF-4)
- betacellulin
- activin A
- transforming growth factor-β (TGF-β1)
- epidermal growth factor (EGF)
- Fms-like tyrosine kinase-3 ligand (Flt-3)
- interleukin-3 (IL-3)
- interleukin-6 (IL-6)
- stem cell factor (SCF)
- insulin-like growth factor-1 (IGF-1)
- hepatocyte growth factor (HGF)
- insulin-like growth factor-2 (IGF-2)
- leukaemia inhibitory factor (LIF)

All factors were tested at concentrations of 20 ng/ml. The authors reported that bFGF is essential for MSC proliferation and survival – 50% cell death occurred when bFGF was absent from the media. Supplementation with TGF-β1 further enhanced the proliferation rate, but only when

bFGF was present. Addition of other growth factors did not enhance proliferation in the presence of bFGF. Cell expansion did not occur unless 50 µg/mL ascorbic acid, 1 g/L fetuin and 50 nM hydrocortisone was present. Addition of bovine gelatine or fibronectin further enhanced cell proliferation 2 fold (133). The finalised media was named PPRF-msc6. MSCs cultured in the PPRF-msc6 maintained trilineage differentiation and stem cell marker expression (133). To summarise, the research shows that cells only require the following for expansion in serum free media: bFGF, TGF-β1, human fibronectin, ascorbic acid, fetuin and hydrocortisone.

P1 human amniotic MSCs (hAMSCs) have been cultured for 5 passages in serum free medium with a DMEM/F-12 base, supplemented with 1x ITS, 0.5% human serum albumin (HSA), 10 ng/mL bFGF and 100 µg/mL L-ascorbic acid. For the control, cells were also expanded in DMEM/FBS (134). Cells cultured in both FBS and ITS media exhibited identical spindle shaped morphologies at P1, P3 and P5 of culture, and expressed stem cell markers (CD73, CD90 and CD105) without haematopoietic, endothelial and leukocyte markers (CD34 and CD45) (134). Proliferation potential, migration capacity and differentiation potential into osteocytes, adipocytes, chondrocytes and keratinocytes were similar in both groups (134). RT-PCR demonstrated that cells cultured in serum free media expressed higher levels of PGDF, VEGF, keratinocyte growth factor (KGF) (1.5 fold vs. cells cultured in DMEM/FBS), higher levels of matrixmetallopeptidase-2 (MMP2) (3 fold vs. DMEM/FBS group) and elevated levels of TGF-B1 (4 fold vs. DMEM/FBS group) (134). This study demonstrates that MSC expansion may not require other ingredients such as TGF-β1, human fibronectin, ascorbic acid, fetuin and hydrocortisone used by *Jung et al.* (2012) (133).

Since ITS was the common ingredient in multiple formulations of serum free expansion media, it can be concluded that ITS is crucial for cell survival in the absence of serum. The project will investigate whether ITS will maintain MSC survival in the absence of FBS in the novel preservation media, with basal media acting as the control.

2.5.3 - Maintaining Physiological pH at Atmospheric Conditions

Maintaining 5% CO_2 during preservation requires gas cylinders, making preservation expensive and inconvenient. Culture media typically contains 44 mM Sodium Bicarbonate, which would rapidly turn the media alkaline at atmospheric (0.04%) CO_2. It is therefore needed to alter the buffer so that an acceptable pH (7.0-8.0) is maintained during 12 days of preservation at atmospheric CO_2.

Mice MSCs have been successfully cultured in α-MEM at atmospheric CO_2 and 37°C using a buffering combination of 12 mM HEPES, 12 mM MOPS and 5 mM bicarbonate. Over a 60 hour

period, cells in the MOPS/HEPES media at atmospheric CO_2 displayed an identical growth pattern to MSCs in media with 44 mM bicarbonate 5% CO_2 (6 fold increase). The HEPES/MOPS media was shown to be superior to Leibowitz media (4 fold increase). Cells in MOPS and HEPES retained their differentiation potential, as they stained positive for osteocytes, adipocytes and chondrocytes. qPCR revealed no significant changes in genes associated with adipogenic differentiation (lipoprotein lipase, PPARγ2 and C/EBPα), osteogenic differentiation (Collagen I, Osteonetcin and CBFα1) and chondrogenic differentiation (Collagen II, Aggrecan and SOX9) between cells in incubator and atmospheric conditions (107). However, pH during culture has not been stated in the publication (107).

Osteogenic differentiation of human BMMSCs have been carried out at atmospheric CO_2 using a combination of 4.88 mM sodium bicarbonate, 4.19 mM disodium hydrogen phosphate dodecahydrate, and 0.5 mM potassium dihydrogen phosphate in DMEM (phosphate medium). Cells cultured in DMEM and 44 mM sodium bicarbonate at atmospheric CO_2 served as the negative control, while osteogenic media at 5% CO_2 served as the positive control. All medias were supplemented with 0 µM dexamethasone, 50 µg/mL ascorbic acid and 10 mM sodium β-glycerophosphate to promote osteogenic differentiation. (108)

After 72 hours of MSC culture, the pH for phosphate media remained between 6.94 and 7.16, while the pH of control media rapidly rose to 9 at the 12 hour time frame (108). Cells cultured in phosphate medium at atmospheric CO_2 yielded similar levels of alkaline phosphatase activity and expressed similar levels of osteogenic genes Runx2, collagen 1α, OCN and ALP in comparison to the osteogenic media at 5% CO_2. The data shows that phosphates can be used as to maintain media pH during MSC differentiation at atmospheric CO_2 (108).

2.5.4 – The Role of Amino Acids in Cell Culture

Amino acids are organic compounds that contain carboxyl (-COOH) and amine (-NH) groups, along with a side chain (-R) unique to each amino acid (135). Amino acids form the basic building blocks of the cell, including the cytoskeleton, enzymes, and receptors and signalling molecules (136), and are included in cell culture media. In total, there are 20 amino acids, 9 of which are essential, where they cannot be synthesized by human cells (112)– histidine, isoleucine, leucine, lysine, methionine, phenylalanine, threonine, tryptophan and valine. The remainder are arginine, cysteine, glutamine, glycine, proline, tyrosine, alanine, aspartic acid, asparagine, glutamic acid and serine (112).

Eagle *et al.* (1958) reported that amino acid supplementation is crucial for the expansion of mammalian cells, leading to the formulation of Dulbecco's modified eagle's medium (DMEM) and minimum essential medium (MEM) that is commonly used in cell culture today (112). They reported that monkey kidney cells also required arginine, cysteine, glutamine, histidine, and tyrosine on top of the essential amino acids, as cells lose the ability to synthesise non-essential amino acids after prolonged in vitro culture (112).

In various cancer cell lines, isotope radio mass spectroscopy (IRMS) has shown that amino acids form the majority of proliferating cell mass (30%), where glucose comprised of 5% (137). Non-proliferating cell mass was predominantly glucose (15%) followed by glutamine, serine and valine at percentages of 7%, 2% and 2% respectively (137). When cells were treated with thiol lysate and tracked for 14C isotopes, they found that all valine (100%) and the majority of glycine and serine (60-70%) contributed to protein formation. When cells were deprived of non-essential amino acids – more carbon from glucose was incorporated into cell mass to compensate (137).

During static monolayer culture, Higuera *et al.* (2009) reported that MSCs produce alanine, cysteine, glutamate, glycine, ornithine and proline. All other amino acids were consumed (138). During the batch fed culture of MSCs, Schop *et al.* (2009) reported that MSCs produce only Glutamate, alanine and glycine, while all other amino acids are consumed (113).

2.5.5 - Role of Vitamins in Cell Culture Media

Culture media is supplemented with B vitamins, which act as a co factors for various biological processes, and are thought to be essential for cell function, proliferation and survival (139).

Choline chloride (Vitamin B4) serves as a phospholipid precursor for phosphatidylcholine and sphingomyelin, which are components of lipid bilayers (140). Choline is transported into cells via an ATP dependent choline transporter, and is converted to phosphatidylcholine via the Kennedy Pathway (141). A portion of the phosphatidylcholine produced donates a phosphocholine group to ceramide (a product of Palmitoyl-CoA and Serine condensation), forming sphingomyelins (141).

Folate (Vitamin B9) is required for the synthesis of guanine monophosphate (GMP) and adenosine monophosphate (AMP) (142), and the methylation of vitamin B12, which is required for the methionine cycle (143).

Nicotinamide (Vitamin B3) is combined with nicotinic acid and nicotinamide riboside to form nicotinamide adenine dinucleotide ($NADH/NAD^+$) – which serves as a coenzyme in various metabolic reactions such as glycolysis, the citric acid cycle and the electron transport chain (144).

Pantothenic acid (Vitamin B5) is required for the synthesis of Coenzyme-A (CoA), consuming cysteine and ATP in the process (145). CoA may combine with carboxylic acid to form thioesters, required to transport fatty acids from the cytoplasm to the mitochondria or converted into Acetyl-CoA, an intermediate of the citric acid cycle (145).

Riboflavin (Vitamin B2) is converted into flavin mononucleotide (FMN) by riboflavin kinase. FMN and its redox-active form flavin adenine dinucleotide (FAD) are required for the formation of Complex I (FMN) and Complex II (FAD) of the electron transport chain (146). Thiamine (Vitamin B1) is converted into thiamine pyrophosphate, a cofactor for sugar and amino acid metabolism or into thiamine diphosphate, a co factor for transketolase in the pentose phosphate pathway. Vitamin B1 is also required as a cofactor for pyruvate dehydrogenase and 2-oxoglutarate dehydrogenase (147).

2.6 – Conclusion

In conclusion, MSCs have been shown to have the potential to treat a wide range of diseases and possess multiple advantages over ESCs. Studies of MSC metabolism has been conducted at normal conditions, where cells were cultured in a monolayer in expansion media. However, there is a need to develop a novel preservation media to keep cells viable at atmospheric conditions without the requirement for cryopreservation, cold temperature or serum. It is important to study metabolic requirements during atmospheric conditions to determine the optimal cell density for storage.

Chapter 3

Materials and Methods

3.1 – Expansion and Characterisation of hTERT Human and Primary Bovine Mesenchymal Stem Cells

3.1.1 - Extraction of Primary Bovine Bone Marrow Mesenchymal Stem Cells (BMMSCs)

The method used for MSC extraction was an established technique in the Tissue Engineering group at the Institute of Biomedical Engineering by Dr Huidong Jia (Oxford University, UK). Bovine feet from 3 week old calves were obtained from a local abattoir within 3 hours of slaughter and used immediately. Hooves, skin and cartilage were removed using a scalpel, and the foot was soaked in diluted bleach for 1 hour. Holes were drilled on both ends of the bone, and marrow was flushed into a petri dish using a 50 mL syringe containing phosphate buffered saline (PBS) (1010023) and 1% penicillin/streptomycin (p/s) (15140122) (all from Gibco, UK). The marrow was then aspirated with a syringe into 50 mL centrifuge tubes (Corning, UK). The sample was shaken vigorously to release the BMMSCs into the PBS, and then incubated at room temperature for 10 minutes to allow a layer of adipocytes to float to the top. The adipocyte layer was then discarded. Bone marrow samples were filtered through a 70 µm cell strainer (CLS431751-50EA, Sigma-Aldrich, UK) to remove bone fragments. In order to wash the tissue sample, cells were centrifuged at 1800 g in 30 mL PBS (1010023) for 5 minutes, and the supernatant was discarded. A total of 3 washes were carried out. All liquid DMEM (11885084) used was purchased from (Gibco, UK), while all powder DMEM (D5030-10X1L) was purchased from (Sigma-Aldrich, UK). All media formulations used in this thesis are listed in **Table 3.1**. The pellets were suspended in 5 mL of DMEM **(Media A)**. A manual cell count was performed using a haemocytometer. Cells were seeded at a density of 5 x 10^6/ cm^2 in T-175 flasks containing 30 mL of the media described previously. Flasks were incubated for exactly 48 hours at 37°C, 5% CO_2, before a media change to remove unwanted cell types. Further media changes were performed twice per week thereafter, and cells were harvested when 80% confluence was reached.

3.1.2 – Media and Cell Culture

Human immortal bone marrow mesenchymal stem cells (BMMSCs) were created by Mihara *et al.* (2003) (148), where human BMMSCs were genetically modified to express human telomerase reverse transcriptase (hTERT) and green fluorescent protein (GFP) (148); primary human BMMSCs from a single healthy donor was obtained by direct plating and expanded using Roswell Park Memorial Institute (RPMI)-1640 medium with 10 % foetal calf serum (FCS) and 10 µM hydrocortisone for 2 months (148).

Cells were then infected with a vesicular stomatitis virus G (VSV-G) retroviral vector containing genes for hTERT to confer immortality and GFP to allow improved visualisation under a fluorescent microscope (148). GFP^+ MSCs were isolated using a fluorescence activated cell sorter and a total of 42 $hTERT^+$ stably growing clones were isolated from 1500 wells. Immortalised cells expressed stem cell markers CD44 and CD73, while lacking expression of leukocyte, haematopoietic and endothelial markers (CD31, CD45 and HLA-DR) (148). hTERT cells proliferated continuously after 800 days of culture without decline in doubling times, while wild-type cells became senescent after 100 days of culture (148). Differentiation potential of $hTERT^+$ and GFP^+ cells were confirmed by western blot, which detected genes associated with osteogenesis (Osteoponin, Bone sialoprotein and Osteocalcin) and chondrogenesis (COMP and collagen type II α) (148).

For expansion, both hTERT human and primary bovine MSCs were seeded at a density of 5000 cells/cm^2 into T175 flasks (Corning, UK) in 30 mL of DMEM/FBS **(Media A)**. Unless stated otherwise, cell culture was carried out in a humidified incubator set to 37^oC and 5% CO_2. Prior to all experiments, the incubator was checked to ensure that water was present.

The hTERT MSCs were used during the development of ITSE based medium and to test the effect of low temperature and low glucose, while both primary and hTERT MSCs were used to test the finalised insulin preservation medium (ITSE) at atmospheric conditions. For all media used component concentrations are at default unless stated otherwise in **Table 3.1**. To prevent background degradation of glutamine into ammonia, all key experiments used 4 mM GlutaMAX (35050061, Gibco, UK) instead of glutamine. All media contained 1% penicillin and streptomycin (p/s) to prevent infection. For all experiments, hTERT MSCs were at P15, while primary bovine MSCs were at P3 unless stated otherwise. For all media containing ITSE (41400045, Gibco UK) in this entire project, the final concentrations of ITSE components in the media were as follows: 10 mg/L insulin, 5.5 mg/L transferrin, 6.7 µg/L sodium selenite and 2 mg/L ethanolamine.

Table 3.1: Media formulations used in various studies.

Experiment	Media formulation	Section
Cell expansion	DMEM, 5.5 mM glucose, 10% FBS **(Media A)**	3.1.1-3, 3.1.5, 3.1.8, 3.4.3, 4.3.4, 6.3.3-5
End point metabolism (24-48h) for hTERT MSCs (37°C, 25°C and 4°C), primary MSCs (37°C), and recovering hTERT MSCs after 4, 8 or 12 days in ITSE (37°C)	DMEM, 2.5 mM glucose, 10% FBS **(Media B)**	3.1.7-8, 3.2.1-4, 3.3, 4.3.3-5, 4.4.4, 5.3.2-5, 6.3.2-5
Change in hTERT MSC metabolism during 72 hours of expansion	DMEM, 1 mm glucose, 10% FBS **(Media C)**	4.3.4
Determine if loss of proliferation in ITSE was due to absence of serum growth factors	DMEM, 2.5 mM glucose, 5 µg/0.5 L basic fibroblast growth factor (bFGF), 5 µg/0.5 L epidermal growth factor (EGF), 2.5 µg/0.5 L of Transforming growth factor (TGF-β1), 1% ITSE **(Media D)**	3.1.5, 5.3.2
Determine if ITSE keeps hTERT MSCs viable at 37°C in the absence of serum over 12 days	DMEM, 5 mM glucose, 1% ITSE **(Media E1)**	3.2.3, 3.3, 5.3
	DMEM, 5 mM glucose, no supplement/serum (control) **(Media E2)**	
Effect of 12 days in ITSE on recovery of proliferation (hTERT MSC, 37°C), End point metabolism (24-48h) for hTERT MSCs in ITSE, autophagy and ROS	DMEM, 2.5 mM glucose, 0.3 mM pyruvate, 1% ITSE **(Media F1)**	3.1.5-6 3.2.1-4 5.3.2-4
	DMEM, 2.5 mM glucose, No pyruvate, 1% ITSE **(Media F2)**	
Effect of 12 days in ITSE on differentiation potential (hTERT MSC, 37°C)	DMEM, 2.5 mM glucose, 1 mM pyruvate, 1% ITSE **(Media G)**	5.3.2, 5.3.4-5
Survival and metabolism of MSC monolayers under atmospheric conditions	DMEM, 2.5 mM glucose , 1% ITSE, 0.9 mM sodium bicarbonate **(Media H)**	3.2.4.4, 3.4.3.8, 6.3.1

12 day preservation of MSCs in suspension above agarose, and in alginate beads	DMEM, 5 mM glucose, 1% ITSE, 0.9 mM sodium bicarbonate **(Media I)**	3.1.8.2, 3.4, 3.4.3.2-5, 6.3.3-4
MSC metabolism in alginate beads	DMEM, 1 mM glucose, 1% ITSE, 0.9 mM sodium bicarbonate **(Media J)**	3.4, 3.4.2.1, 6.3.2, 6.3.7
Stempro Osteogenesis (**Media K1**), Adipogenesis (**Media K2**) and Chondrogenesis (**Media K3**) Kit	Basal Medium + proprietary supplement	3.1.8, 4.3.2, 5.3.5, 6.3.5

3.1.3 - Study of MSC Surface Marker Expression for hTERT Human and Primary Bovine BMMSCs

P14 MSCs were defrosted and cultured in a T175 culture flask in DMEM/FBS **(Media A)**, and expanded to 80% confluence to allow recovery. Cells were detached using trypsin-EDTA (0.25%) (all from Gibco, UK), followed by washing and suspension in phosphate buffered saline (PBS) with 1% FBS (PBS/FBS). Freshly extracted P1 bovine MSCs were used for flow cytometry.

For hTERT MSCs, stemness was assessed using positive markers (10); anti-human CD73 – PE (Thermo Fisher, UK), anti-human CD105 - PE (Thermo Fisher, UK), anti-CD90 - APC (R&D Biosystems, USA). Negative markers were included to ensure the absence of haematopoietic cells, endothelial cells and leukocytes, which included rat anti-CD45 – FITC (Miltenyi Biotech, Germany) and human anti-CD34 - PE (R&D Biosystems, USA) (10). For primary cells, all markers were assessed using newly purchased anti-human CD73, CD90, CD105, CD34 and CD45 (all PE), as antibodies used for hTERT MSCs had expired at the time of primary cell extraction.

To study surface marker expression, a total of 10 µL of antibody was added to 90 µL (100,000 cells) of MSC suspension and left in the fridge for 1 hour in the dark. Afterwards, cells were washed with PBS/FBS and fixed in PBS/FBS with 3.6% formaldehyde (Sigma Aldrich, UK). Flow cytometry was conducted using the FACScalibr (Becton-Dickinson, USA), where a total of 10,000 events were collected. Data was acquired using CellQuest Pro (BD Biosciences, USA) and analysed using FlowJo (FlowJo LLC, USA).

3.1.4 - Cell Proliferation and Survival

For all monolayer experiments, cell counts were acquired using the Spectramax i3X plate reader (Molecular Devices, USA) and Softmax Pro analytical software (Molecular Devices, USA). The

population doubling time (PDT) was calculated as:

$$PDT\ (h) = \frac{24h \times \log\ (2)}{\text{Log (cellstart)} - \text{Log (cellend)}}$$

Cellstart represented the number of cells/well at 24h, while *cellend* represented the number of cells/well at 48h for endpoint experiments. For experiments where cell proliferation was assessed at 12 hour intervals, *Cellstart* represented the number of cells/well at 24h, while *cellend* represented the number of cells/well at 72h. Proliferation was also reported as fold change vs. control, or fold change vs the first count.

A protocol was created to adapt the Spectramax i3X plate reader to read 24 well plates by selecting a 384 well plate, and choosing panels that correspond with the wells that were used in the 24 well plate, as shown in **Figure 3.1**. The plate reader was set to read 4 fields of view per panel to maximise the area of cell count per well, as indicated in **Figure 3.2**.

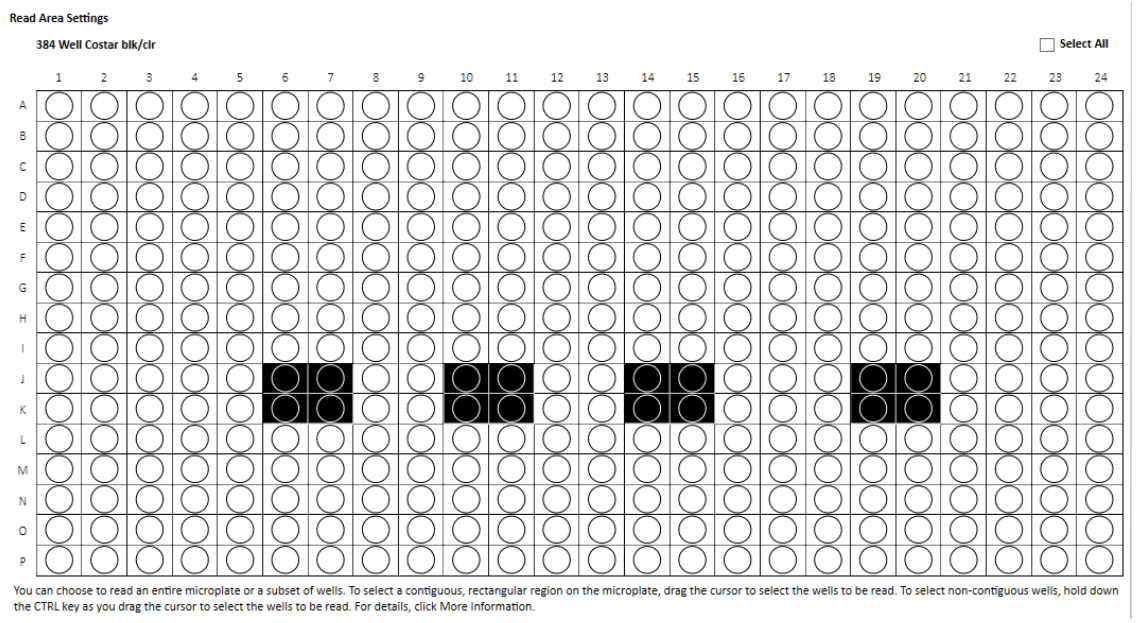

Figure 3.1: Adaptation of the 384 well protocol to a 24 well plate. For cell counts, the Spectramax i3x plate can only read 96 and 384 well plates. Corresponding panels to the 24 well plate were selected and shown as black squares on the map of a 384 well plate. Panel J14 was excluded as it covers the well edge.

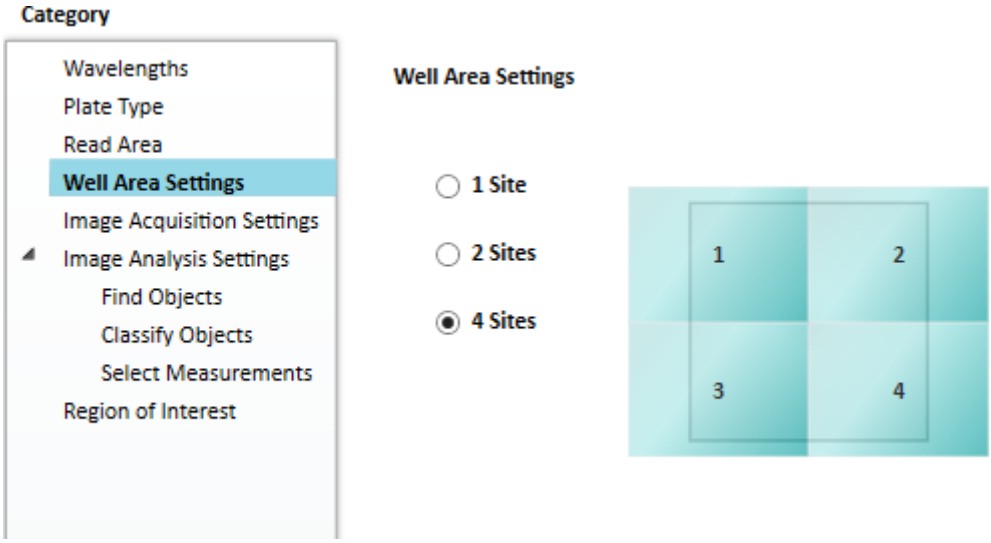

Figure 3.2: Selecting the number of fields of view per panel. A total of 1, 2 or 4 sites per panel could be selected for cell counting. 4 sites were chosen in this project.

Cells and blank spaces were selected and preliminary reads were repeated until the software correctly differentiated between them. Cells were detected via transmitted light – if a cell is present, light fails to pass through and that is detected as an object shown by purple spots – this is illustrated in **Figure 3.3**.

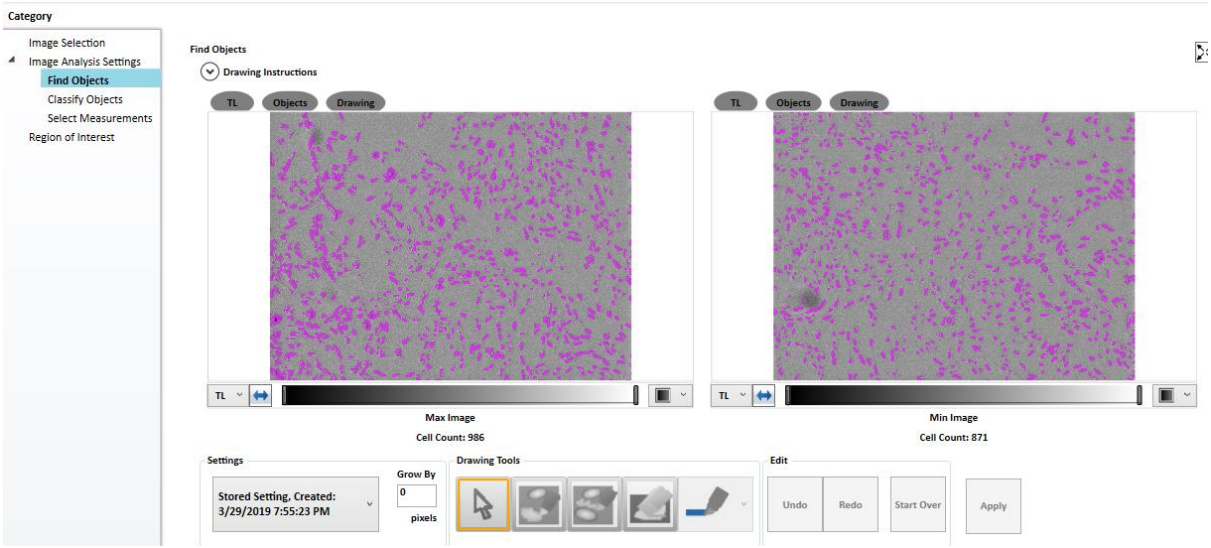

Figure 3.3: selecting cells for counting. Cells prevent light passing through, and are highlighted as purple.

According to Molecular Devices technical support, each field of view has an area of 0.0432 cm^2. Since 4 fields of view were read per panel, the area per panel is therefore 0.1728 cm^2. The total area of a single well in a 24 well plate is 1.9 cm^2. Therefore, the average cell count per panel was

multiplied by 11 (1.9 divide by 0.1728) to give the number of cells per well. Panels were excluded if they covered the edge of the well, as the edge can be mistaken for additional cells.

3.1.5 – Measurement of hTERT MSC Proliferation and Metabolic activity at 12 Hour Intervals.

P15 hTERT MSCs were seeded in 4 wells of a 24 well culture plate (3337, Corning, USA) at a density of 7500 cells/cm^2 for culture in DMEM/FBS at varying glucose concentrations (1-5.5 mM) **(Media A, B or C)** and 10,000 cells/cm^2 in ITSE based media **(Media F1, F2 or G)**. ITSE based medium was also supplemented with growth factors **(Media D)** to determine if loss of proliferation in ITSE based medium vs. DMEM/FBS was due to growth factors epidermal growth factor (EGF) (PHG0315, Gibco, UK), transforming growth factor beta 1 (TGF-β1) (ab50036, Abcam, UK) or basic fibroblast growth factor (bFGF) (PHG0264, Gibco, UK). Cell counts were performed at 12 hour intervals, and proliferation was reported as fold change vs. the number of cells at 12 h. Media changes were also performed at 12 hour intervals following cell counts, and glucose consumption or lactate production at each interval was calculated as (change in the amount of glucose or lactate in spent media/the average number of cells during that 12 hour interval/0.5) and reported as pmoles/cell/day. The total media volume was 500 µL and free of phenol red, as it was previously observed that cell death occurs when phenol red is used in ITSE based media.

Media without cells were incubated for the same amount of time to account for the starting metabolite concentration and background reactions between metabolites in media and metabolic enzymes in FBS. Outer wells were filled with PBS to prevent media evaporation. Cell counting, sample collection and media changes were all performed at 12 hour intervals for a total of 72 hours. For media samples with FBS, harvested culture media was deproteinsed by centrifugation at 10,000 g for 10 minutes in 10 kDa spin columns (ab93349, Abcam, UK) to remove interfering enzymes prior to being frozen at -20°C. For those in ITSE based media **(Media F1 and F2)**, harvested culture media was centrifuged at 10,000 g for 10 minutes to remove dead cells, before being frozen at -20°C. For all experiments, batch number 08G8380RK (Gibco, UK) was used for FBS. Glucose levels in media samples were measured using a glucose assay kit (ab102517, Abcam, UK) according to the manufacturer's instructions.

A standard calibration curve was created with known glucose concentrations (0, 80, 160, 240, 320 and 400 µM) in basal glucose free DMEM (A1443001, Gibco, UK) to account for interfering substances in media. For each experimental condition, media blanks were included to account for background absorbance of DMEM and FBS. Dilutions of media samples using DMEM were made prior to measurement to ensure that absorbance values lie within detectable range. Standards,

samples and blanks were incubated with the reagent for 30 minutes at room temperature in the dark before absorbance was measured at 450 nm. Absorbance measurements were performed in triplicate to reduce the margin of error. The procedure was repeated with the lactate assay kit (ab65331, Abcam, UK). During the development of the ITSE media, single measurements of metabolic activity were performed between 24-48h using an ARCHITECT bio analyser (Abbott Core Laboratory, USA) due to the extremely high cost of 6x 12 hour measurements with colorimetric assays.

3.1.6 – Validation of the ARCHITECT Bioanalyser

The ARCHITECT bioanalyser, used for glucose and lactate measurements from 24-48 hours, was validated using known glucose concentrations in DMEM (1 mM, 2.5 mM and 5 mM) and comparing them with values produced by the device.

3.1.7 – Measurement of Proliferation, End Point Glucose Consumption and Lactate Production in MSC Monolayers

Both P15 human hTERT and P3 primary bovine BMMSCs were seeded in a 24 well plate (3337, Corning, UK) at a density of 7500 cells/cm^2. Cell counts were performed at 24 and 48 hours, and media was harvested for analysis from 24-48h. Photographs were taken at 48h using a fluorescent microscope (Nikon, Japan), where the green filter was used for hTERT MSCs, and the phase contrast (∞Ph1) for primary MSCs.

In this study, single end point measurements were carried out at the 24-48 hour timeframe, where proliferation was reported as fold increase in cell count at 48h vs 24h. Media changes were also performed at 24 hour intervals and glucose consumption or lactate production was calculated as (change in the amount of glucose or lactate in spent media from 24-48h/the average number of cells during from 24-48h) and reported as pmoles/cell/day.

Measurements were carried out for the following experimental conditions (all of which as cell monolayers):

- hTERT MSCs in DMEM/FBS **(Media B)** under standard culture conditions
- hTERT MSCs in DMEM/FBS **(Media B)** at 25°C and 4°C
- hTERT MSCs in **(Media B)** after 4, 8 or 12 days preservation as monolayers in ITSE based media **(Media F1)** or 12 days in ITSE based media **(Media G)** at standard culture conditions
- hTERT MSCs in ITSE based media **(Media F1)**

- primary bovine MSCs in DMEM/FBS **(Media B)** after 1 month cryopreservation
- primary bovine MSCs in DMEM/FBS **(Media B)** after 1 year cryopreservation

3.1.8 – Methods Used to Assess Differentiation

The StemPro Adipogenesis (**Media K2**) (A1007001), Ostengenesis (**Media K1**) (A1007201) and Chrondrogenesis (**Media K3**) (A1007101) kits (Gibco, UK) served as differentiation media, which was changed twice weekly. As cell counts cannot accurately be made once differentiation occurs, differentiation media was added upon reaching 90% confluence after culture in DMEM/FBS **(Media A)**, to ensure little/no further change in cell number over a 2-3 week period. Pictures were taken using a Nikon fluorescent microscope (Nikon, Japan) using the red filter, or far red for the Oil Red O group. The 96 well plates were used during the study of adipogenesis and ostengenesis, and 24 well plates were used during the study of chrondrogenesis (all from Corning, UK). After 12 days of 2D preservation at 37°C, cells were seeded at a density of 7500 cells/cm^2 for the assessment of differentiation. For cells released after 8 days in alginate, cells were seeded at a higher density of 30,000 cells/cm^2, as <60% of viable cells successfully attached for some experimental groups.

For each replicate, the extent of differentiation was first calculated as fluorescence or absorbance divided by number of cells (OD/cell or RFU/cell), which was then reported as fold change vs the mean extent of differentiation at day 7 for non-preserved cells in DMEM/FBS **(Media A)**.

3.1.8.1 – Differentiation of hTERT and Bovine MSCs Monolayers at 37°C in DMEM/FBS (Media A) and hTERT MSCs Monolayers After 12 Days in ITSE Based Media (Media G) at 37°C

For hTERT MSCs, differentiation potential for recovering cells were assessed before and after 12 days of culture in ITSE based medium **(Media G)** at day 7, 14 and 21. For primary bovine MSCs, P1 cells were harvested from the flask and differentiation potential was assessed at day 7 and day 14. Nile red, alizarin red and safranin-O were used to confirm adipogenesis, osteogenesis, and chondrogenesis respectively. At day 21 for hTERT human BMMSCs and at day 14 for primary bovine BMMSCs, chondrocyte layers formed clumps, while calcium covered the walls of the well in the osteogenesis group. There data at week 3 and week 2 respectively was not included.

3.1.8.2 – Differentiation of hTERT and Bovine MSCs After 8 Days in Alginate at Atmospheric Conditions

The differentiation potential of both primary and hTERT MSCs were also assessed after 8 days of alginate preservation in ITSE based medium **(Media I)**. For the 3D alginate experiments, Oil Red O (MAK-194, Sigma-Aldrich, UK) was used for lipid quantification, to measure the total lipid content per well rather than the surface fluorescence. Cells were treated with differentiation media (**Media K1, K2 or K3**) for 2 weeks, and performed quantification of osteogenesis at week 1 and week 2. Non-preserved cells cultured in DMEM **(Media A)** served as the control. The extent of chondrogenesis was not quantified due to a sudden malfunction in the plate reader, nor adipogenesis as the amount of oil red O stained lipids in a 96 well was too low to detect by week 2.

3.1.8.3 - Testing of Chondrogenic Potential Using Safranin-O (SO)

MSCs stained with SO emit fluorescence at Ex530/Em570 nm proportional to the amount of sulphated glycosaminoglycans (SG) that are present in the well following treatment with 75% ethanol. Chondrogenic media was aspirated and cells were washed 1x with 1% acetic acid (Sigma-Aldrich, UK), and then stained with 500 µL of Safranin-O solution (TMS-009-C, Sigma Aldrich, UK) for 5 minutes at room temperature away from light. MSCs were then washed 4x with 500 µL PBS and gentle shaking, where cells were submerged for 5 minutes at each wash. In the final step, cells were treated with 75% ethanol (Sigma-Aldrich, UK) for 5 minutes at room temperature before reading fluorescence at Ex530/Em570 nm (149).

3.1.8.4 - Testing of Osteogenic Potential Using Alizarin Red

Osteogenic medium was aspirated and MSCs were washed 1x with PBS before fixing with 4% formaldehyde (Sigma-Aldrich, UK) for 30 minutes at 4°C in the dark. Following fixation, MSCS were stained with alizarin red (40 mM) (TMS-008-C, Sigma-Aldrich, UK) for 10 minutes at room temperature away from light. Afterwards, the dye was aspirated and MSCs were washed 1x with PBS. In the final step, cells were treated with 200 µL destaining solution (20% methanol, 10% acetic acid and 70% distilled water) (Sigma-Aldrich, UK) for 20 minutes before reading absorbance at 405 nm, which is proportional to the amount of Ca^{2+} in the well (150).

3.1.8.5- Testing of adipogenic potential using Nile Red

The Nile Red Staining Kit (ab228533, Abcam, UK) was used according the manufacturer's instructions. To account for background fluorescence of green fluorescent protein from hTERT MSCs at Ex550/Em640 nm, the fluorescence of unstained cells was measured and subtracted from the fluorescence after staining. Each well was filled with 200 µL of Nile Red Staining Solution (Abcam, UK) and incubated for 37°C for 30 minutes in the dark at 5% CO_2. After incubation, the media was aspirated and the fluorescence was measured again at Ex550/Em640 nm to quantify the amount of lipid droplets in the well.

3.1.8.6 - Testing of Adipogenic Potential Using Oil Red O

The Lipid (Oil Red O) staining kit (MAK194-1KT, Sigma-Aldrich, UK) was used according the manufacturer's instructions. All chemicals in this subsection were from Sigma-Aldrich, UK. Media was aspirated and cells were washed 2x with PBS. 10% (v/v) formalin was then added to each well, and incubated at room temperature for 1 hour away from light. At the 50 minute mark the following reagents were prepared: 60% isopropanol, and Oil Red O dissolved in 100% isopropanol, the latter serving as the stock solution. The Oil Red O stock was diluted to a concentration of 60% (v/v) in distilled water. All reagents were vortexed for a few seconds and used within a 2 hour period.

Formalin was then removed and cells were washed twice with distilled water, followed by staining with 60% isopropanol for 5 minutes at room temperature in the dark. The 60% isopropanol was aspirated and cells were submerged in 60% oil red o for a further 20 minutes at room temperature in the dark. After the oil red o was removed, wells were washed 5x with distilled water, then stained with haematoxylin for 1 minute. After haematoxylin staining, cells were further washed 5x with distilled water and submerged with 100 µL distilled water. Lipid droplets were shown as red patches and visualised using the far red filter of the fluorescent microscope.

3.2 – Examination of Metabolism of MSC Monolayers in ITSE Based Media at 37°C and 5% CO_2

3.2.1 – Measurement of Intracellular Reactive Oxygen Species (ROS) Production with DCFDA

As mentioned in **chapter 2**, it is important to ensure that ROS levels do not reach harmful levels during cell preservation. In order to determine if ITSE based medium preservation increased oxidative stress, MSCs were seeded in a 96 well plate (Corning, UK) at a density of 15,000 cells/cm^2 (5,000 cells/well) in 200 µL of DMEM/FBS **(Media B)** and cultured for 24 hours to bypass the lag phase of growth. MSCs in DMEM/FBS **(Media B)** acted as the control. MSCs were also seeded in ITSE based medium **(Media F1 and F2)** to determine if preservation in ITSE increased intracellular reactive oxygen species (ROS) production. After 24 hours, cells were stained with 25 µM 2',7'-dichlorodihydrofluorescein diacetate (DCFDA) (ab113851, Abcam, UK) in dimethyl sulfoxide (DMSO) and a proprietary buffer for 45 minutes at 37°C in the dark. Cells were then washed with a proprietary buffer before replacement with the original culture media prior to staining. Fluorescence at Ex485/Em535 nm was measured at 2h, 4h and 6h and a final cell count was performed using the Spectramax i3X plate reader (Molecular Devices, USA).

Intracellular oxidative stress was calculated as a fold increase in relative formula units (RFU) per cell vs 0h. Experiments were repeated in the presence of ITSE based medium **(Media F1)** with 10 mM N-Acetyl cysteine (NAC) (A7250-10G, Sigma Aldrich, UK) to confirm that fluorescence increase was due to ROS production. Total ROS production was calculated as the area under curve (AUC), which was the sum of ROS production from 2-6h multiplied by 4.

3.2.2 – Measurement of Autophagy Using a Proprietary Autophagosome Marker

To compare the extent of damaged cellular components between cells in DMEM/FBS and in ITSE based media, autophagy was studied using an autophagy assay kit (MAK138, Sigma-Aldrich, UK), which consists of a proprietary autophagosome marker that gives fluorescence at Ex360/Em520 nm proportional to the number of autophagosomes in the cell. P15 hTERT MSCs were seeded in a 96 well plate (Corning, UK) at a density of 12,000 cells/cm^2 (4,000 cells/well) in 200 µL of DMEM/FBS **(Media B)** or ITSE based media **(Media F1 or F2)** to see if autophagy is increased in the presence of ITSE. Cells were cultured for 24 hours to bypass the lag phase of growth and the number of cells was counted. MSCs were then stained with the proprietary autophagosome marker for 1 hour at 37°C, before washing with a proprietary buffer three times followed by replacement with the original culture media. Fluorescence at Ex360/Em520 nm was measured immediately after and a final cell count was performed using the Spectramax i3X plate reader (Molecular Devices, USA).

3.2.3 – Effect of ITSE Supplement on hTERT MSC Survival over a 12 Day Period

P15 hTERT MSCs were seeded into 24 well plates (Corning UK, lot number 3337) at a density of 7500 cells/cm^2 in 1 mL of blank DMEM **(Media E2)** or ITSE based media **(Media E1)** at 37°C and 5% CO_2 for 12 days without media change. The number of attached cells per well were counted at day 1 , day 2 and at 2 day intervals thereafter using the SpectraMAX i3x plate reader (Molecular Devices, USA). At day 12, the ITSE based media **(Media E1)** group was collected and frozen at -20°C prior to analysis of amino acid consumption. Data was reported as fold change in attached cells vs. day 1.

3.2.4 –hTERT BMMSC Proliferation and Metabolism after Culture in ITSE Medium at Incubator Conditions

hTERT MSCs were seeded at a density of 4000 cells/cm^2 in a T75 flask in 10 mL of ITSE based medium **(Media F1)** for either 4 days, 8 days or 12 days or **(Media G)** for 12 days at 37^{o}C and 5% CO_2 before transfer to a 24 well plate to determine if proliferation and differentiation is maintained after preservation. After ITSE culture, MSCs were transferred to DMEM/FBS **(Media B)** where cell counting and media changes were performed at 24 hour intervals for a total of 48 hours. Media samples between the 24h and 48h time frame in both DMEM **(Media B)** and ITSE **(Media F1)** were frozen at -20°C until analysis.

3.3 – Amino acid Consumption of hTERT MSC in DMEM and in ITSE Based Media

Cell culture was carried out at 37°C and 5% CO_2 for all gas chromatography and mass spectroscopy (GC-MS) experiments. For all experiments, P15 human hTERT MSCs were seeded into 24 well plates at a density of 7500 cells/cm^2. For cells cultured in DMEM/FBS **(Media B)**, a total of 500 µL of media was used and cells were incubated for 3 days (when 80% confluence was reached). For cells cultured in ITSE based media **(Media E1)**, a total of 1 mL media was used, and cells were incubated for 12 days (target preservation time). For the DMEM/FBS group, the sample purification, derivatisation and data analysis was carried out by myself under the supervision of Dr Zhanru Yu (Nuffield Department of Medicine, University of Oxford, UK) while the operation of the GC-MS instrument was conducted by Dr Zhanru Yu alone. For the ITSE based media **(Media E1)** group, all experimental procedures were conducted by Dr Zhanru Yu alone due to COVID-19 restrictions at the time.

3.3.1 – Sample Purification

Sample preparation was undertaken for both the fresh media and spent media so that metabolite changes could be determined. A total of 200 µL of media sample was mixed with 200 µL of 100% methanol and placed in a bead beater (Precellys, UK) for a total of 4 cycles, where each cycle was set to 6500 Hz for 45 seconds. The sample was then placed in dry ice for 5 minutes, and mixed with 800 µL methyl-tert butyl ether prior to further treatment with the bead beater, using the same protocol as before. Afterwards, samples were centrifuged at 13,000g for 20 minutes at 4^{o}C to separate the organic phase (top layer) and aqueous phase (bottom layer). 700 µL of the organic phase was transferred to glass vials. A total of 800 µL ethanol was then added to the remaining aqueous phase. Both the organic and aqueous phases were dried using a speed vac. Following drying, the aqueous phase was frozen at -80^{o}C for 1 hour to remove proteins and ionic compounds by precipitation. The remaining supernatant from the aqueous sample was mixed with the dried organic sample prior to a further round of drying. In the final step, samples were frozen at -80^{o}C until the derivatisation.

3.3.2 – Sample Derivatisation

A total of 20 mg O-methoxyamine hydrochloride was dissolved in 1 mL pyridine, and 50 µL was added to both the fresh and spent media samples. Samples were then shaken once at 1200 rpm for 90 minutes at 30^{o}C (Bioshake IQ, Germany) before treatment with 30 µL pyridine and 70 µL N-methyl-N-trimethylsilyl tri fluoro acetamide with 1% trimethylchlorosilane (MSTFE). The mixture was shaken once more at 1200 rpm, for 60 minutes at 60^{o}C before centrifugation at 5000 g for 20 minutes at 4^{o}C to ensure that insoluble matter was sedimented. The whole sample was measured immediately by Dr Zhanru Yu (Target Discovery Institute, Oxford University, UK), where samples were injected both at a ratio of 1:10 for amino acids (low starting concentration) and 1:100 for pyruvate analysis (which has a high starting concentration).

3.3.3 – Analysis of GC-MS Data

Data files were first opened in GC-MS postrun analysis, which was used to identify saturated metabolites in samples injected at a 1:10 ratio, and were subsequently analysed in the 1:100 ratio samples instead. Data analysis was performed using Chromebit 2.2 which plotted retention time in non-polar column vs. retention time in the polar column vs. relative abundance in the mass spectrometer as blobs. Having 2 columns allowed further separation of compounds in column 2 after column 1. Metabolites were identified using the following built in databases: NIST11, NIST 11s,

AA_EZfaast, OA_TMS and YUTPI. Data was checked to ensure that 2 blobs were not seen as 1 object during blob identification. Chromebit 2.2 calculated the relative concentration of all amino acids and pyruvate in both the spent and fresh media, and metabolites were displayed as fold change in spent media vs. fresh media. If the relative concentration was displayed as -1, which indicates a calculation error, the fold change was calculated manually as:

Volume of blob from spent media / Volume of blob from fresh media.

For media components in spent media, fold changes at 0.66x or lower, and 1.5x or higher were regarded as substantial; this was an established threshold used by Dr Zhanru Yu at the Nuffield Department of Medicine in published work, to ensure that any metabolite differences between spent media and control media are not due to error margins (151) (152).

3.4 – ITSE Preservation of MSCs Under Atmospheric Conditions for 12 Days

For all experiments at atmospheric conditions, the temperature of the laboratory was 20°C and atmospheric CO_2 levels were 0.04%. Cells were stored in cardboard boxes with small punctured holes to allow air exchange, but to prevent light degradation of culture media. Additional spaces were filled with 24 well plates containing water to prevent media evaporation. Fresh ITSE based medium **(Media J for monolayer experiments or to assess cell metabolism in alginate, Media I for 3D preservation for survival experiments in suspension or in alginate)** alone (2D experiments) or with empty alginate beads (3D experiments) was used as the negative control to account for any background metabolite degradation at room temperature.

3.4.1 – Determine the Optimum Buffer for Atmospheric Preservation

P15 human hTERT BMMSCs were cultured in ITSE based medium **(Media G)** at 20°C and 0.04% CO_2 using the following buffer formulations listed below to replace the 44 mM $NaHCO_3$:.

- ITSE-bicarb: 0.9 mM $NaHCO_3$
- ITSE-glycerophosphate: 0.9 mM $NaHCO_3$ + 20 mM Sodium β-glycerophosphate

- ITSE-HEPES: 5 mM $NaHCO_3$ + 12 mM HEPES + 12 mM MOPS
- ITSE-Phosphate: 4.88 mM $NaHCO_3$ **+** 4.19 mM NaH_2PO_4 + 0.5 mM KH_2PO_4

Each media combination was adjusted to pH 7.4 using 20-50 µL droplets of HCL and NaOH (Sigma-Aldrich, UK) (1 mM) and sterile filtered at 0.22 µm (Sigma-Aldrich, UK) before culturing MSCs at 20°C and 0.04% CO_2. Cells were seeded in T175 flasks (Corning, UK) with 15 mL of medium, at a density of 2850 cells/cm^2, equivalent to 500k cells/flask. Photographs were taken on day 2, and the buffer was regarded as suitable if MSCs attached and elongated. Cells in ITSE based medium with 44 mM $NaHCO_3$ **(Media G)**, which cultured MSCs at 37°C and 5% CO_2, served as the positive control. This ensured that there were no faults with the cell line used or the DMEM powder.

3.4.2 – hTERT and primary MSC Monolayers Under Atmospheric Conditions

3.4.2.1 – Assessment of Survival and Metabolism During Preservation Over a 12 Day Period

Both P15 hTERT human and P3 primary bovine BMMSCs were seeded in 24 well plates (Corning, UK) at a density of 25,000 cells/cm^2 for P3 primary bovine MSCs and 17,500 cells/cm^2 for hTERT human MSCs in 500 µL ITSE based media **(Media J)**. The media glucose concentration was lowered from 2.5 mM to 1 mM and seeding density was increased from 15,000 cells/cm^2 because glucose concentration changes were too small to measure from the prior cell density and glucose starting value. The number of attached cells were counted using the SpectraMAX i3x plate reader (Molecular Devices, USA), at day 2, 4, 6, 8 and 12, where cells each time point were seeded in a separate group of wells. For each time point, the number of attached cells at day 1 was also counted.

Percentage survival was calculated as ((attached cells on current day/attached cells on day 1) x 100). Spent media was harvested at 4 day intervals and centrifuged to separate dead cells. The supernatant was transferred to 1.5 mL Eppendorf tubes and frozen at -20°C until analysis. Glucose consumption and lactate production was only displayed at day 12, as larger concentration changes are more accurately measured. Surrounding wells were filled with 1 mL PBS to prevent media evaporation.

3.4.2.2 – Assessment of Morphology and Recovery After 12 days Monolayer Preservation at Atmospheric Conditions

HTERT MSCs were seeded in ITSE based medium **(Media H)** in T75 flasks (Corning, UK), at a density of 4000 cells/cm^2 in 10 mL media for 12 days, detached with Trypsin-ETDA (0.25%) (Gibco,

UK), and then transferred to DMEM/FBS **(Media B)** in 24 well plates at a density of 7500 cells/cm^2 to assess recovery. However primary MSCs could not be detached using trypsin after 12 days preservation. Photographs were taken at 100x magnification using a fluorescent microscope (Nikon, Japan) from the flask during preservation. A 2 mL sample of media was taken from the flask at 4 day intervals for pH measurement with a pH213 reader (Hannah Instruments, UK). The pH meter was calibrated with a pH7 standard prior to measurement.

3.4.3 – Preservation of hTERT and Primary MSCs at Atmospheric Conditions in 3D Culture in Suspension and in Alginate Beads

In all experiments involving preservation and recovery of proliferation, cells were preserved in 24 well cell culture plates (3337, Corning, UK), where cells were placed in the central 8 wells, and surrounding wells and spaces between wells were filled with phosphate buffered saline (PBS) to prevent evaporation. For all 3D experiments, cells were passaged twice after defrosting to ensure full recovery from DMSO. Survival rates were measured at regular intervals for up to 12 days using Trypan Blue Solution at 0.4% (Thermo Fisher Scientific, USA).

3.4.3.1 – Reagents Used to Make and Dissolve Alginate Beads

A total of 12 g of sodium alginate (71238, Sigma-Aldrich, UK,) was dissolved in 500 mL of PBS (10010023, Gibco, UK) creating a stock solution of 2.4% (w/v). The solution was stirred on a hot plate at 60°C for 6 hours to facilitate dissolving, and then autoclaved at 121°C. For alginate crosslinking, 102 mM of $CaCl_2$ (Sigma-Aldrich, UK) was dissolved in distilled water. For the dissolution of beads, 55 mM of sodium citrate (Sigma-Aldrich, UK) was dissolved in PBS (10010023, Gibco, UK). All solutions were adjusted to pH 7.4 using droplets of concentrated NaOH and HCl and then sterile filtered through a 0.22 µM filter bottle (Corning, UK), and stored at room temperature (20°C) in the dark when not in use.

3.4.3.2 – Coating well Plates with Agarose

Agarose coating was carried out under sterile culture conditions. 7.5 g agarose (Sigma-Aldrich, UK) was dissolved in 500 mL of distilled water, making a final concentration of 1.5% (w/v). The solution was sterilised by autoclaving at 121°C, and left for 1 hour 30 minutes inside the autoclave to cool the solution down to 60°C. The middle 8 wells of a 24 well plate were coated with 300 µL agarose and left to set for 1 hour. Each well was washed 1x with 70% ethanol and then 3x with sterile PBS, the latter of which was evaporated by air drying in a cell culture hood. Well plates

were stored inside a plastic jar and kept in the fridge until use, or for a maximum time of 1 month. Cells were preserved at a density of 300k cells/mL of ITSE based media **(Media I)**.

3.4.3.3 - Creating the Cell Suspension in Alginate

P15 hTERT human MSCs or P3-4 primary bovine MSCs were suspended at a density of 750,000 cells/0.5 mL in a mixture of 50% v/v ITSE based media **(Media I)** and 50% v/v alginate stock solution (2.4% w/v), making a final concentration of 1.2% w/v alginate (49k cells per bead). For the 2-2.7 mm beads, a lower density of 600,000 cells/0.5 mL (12k cells per bead) was used. The cell suspension was pipetted up and down, and vortexed for a few seconds to ensure even mixing. 2 mL of cell suspension was pipetted into a spare well in a 24 well plate (3337, Corning UK) and 1 mL was aspirated into a 1 mL syringe. Air bubbles were removed and a 30G needle was fitted onto the end (AN-3013R, Terumo AGANI, UK).

3.4.3.4 - Creating 4 mm Beads with a Syringe Pump

The syringe with cell suspension was fitted onto a syringe pump (Harvard Apparatus, USA) according to the manufacturer's instructions. To create the 4 mm beads, the following settings were used: flow rate of 1 ml/min, internal diameter of 4.78 mm and target volume of 400 µL of alginate in 1.5 mL of ITSE based media **(Media I)** equivalent to 400k cells per mL of ITSE media. A 24 well plate was filled with 1.5 mL of $CaCl_2$ per well, and held up high using a pipette box. The tip of the needle was aligned above the well, and alginate droplets were collected into the wells with calcium chloride. For every two wells, the calcium chloride was aspirated, washed 2x with PBS (Gibco, UK) and replaced with 1.5 mL of ITSE based medium mentioned previously.

3.4.3.5 - Creating 2-2.7 mm Beads by Hand

Smaller beads could not be created using the syringe pump, as the bead would not be heavy enough to drop into the $CaCl_2$ well until it reached a diameter of 4 mm. The 1 mL syringe was taken by hand – the thumb of the right hand was placed above the plunger, while the second and third finger of the right hand was placed beneath the wings as support. Fingers 1, 2 and 3 of the left hand held onto the stem of the plunger for additional support. The plunger was pushed slowly and gently, and the needle was dunked into the $CaCl_2$ and immediately back out of the well as soon as the smallest droplet was seen coming out of the needle. A different site of the well was chosen for each downward movement to prevent beads forming a chain. For every single well completed, $CaCl_2$ was aspirated, washed 2x with PBS and then filled with 1 mL ITSE based medium **(Media I)** to prevent prolonged exposure to $CaCl_2$. A total of 300 µL alginate was used per well, equivalent to 360,000

cells per mL of ITSE media. Primary MSCs were also preserved in ITSE based medium **(Media I)** supplemented with 10% FBS for 12 days, to determine if lack of serum components was the limiting factor preventing long term survival at atmospheric conditions.

3.4.3.6 - Release of Cells from Alginate Beads to Assess Survival and Recovery

Prior to release, DMEM/FBS **(Media A)** was added to well plates in advance, and pre warmed to 37 °C. At 2 days intervals for the 4 mm beads and 4 day intervals for the 2-2.7 mm beads for a total of 12 days, cells were extracted to test survival rates. ITSE based medium was aspirated and beads were washed 2x with PBS and treated with 1 mL of 55 mM sodium citrate for 5-10 minutes at room temperature, with constant shaking of the well plate by hand. Afterwards the cell suspension was transferred to a 15 mL falcon tube (Corning, UK), washed 1x with 10 mL of PBS and suspended in 0.5 mL of PBS to count cell survival rates with trypan blue and the Countess automatic cell counter (Thermofisher, UK). For the 2-2.7 mm beads, PBS with 10% FBS was used, which may reduce the possibility of cell death during washing.

During counting, the minimum cell size was increased to 10 µm, and sensitivity was reduced from 5 to 3, to prevent cell debris and background from being mistaken for cells. If live cells were missed out, minimum cell size was reduced by intervals of 1 µm until all cells were counted. After counting, all pellets were combined and washed 1x more with PBS and FBS, before suspension in 1 mL DMEM/FBS **(Media A)**. To assess recovery of proliferation, cells were seeded in 24 well plates in 1 mL of DMEM/FBS **(Media A)**. For the primary MSCs from 4 mm beads cells were seeded at a density of 15,000 cells/cm^2 as cell survival rates were low, and it was not expected that many cells would attach. For all other groups, cells were seeded at densities of 7500 cells/cm^2. Media changes were performed at the 24 hour timeframe and every 48 hour intervals thereafter. Cells were counted at 24 hour intervals using the SpectraMAX i3X plate reader until the cell number per well reached around 40-50k. Proliferation was reported as fold change vs number of attached cells at 24h. At the 96h timeframe, photos were taken at 100x magnification using a fluorescent microscope (Nikon, Japan). Primary MSCs were taken using the phase contrast setting (∞Ph1), while hTERT MSCs were taken using the green filter. Additional cells were seeded into well plates and cultured to 90% confluence using DMEM/FBS **(Media A)** to assess differentiation potential, as described in **Section 3.1.7**.

3.4.3.7 – Staining of Late Apoptotic Primary cells in 4 mm Beads with Propidium Iodide

After day 12, media was aspirated and a handful of 4 mm beads containing bovine MSC (49k cells/bead) were scooped with a spatula into an empty well of a 24 well plate. Beads were washed 2x with PBS, before treatment with 70% ethanol and 30% distilled water (1 mL) for 30 minutes at 4°C in the dark. Afterwards, the ethanol was aspirated and washed 2x more with PBS before submerging in propidium iodide (PI) (1 mg/mL) stock solution diluted in PBS (850 µL of PBS and 50 µL of PI) (P4864-10ML, Sigma Aldrich, UK). The beads were incubated for a further 15 mins at 4°C in the dark. Without aspirating PI, beads were visualised under the red filter of a fluorescent microscope at 10x magnification.

3.4.3.8 – Assessment of MSC Metabolism in Alginate Beads

To ensure that pH acidity did not become a limiting factor during metabolic study, both hTERT and primary MSCs were encapsulated in 4 mm beads at low densities of 60k or 15k cells per mL of ITSE based media **(Media J)**, with a total of 300 µL of alginate in each well. For the 60k group, there were 100,000 cells/0.5 mL of alginate, while there were 25,000 cells/0.5 mL of alginate for the 15k group. A total of 4 wells were allocated for each replicate.

To overcome higher error margins when counting low densities of suspended cells, 4 wells for each replicate were combined and the mean was calculated. After 6 days under atmospheric conditions, media from 1 well was frozen at -20°C for glucose and lactate measurements, while spent media from the remaining 3 wells were combined and measured for pH. For each replicate, the released cells from 4 wells were combined together and suspended in 300 µL of PBS/FBS after washing (filling to 10 mL PBS/FBS, centrifugation followed by supernatant removal). Cells in each well were counted twice and the average was calculated. For each replicate, the mean cell count and survival rates from 4 wells was combined and averaged to yield a single value for the number of viable cells. Glucose and lactate levels in spent and fresh media were measured using the ARCHITECT bio analyser by staff at the John Radcliffe Hospital. Glucose consumption and lactate production (pmoles/cell/day) was calculated as the mean change at day 6, divided by 6x the mean number of viable cells. Fresh media with empty beads served as the negative control.

3.4.3.9 – Characterisation of Bead Size, Pore Size and Porosity

Photographs of both 4 mm and 2-2.7 mm beads from day 4-12 were expanded on Microsoft Word until the 2000 µm scale bar reached a length of 35 mm. For each bead a red line was created along its longest length, and then measured with a ruler. Each bead was numbered and the measured length was recorded in Microsoft Excel. Actual length was calculated as (measured length ÷ 35) x2, and displayed in mm. Box and whisker plots were created for both 4 mm and 2-2.7 mm beads, where the length of each individual bead was displayed to show the size distribution. Since bead size is not determined by the source of MSCs, for both 4 mm and 2-2.7 mm beads the sizes from both hTERT human MSCs and primary bovine MSCs were combined.

Although pore size and porosity was not studied in this project, the published literature shows that alginate pore size and porosity vary enormously depending on the technique, alginate concentration and elemental composition. Pore sizes of ≈ 5.5 nm have been reported using thermoporometry when high molecular weight sodium alginate (M_w=5.3×10^4 g/mol) was diluted to a concentration of 1% (w/v) in distilled water, and gel disks were formed by 30-40 minute incubation in 3 M $CaSO_4$ solution (153). In another study, when 0.5-2% (w/v) alginate was dissolved in DMEM, and cross-linked to form 1.5 mm beads in 100 mM $CaCl_2$, the pore sizes were reported as 10.9 µm at 0.5% alginate, 5 µm for 1% and 3.4 µm at 2% (154). When incubated with FITC (327 Da), the beads were fully penetrated within 1 hour irrespective of alginate concentration. For the beads with 0.5% alginate, FITC-IgG (150 kDa) and FITC-dextran (500 kDa) required 24 hours to fully penetrate the bead. FITC-IgG failed to penetrate the 2% alginate beads within 24 hours, while FITC-dextran failed to penetrate both the 1% and 2% beads (154).

When 2-2.26 mm alginate beads are created using a syringe pump, bead surface area, pore size and porosity depended on the elemental composition, which varies between different brands (155):

- CR8133 beads contained 46% oxygen, 27% carbon, 16% chlorine, 11% calcium, 0% sodium surface area = 21.8 m^2/g, total pore volume = 0.055 m^3/g, and pore diameter = 3.94 nm.

- CR8223 beads contained the same formulation as CR8133 above + 1% sodium surface area = 36.1 m^2/g, total pore volume = 0.068 m^3/g, and pore diameter = 3.94 nm.

- LFR5/60 beads contained 36% oxygen, 16% carbon, 29% chlorine, 18% calcium, 0.6% sodium surface area = 13.7 m^2/g, total pore volume = 0.034 m^3/g, and pore diameter = 2.52 nm.

Porosity of 4.3 mm diameter alginate beads (1.5 % (w/v) in distilled water, cross-linked in 20 mM $CaCl_2$ have been assessed in the literature by leakage of trapped rhodamine-BSA (rh-BSA) protein into the surrounding 25 mM HEPES buffer (pH 7.4) (156). For untreated beads, the rate of rh-BSA leakage was reported as 34% at 6 h and 63% at 24 h, while leakage rates for a FITC-labelled insulin hexamer (M_w 36 kDa) was 61% at 24 h and 91% at 72 h (156). When the pores were filled by treatment with 2% low molecular weight polyvinyl alcohol (60 minutes) followed by additional crosslinking with 1,3-benzenediboronic acid, the leeching of insulin was reduced to 6.5% at 24h and 23% at 72h (156).

3.5 – Statistical Analysis

All data was reported as mean ± standard deviation. When comparing two data sets with multiple points, area under curve was calculated. When comparing two experimental conditions, significance was calculated using Welch's T-test using Microsoft Excel, where differences were considered significant if $p \leq 0.05$. To determine if cells proliferated or died from day 6-12 in ITSE **(Media E1)** and basal DMEM **(Media E2)** at 37°C, the Pearson correlation coefficient was used, which was calculated using Graphpad Prism. For comparison of 3 experimental groups, such as autophagosome production of hTERT MSCs in DMEM, ITSE and ITSE with no pyruvate, the one way analysis of variance (ANOVA) was determined using Graphpad Prism. For both the correlation coefficient and ANOVA, differences were also considered significant if $p \leq 0.05$.

Chapter 4

Characterisation and Metabolism of Primary Bovine and Immortal Human Mesenchymal Stem Cells During Expansion

4.1 – Introduction

Multiple metabolic studies have been carried out on mesenchymal stem cell (MSC) monolayers under incubator conditions (37°C and 5% CO_2), primarily involving glucose consumption, lactate production and amino acid consumption (60) (113). The majority of these studies have been end point measurements, which did not show how metabolism changed between seeding and 80% confluence. Currently there are no studies investigating MSC metabolism in preservation media at temperatures far lower than 37°C. During preservation at room temperature (20°C) and cold storage (4°C), metabolic activity is expected to be considerably lower than at 37°C. Therefore, trying to measure small glucose changes from 5.5 mM may not be accurate.

Firstly MSCs were characterised by conducting flow cytometry and trilineage differentiation assays. At incubator conditions (37°C, 5% CO_2) and as 2D monolayers, the proliferation, glucose consumption and lactate production of both immortal (hTERT) human and primary bovine MSCs have been studied, and gas chromatography and mass spectroscopy (GC-MS) was performed to determine which B vitamins and amino acids were consumed by cells in the DMEM culture medium. Primary MSCs were sourced from healthy 3 week old calves. Unlike the purchase of frozen human cells, or patient samples, no ethical approval was required and MSCs from younger animals tend to have higher proliferation rate, attachment rate and therapeutic potential (34). In later chapters, studies of metabolism are reported under the following conditions for comparison:

- 2D monolayers at incubator conditions (37°C and 25°C at 5% CO_2) in Dulbecco's Modified Essential Medium with foetal bovine serum (DMEM/FBS) (This chapter)
- 2D monolayers at incubator conditions (37°C) in the novel insulin based preservation medium (ITSE) (Chapter 5)
- 2D monolayers at atmospheric conditions (20°C at 0.04% CO_2) in ITSE (Chapter 6)
- 3D structures at atmospheric conditions in ITSE (Chapter 6)

At 37°C for hTERT MSCs, the project aimed to measure proliferation, glucose consumption and lactate production at 12 hour intervals for a total of 72 hours. It was expected that metabolism will be low during the lag phase, increase during the log phase and fall again once the cells reach 80% confluence. This was be carried out at 1 mM, 2.5 mM and 5.5 mM media glucose. The Michaelis Constant (K_m) was estimated by creating a Lineweaver-Burk plot, which indicated the glucose transporter isoform that are present on MSCs.

The data produced has determined whether single end points measurements of glucose consumption and lactate production rates in the published literature are an accurate reflection of metabolism during expansion. Comparing growth and metabolism between proliferating cells cultured in DMEM/FBS at incubator conditions with those in ITSE at atmospheric conditions has revealed the effect of temperature, 3D culture and preservation.

4.2 – Experimental Plan

The experimental techniques used are mentioned in **Chapter 3: Materials and Methods.** In summary, primary bovine MSCs were extracted from the marrow of young calves (**Section 3.1.1**), while the hTERT human BMMSCs were initially created by Mihara (148), where cells were genetically modified to express telomerase to confer immortality, and green fluorescent protein (GFP) to allow easier visualisation. Cells were characterised using flow cytometry (**Section 3.1.3**), where the presence of MSC markers (CD73, CD90 and CD105) and absence of haematopoietic, leukocyte and endothelial markers (CD34 and CD45) was verified. Proliferation (**Section 3.1.4**), glucose consumption and lactate production (**Section 3.1.5, 3.1.7**) of P15 hTERT human BMMSCs and P3 primary bovine MSCs between the 24-48 hour timeframe were studied. For the primary cells, the metabolism between cells frozen for 1 month, and cells frozen for 1 year were compared to determine the effect of prolonged cryopreservation. For the hTERT cells, metabolic measurements at 12 hour intervals were carried out, at 1 mM, 2.5 mM and 5.5 mM media glucose **(Section 3.1.5)**. For hTERT cells, experiments for growth and metabolism were repeated between 24-48 hours at 25°C and 4°C (**Section 3.1.5, 3.1.7**). The experimental plan is illustrated using a flow chart in **Figure 4.1.**

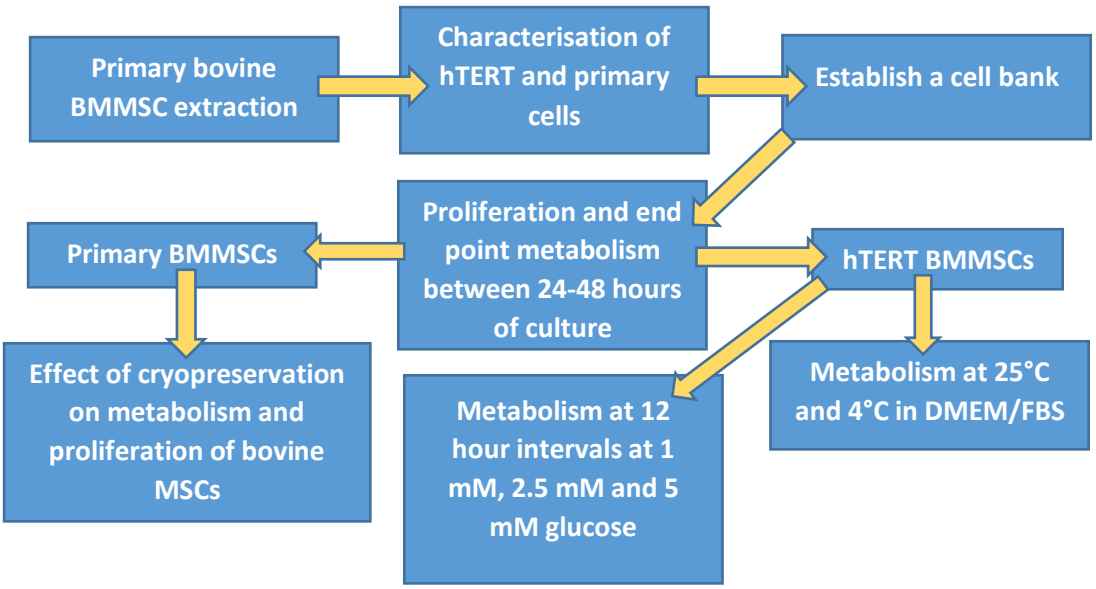

Figure 4.1: Experimental outline for MSC characterisation and the study of MSC metabolism under standard culture conditions, room temperature and cold storage.

All media, unless stated otherwise, contained 44 mM sodium bicarbonate, 1 mM pyruvate and 10% FBS. The media formulations used this chapter have been listed in **Table 4.1.**

Table 4.1: Media formulations used to study MSC proliferation and metabolism at standard culture conditions.

Media Formulation	Experiment
DMEM, 5.5 mM glucose **(Media A)**	MSC expansion, effect of glucose concentration on proliferation and metabolism
DMEM, 2.5 mM glucose **(Media B)**	MSC metabolism at 37°C, 25°C and 4°C, effect of glucose concentration on proliferation and metabolism
DMEM, 1 mM glucose **(Media C)**	Effect of glucose concentration on proliferation and metabolism

4.3 – Results and Analysis

4.3.1 – Validation of the ARCHITECT Bioanalyser

The bio analyser read 1 mM glucose as 0.96 mM, 2.5 mM glucose as 2.47 mM and 5 mM glucose as 4.98 mM (n = 1) in DMEM, as shown in **Figure 4.2**.

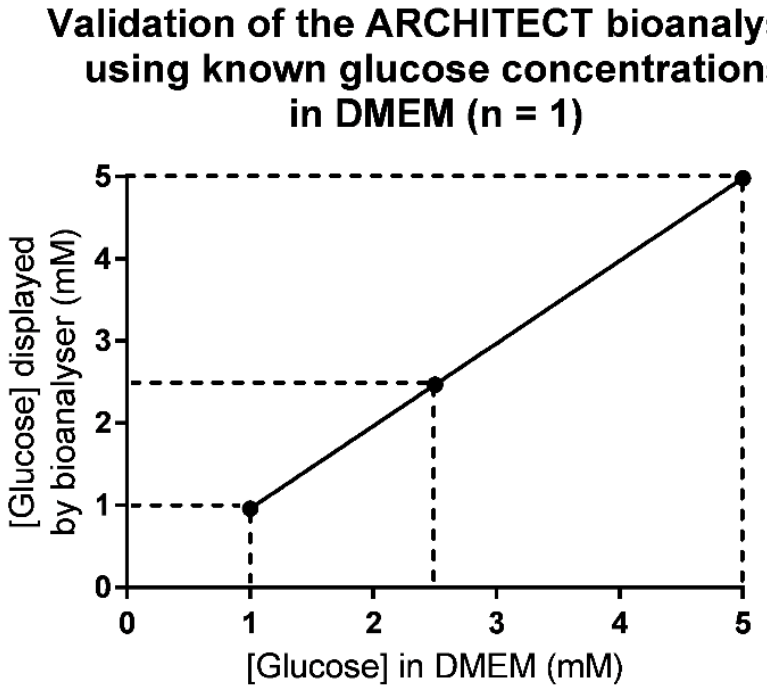

Figure 4.2: Calibration of the Architect Bioanalyser. The bioanalyser accurately measures glucose concentrations of 1 mM, 2.5 mM and 5 mM in DMEM. n = 1 per group.

4.3.2 – Characterisation of Primary Bovine MSCs

Bovine MSCs stained positive for Nile red (lipids), safranin-O (glycosaminoglycans) and alizarin red (calcium) after treatment with differentiation media (**Media K1, K2 or K3 – Table 3.1**), confirming that plating marrow directly in flasks generated MSCs, as shown in **Figure 4.3a-c,** which also demonstrated that freshly extracted BMMSCs exhibited a spindle morphology in **Figure 4.3d** when cultured in DMEM/FBS at 37°C. Despite demonstrating trilineage differentiation potential, primary bovine BMMSCs did not bind to human antibodies for CD73, CD90, CD105, CD34 or CD45, as shown in **Figure 4.4**.

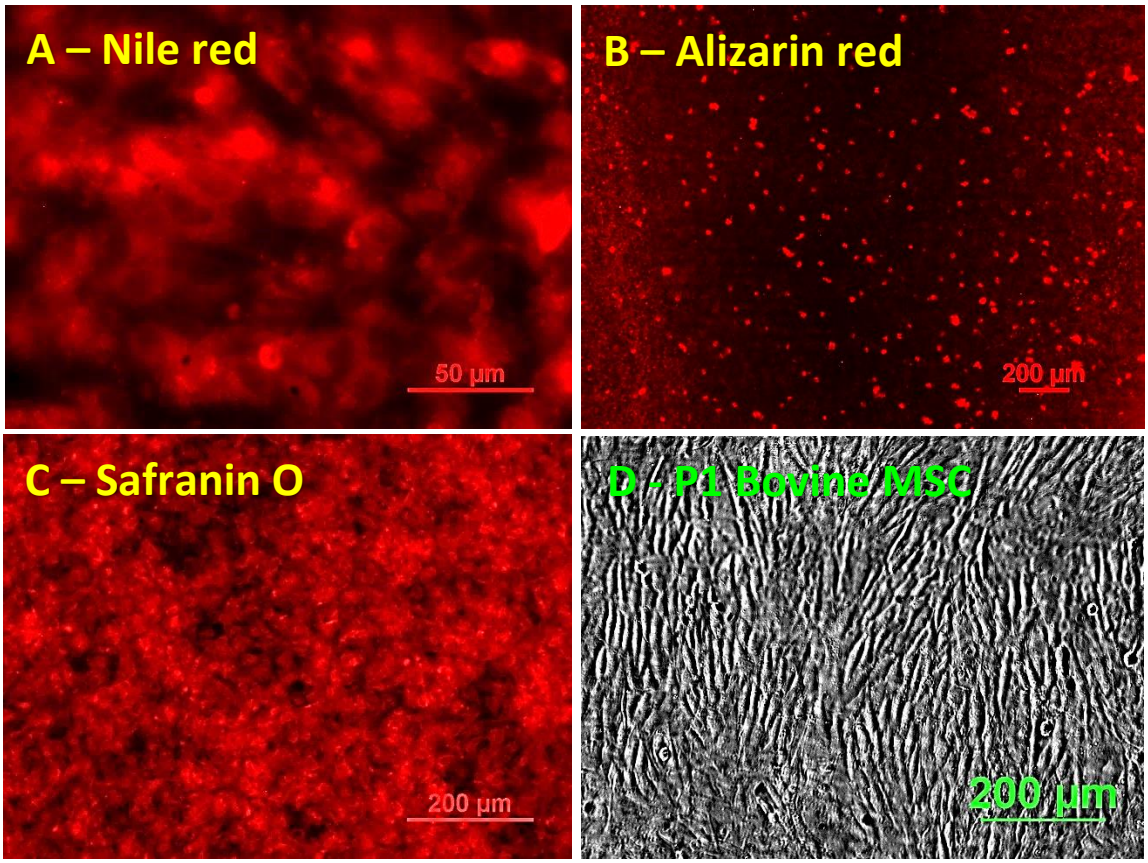

Figure 4.3 – Trilineage differentiation and morphology of primary bovine MSCs. After 7 days of treatment of differentiation media (**Media K1, K2 or K3 – Table 3.1**), primary bovine BMMSCs stained positive for lipids (A), calcium (B) and glycosaminoglycans (C), indicating that they were able differentiate into adipocytes, osteocytes and chondrocytes respectively. (D) Freshly extracted BMMSCs at P1 displayed a spindle morphology, where all cells faced a similar direction.

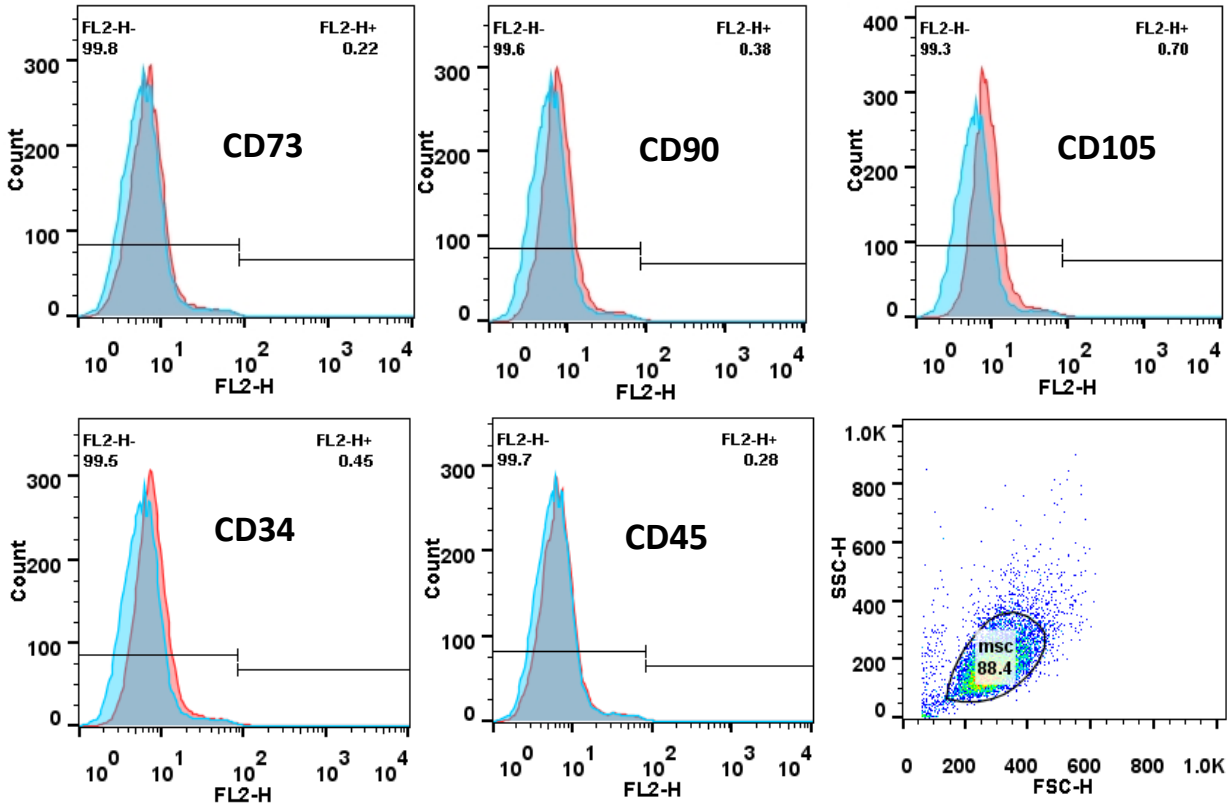

Figure 4.4: Stem cell surface marker expression of P1 primary bovine BMMSCs. Primary bovine BMMSCs did not bind human antibodies (positive markers CD73, CD90 and CD105), as shown by the lack of difference in fluorescence between unlabelled cells (blue peak) and cells treated with antibodies (red peak).

4.3.3 – Characterisation of Human hTERT MSCs

Human hTERT BMMSCS stained positive for Nile red (lipids), safranin-O (glycosaminoglycans) and alizarin red (Calcium) after treatment with differentiation media (**Media K1, K2 or K3**) as shown in **Figure 4.5a-c**, and hTERT MSCs adopt a spindle morphology in **Figure 4.5d** when cultured in DMEM/FBS at 37°C. When stained with human MSC antibodies (CD73, CD90 and CD105) along with antibodies for endothelial cells, haematopoietic stem cells and leukocytes (CD34 and CD45), >90% of the cells bound to to CD73, CD90 and CD105, while almost 0% bound to CD34 and CD45 (rounded to the nearest 2 significant figures). This indicated a high purity of MSCs. The flow cytometry data is shown in **Figure 4.6**.

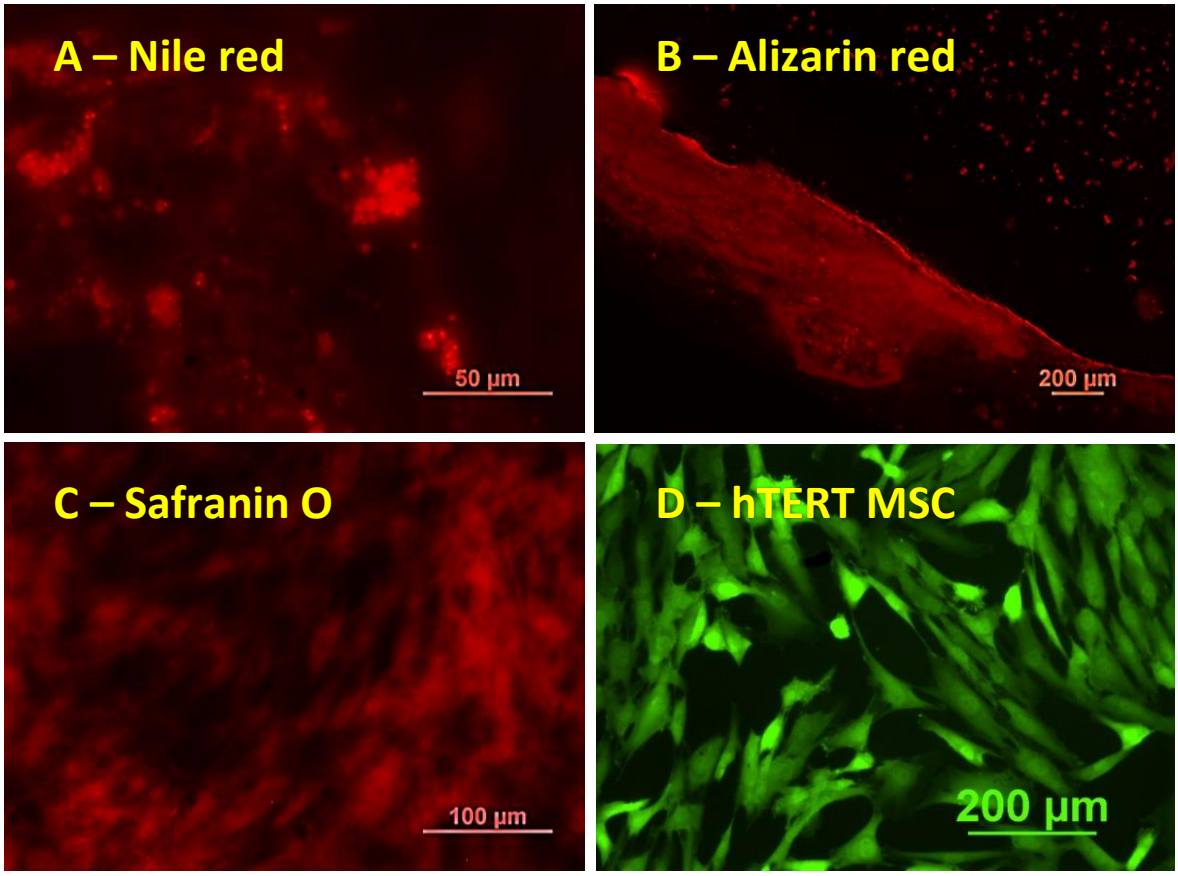

Figure 4.5: Trilineage differentiation and morphology of P15 human hTERT MSCs. After 14 days of treatment of differentiation media (**Media K1, K2 or K3 – Table 3.1**), hTERT BMMSCs stained positive for lipids (A), calcium (B) and glycosaminoglycans (C), indicating that they can differentiate into adipocytes, osteocytes and chondrocytes respectively. (D) hTERT displayed a spindle morphology, where cells face various directions.

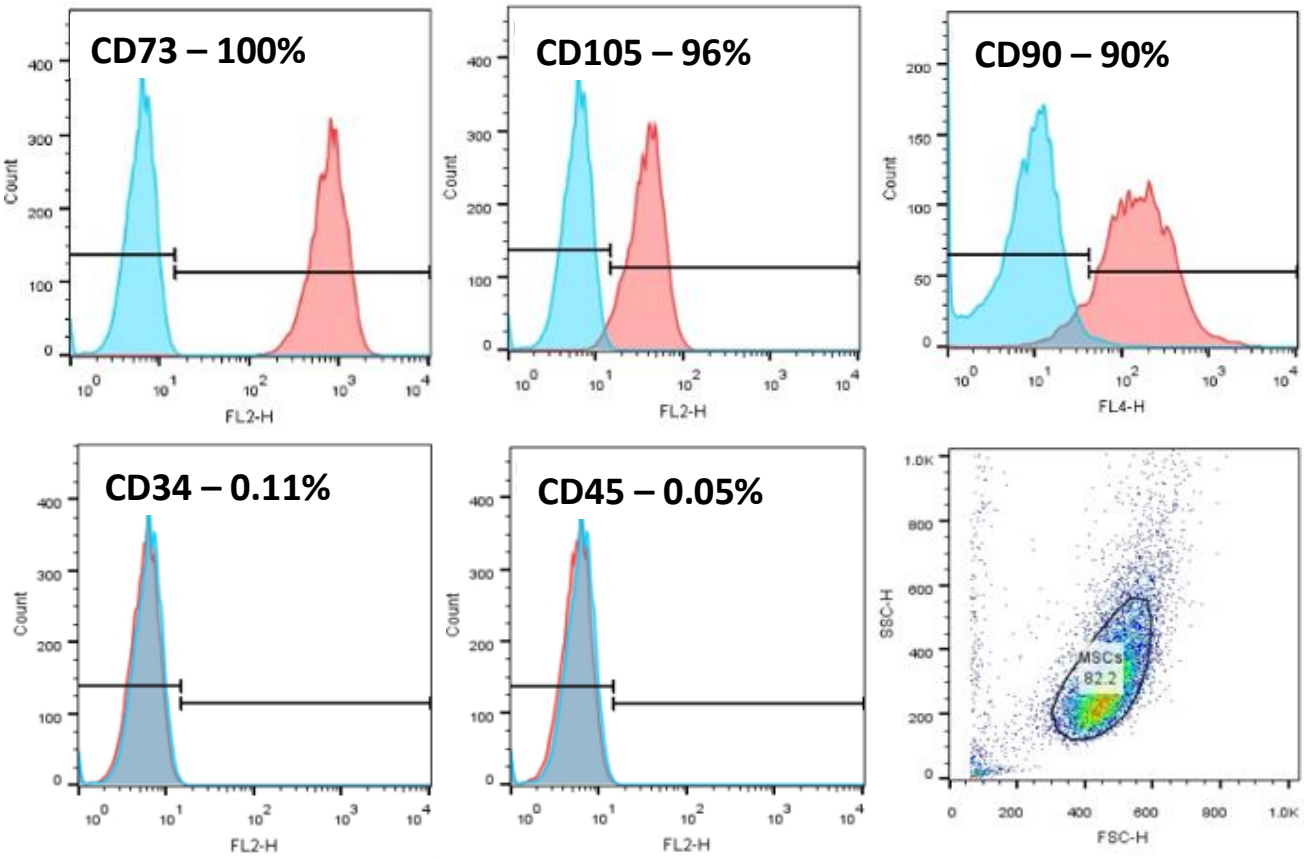

Figure 4.6: Stem cell surface marker expression of human hTERT BMMSCs. Human hTERT BMMSCs expressed MSC markers CD73, CD90 and CD105, as demonstrated by the fact that cells treated with antibodies (red peak) display higher fluorescence than unlabelled cells (blue peak). Cells did not bind CD34 and CD45, indicating absence of leukocytes, endothelial and hematopoietic stem cells.

4.3.4 –Effect of Passage and Cryopreservation on the Metabolism of MSC Monolayers under Incubator Conditions (37°C, 5% CO_2) in DMEM/FBS (Media B)

Both hTERT and primary MSCs (frozen for 1 month) had a similar doubling time of 24 hours. P15 hTERT MSCs proliferated significantly more quickly than P3 primary bovine MSCs (frozen for 1 month) over a 24 hour period (2.07 ± 0.03 fold change for hTERT MSCs, n = 12 vs 1.94 ± 0.05 fold change for primary MSCs, n = 4, p = 0.01). The glucose consumption (measured by ARCHITECT bioanalyser from 24-48h) was significantly and substantially (≈4x) higher in hTERT human MSCs vs. primary bovine MSCs (frozen for 1 month) (16.47 ± 0.13 pmoles/cell/day for hTERT human MSCs, n = 4, 4.63 ± 0.39 pmoles/cell/day for primary bovine MSCs, n = 3, p = 0.0001). The lactate production was significantly higher in hTERT human MSCs vs. primary bovine MSCs (frozen for 1 month) (34.29 ± 0.25 pmoles/cell/day for hTERT human MSCs, 10.17 ± 0.41 pmoles/cell/day for primary bovine MSCs, n = 3-4, p = <0.0001). Over 3 days of culture at a seeding density of 7500 cells/cm^2 in

DMEM/FBS, hTERT MSCs consumed extracellular pyruvate (87% decrease vs fresh media), threonine (15% decrease vs fresh media) and cysteine (45% decrease vs fresh media).

After 1 year of cryopreservation in 90% FBS and 10% DMSO, primary P3 bovine MSCs displayed a small (roughly 15%) but significant decline in proliferation potential compared to those frozen for 1 month (1.94 ± 0.05 fold change for 1 month old, 1.70 ± 0.08 fold change for 1 year old, n = 4, p = 0.0002). For the 1 year frozen group, a 1.55 ± 0.02-3 fold change was reported at P4 and 1.55 ± 0.03 fold change at P5, where there was no significant correlation between passage number and proliferation rate (r = -0.88, p = 0.31). Between primary cells frozen for 1 month and 1 year, there was no significant change in glucose consumption (4.63 ± 0.39 pmoles/cell/day, n = 3 for 1 month group vs 4.27 ± 0.98 pmoles/cell/day for 1 year group, n = 4, p = 0.53) or in lactate production (10.17 ± 1.06 pmoles/cell/day for 1 month group vs 9.43 ± 0.89 pmoles/cell/day for the 1 year group, n = 4, p = 0.33). Primary MSC morphology at P3, P4 and P5 is shown in **Figure 4.7**, where cells became larger and flatter at higher passage numbers. From P3 to P5, passaging had no significant effect on bovine MSC proliferation (r = -0.88, p = 0.31). Proliferation and metabolic data is shown in **Figure 4.8**.

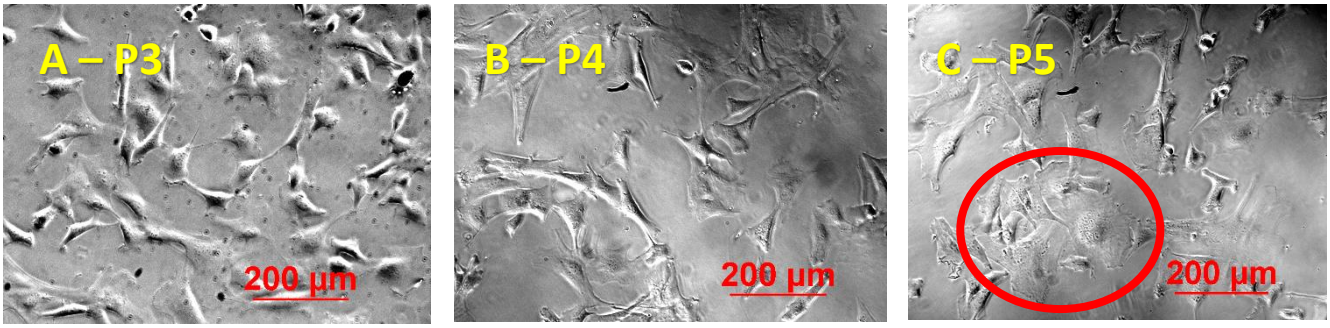

Figure 4.7: Effect of passaging on primary bovine BMMSCs in DMEM/FBS (Media B). At P5, primary MSCs become large and flat, despite no major change in proliferation potential compared to P4. Cells adopted a flat morphology at higher passages, shown by a red circle.

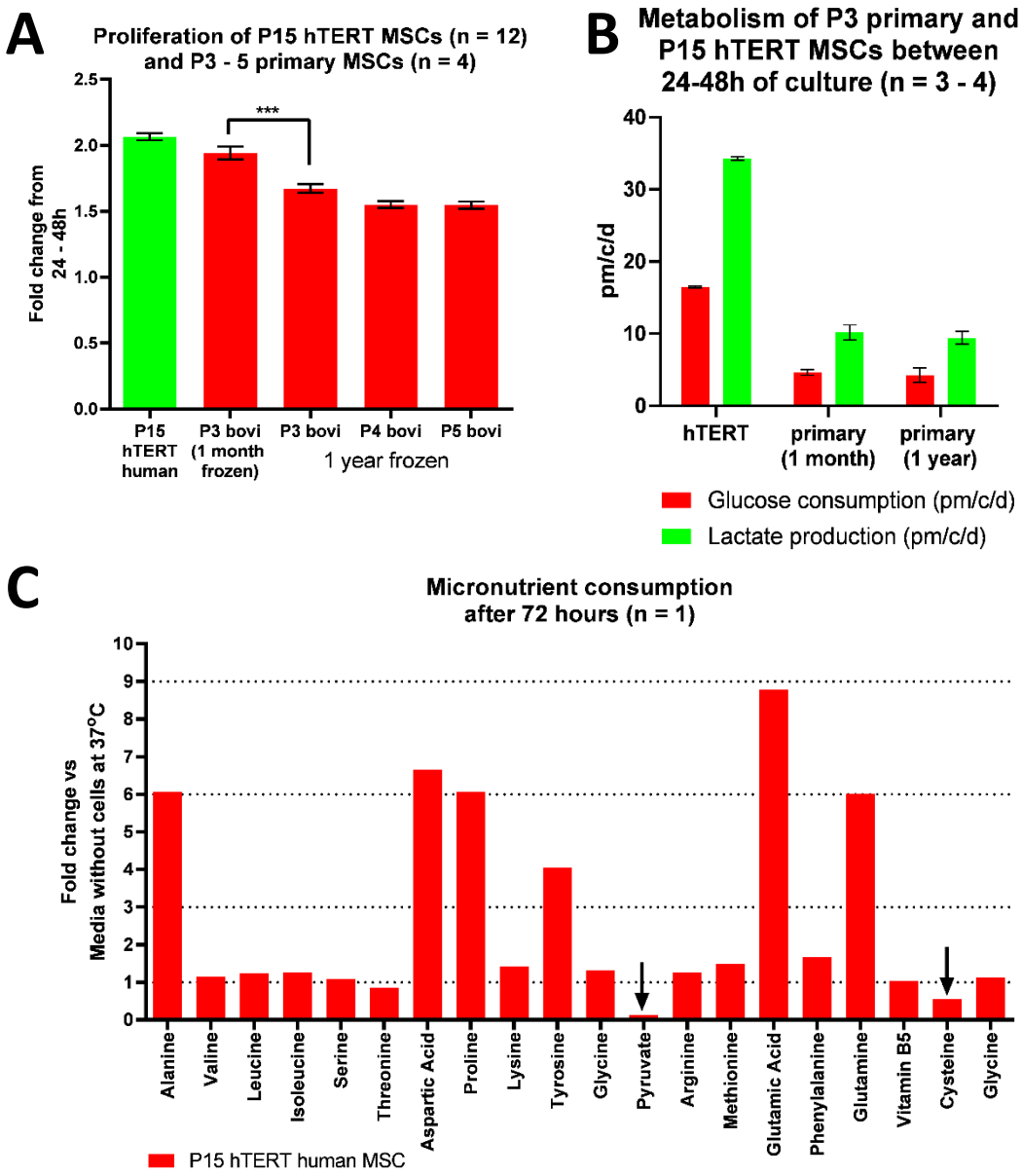

Figure 4.8: A comparison of proliferation and glycolysis between P15 hTERT human and P3 primary bovine BMMSCs and GC-MS data for hTERT MSCs. (A) Prolonged freezing of primary MSCs lowered proliferation potential (fold change in cell count between 24-48h), values are mean ± SD, n = 12 for P15 hTERT human group, n = 4 for all other groups. (B) Both primary bovine and hTERT human BMMSCs favoured glycolysis, where the lactate: glucose ratio was over 2, and cryopreservation had no effect on metabolism. The glucose consumption and lactate production in hTERT MSCs was 3-4x higher than primary bovine MSCs values are mean ± SD, n = 3 for primary MSC glucose consumption after 1 month freezing, n = 4 for all other groups. (C) GC-MS analysis of spent media (DMEM and 10% FBS) demonstrated that hTERT BMMSCs required extracellular cysteine and pyruvate, despite being able to make pyruvate from glucose and cysteine from serine, n = 1 for all groups, substantial decreases (≤0.66x change compared to media without cells incubated for 72 hours) are marked by arrows. Fold change in (C) was calculated vs media without cells incubated for the same time under the same conditions (37°C for 72 hours). *** = $p \leq 0.001$ by way of Welch's t-test.

4.3.5 – Effect of Media Glucose on Proliferation, Total Metabolic Activity and Trending Metabolic Activity in hTERT Human MSCs in DMEM/FBS

Regardless of glucose concentration, hTERT MSCs experienced a 24 hour lag phase followed by log phase growth. MSCs proliferated rapidly regardless of glucose concentration in DMEM/FBS (1, 2.5 or 5.5 mM) **(Media A, B and C)**, with all doubling times faster than 48 hours. There was no significant difference in doubling time between DMEM with 5.5 mM glucose **(Media A)** and 2.5 mM glucose **(Media B)** (28.43 ± 1.38 hours PDT for 5.5 mM glucose, 29.66 ± 0.39 hours for 2.5 mM glucose, n=8, p = 0.06). For hTERT MSCs cultured in 1 mM glucose **(Media C)**, growth became inhibited between the 60-72h time frame. Doubling time for MSCs cultured at 1 mM glucose was significantly slower than 5.5 mM glucose (38.63 ± 1.63 hours for 1 mM glucose, n=8, p = <0.0001) and compared to 2.5 mM glucose (n=8, p = <0.0001). The data is shown in **Figure 4.9.**

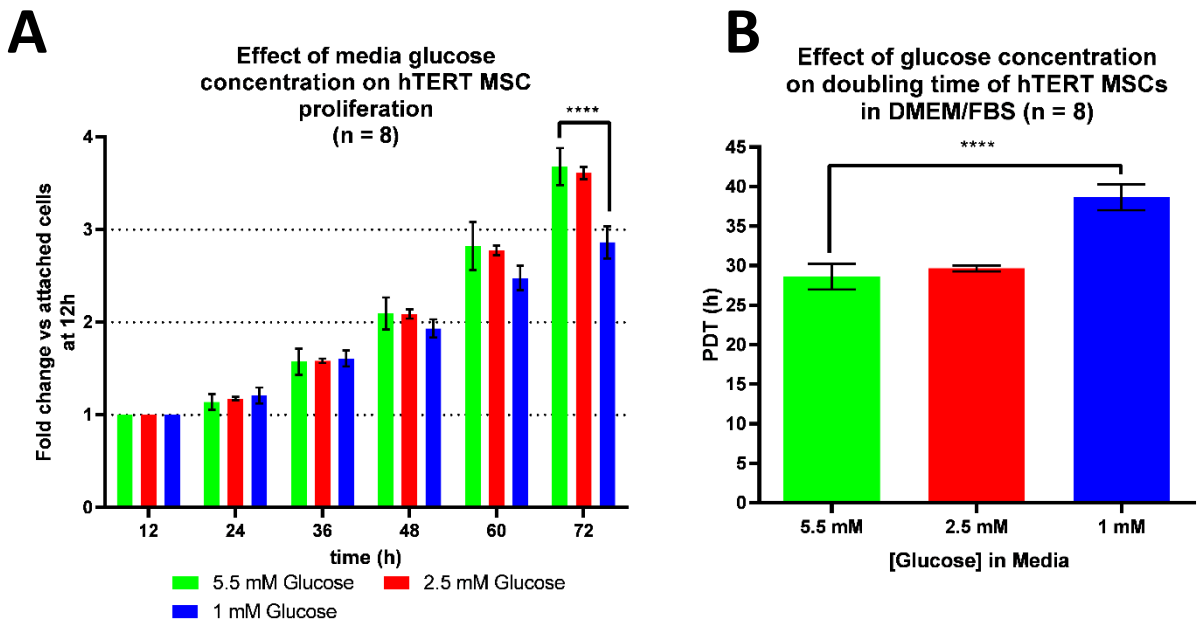

Figure 4.9: effect of glucose concentration on proliferation of hTERT MSCs. hTERT MSC fold change in cell count vs at 12h under different media glucose concentrations (A). Regardless of glucose concentration, hTERT MSCs experienced a 24 hour lag phase followed by exponential growth (A). Differences in proliferation between 5.5 mM and 1 mM glucose has been seen at 60-72h. The population doubling time of hTERT MSCs was significantly slowed at 1 mM glucose, but not at 2.5 mM (B). Values displayed as mean ± SD, n = 8 for all groups, **** = p ≤ 0.0001 by way of Welch's t-test.

Total glucose consumption/lactate production per cell per day was calculated as the sum of glucose consumption rate at each time point x12, since each interval is 12 hours long. Under normal culture conditions in DMEM/FBS with 5.5 mM glucose **(Media A)**, the total glucose consumption for MSCs over 72 hours was 1280 ± 154 pmoles/cell, while the total lactate production was 2519 ± 189 pmoles/cell (n = 4). In DMEM/FBS with 2.5 mM glucose **(Media B)**, the average glucose consumption was (1111 ± 90 pmoles/cell, n = 4, p = 0.1 vs 5.5 mM), while the lactate production was (1962 ± 50 pmoles/cell, n = 4, p = 0.007 vs 5.5 mM). In comparison to 5.5 mM glucose, the total glucose consumption in DMEM/FBS with 1 mM media glucose **(Media C)** was (802 ± 29 pmoles/cell, n = 4, p = 0.007 vs 5.5 mM glucose) while the total lactate production was 1513 ± 86 pmoles/cell, n = 4, p = 0.0005 vs 5.5 mM glucose). Regardless of glucose concentration in media, no major fluctuations occurred in either glucose consumption or lactate production. The data is shown in **Figure 4.10**. A Lineweaver-Burk plot detailing the effect of media glucose on total glucose consumption is shown in **Figure 4.11**. The data showed that the maximal glucose consumption was 19.8 pmoles/cell/day and half maximal consumption was achieved at 0.86 mM glucose for P15 hTERT MSCs. Irrespective of glucose concentration, MSC morphology remained spindle shaped throughout, so photos were not taken.

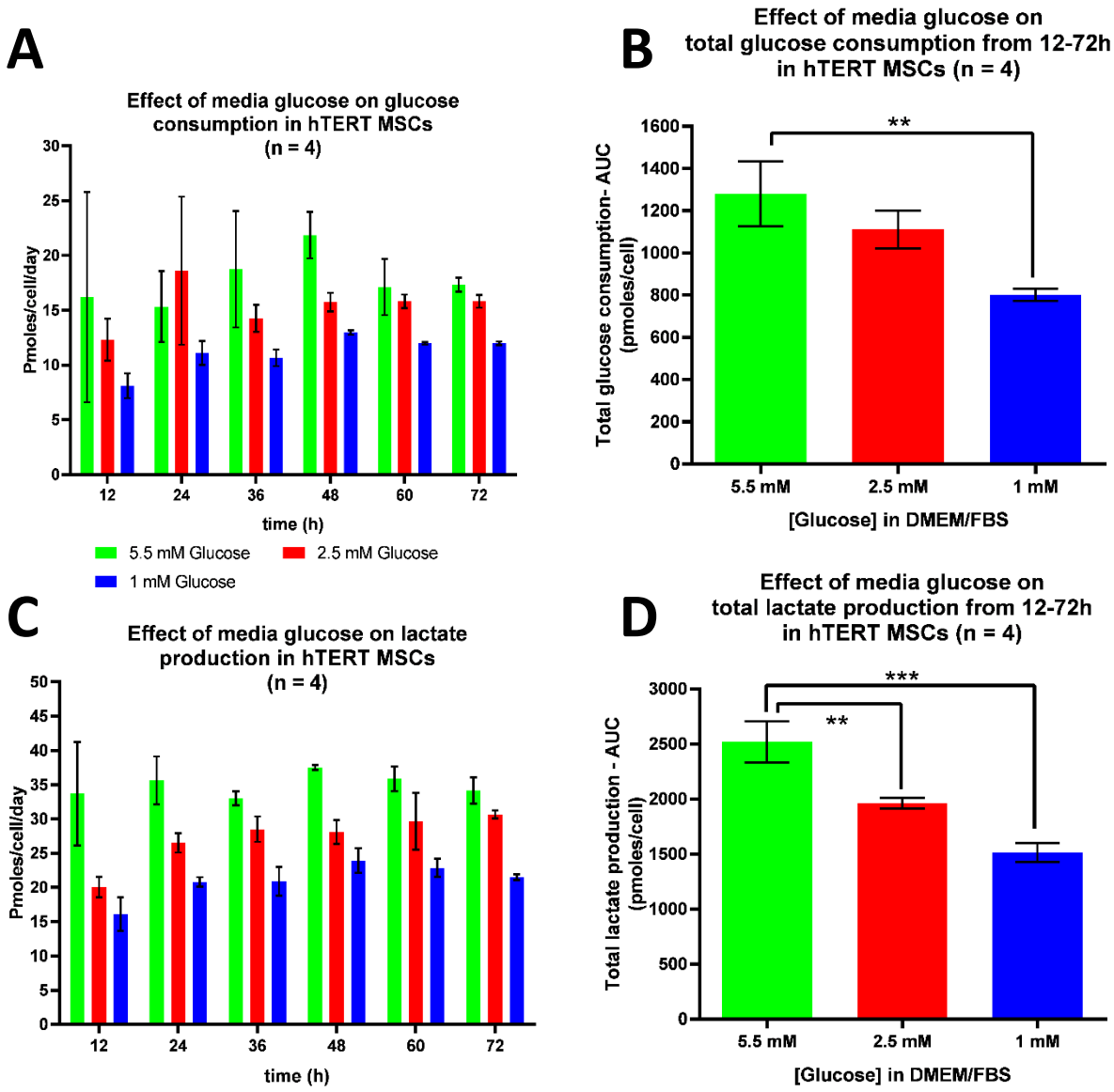

Figure 4.10: effect of media glucose concentration on metabolism in hTERT MSCs. No major changes occurred in glucose consumption (A) or lactate production (C) during the 72 hours of culture. (B) Total glucose consumption was not significantly altered by 2.5 mM glucose, but only when 1 mM glucose was used. Total lactate production was significantly lowered by both 2.5 and 1 mM glucose (D). Values displayed as mean ± SD, n = 4 for all groups, ** = $p \leq 0.01$, *** = $p \leq 0.001$, all by way of Welch's t-test.

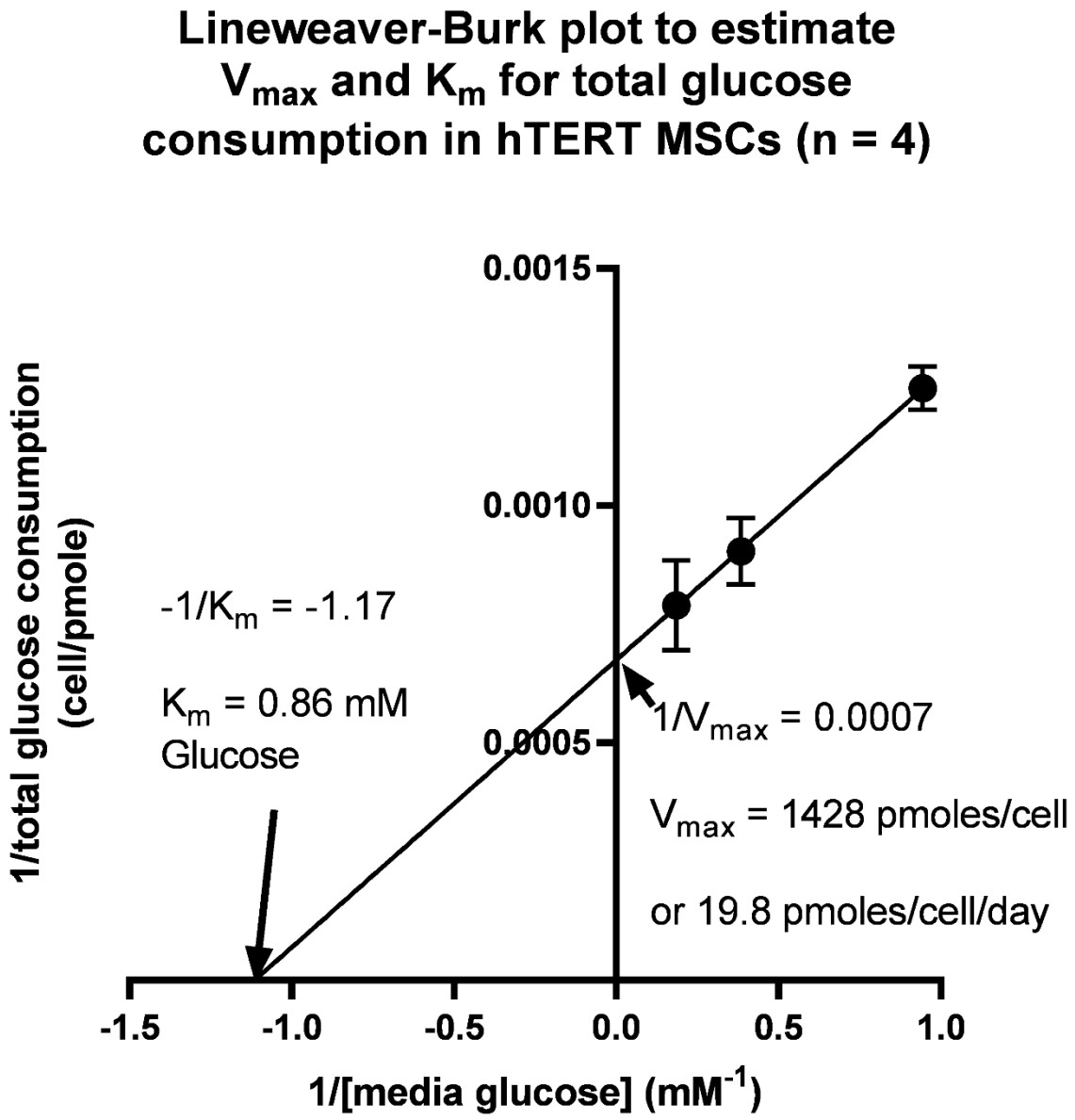

Figure 4.11: Lineweaver-Burk plot for the effect of media glucose concentration on total glucose consumption in hTERT MSCs. Maximal glucose consumption was almost reached at 5.5 mM media glucose. Half maximal total glucose consumption was achieved by a media concentration of 0.86 mM. Values displayed as mean ± SD, n = 4 for all groups. Data was analysed using the method of Lineweaver-Burk (157) to estimate the K_m and V_{max} for glucose total glucose consumption per cell over 72 hours. Plotting 1/[media glucose] on the x-axis against 1/[total glucose consumption] on the y-axis at media glucose concentrations of 1 mM, 2.5 mM and 5.5 mM yielded a straight line, which suggests that glucose consumption in hTERT human MSCs follows Michaelis-Menten kinetics (158).

4.3.6 – Effect of Low Temperature on hTERT MSC Proliferation and Metabolism

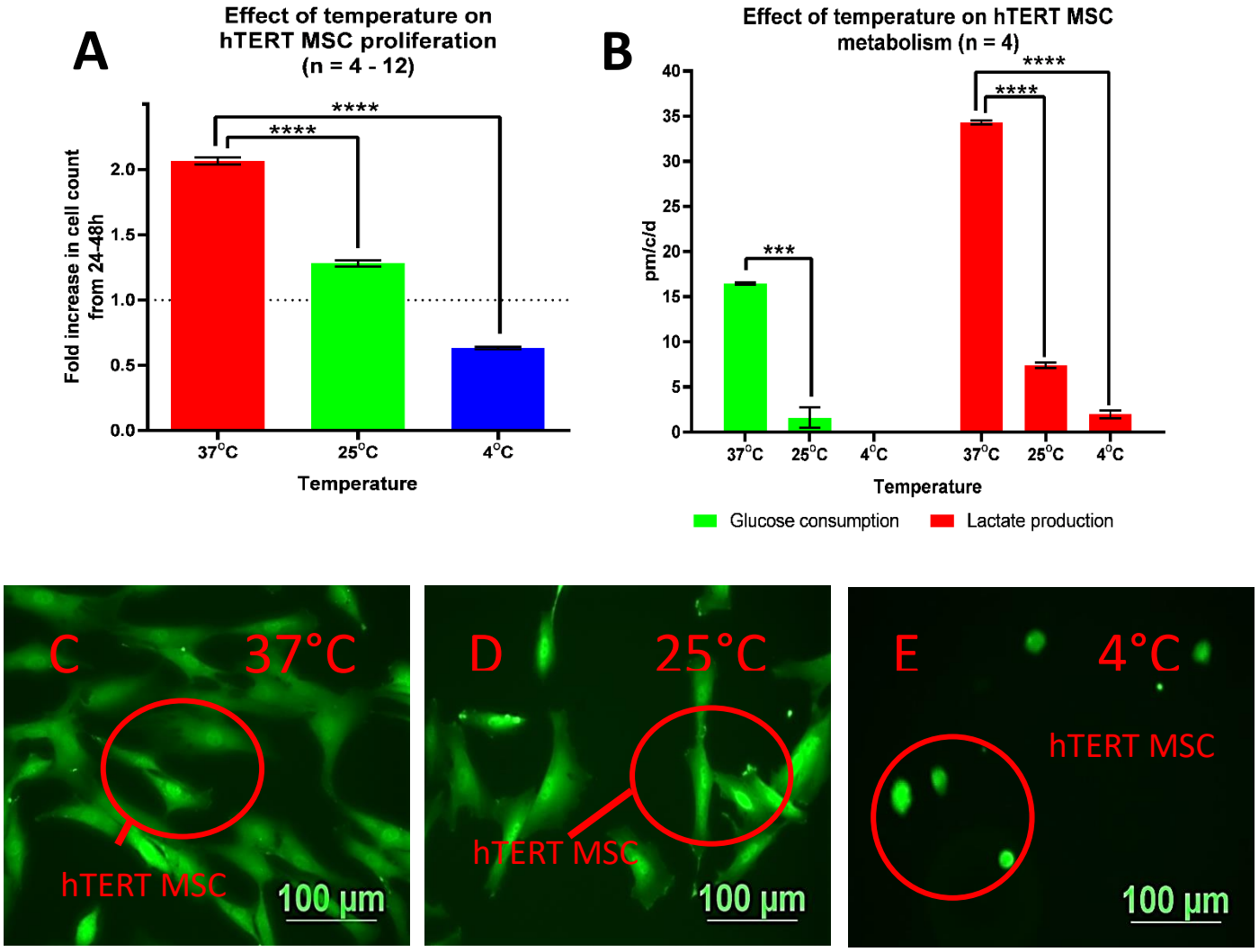

Figure 4.12: Effect of temperature on hTERT MSC proliferation and metabolism in DMEM/FBS (Media B). (A) MSC proliferation was halted at room temperature, but cell death occurred at cold storage, values displayed as mean ± SD, n = 12 for hTERT MSCs at 37°C, n = 4 for all other groups. (B) Lactate production and glucose consumption fell with decreasing temperature (B). Glucose consumption at 4°C was too low to accurately measure, values displayed as mean ± SD, n = 4 for all groups. (D) hTERT MSCs retained their spindle morphology at 25°C (D), but failed to elongate at 4°C (E). *** = $p \leq 0.001$, **** = $p \leq 0.0001$, all by way of Welch's t-test.

At 37°C, the PDT of hTERT MSCs in DMEM/FBS **(Media B)** was 23h (2.07 ± 0.03 fold change in cell count over 24 hours, n = 12). At 25°C in the same media, the PDT slowed significantly to 67h (1.28 ± 0.02 fold change over 24 hours, n = 4, $p < 0.0001$ vs fold change at 37°C). Cell death occurred at 4°C in **Media B** on day 2, with cell count falling by an average of 37%. At 37°C, glucose consumption in hTERT MSCs was 16.47 pmoles/cell/day and lactate production was 34.29 pmoles/cell/day. MSCs favoured glycolysis, with the Lactate/Glucose ratio being 2.08. When

cultured at 25°C, glucose consumption fell significantly (1.60 pmoles/cell/day, n = 4, p = 0.000103 vs 37°C) along with lactate production (7.39 pmoles/cell/day, n = 4, p < 0.0001). At 4°C, there was no clear change in glucose concentration (2.4 mM glucose for both fresh and spent media), while lactate production further fell compared to 25°C (1.98 pmoles/cell/day ± 0.43, n = 4, p < 0.0001). The data is shown in **Figure 4.12**.

4.4 – Discussion

4.4.1 – MSC Characterisation

Both hTERT and human bovine MSCs were validated by trilineage differentiation potential (**Figure 4.3** and **Figure 4.5**), yet human antibodies did not bind to bovine cells during flow cytometry, possibly due to incompatibility in protein structure between bovine MSC surface markers and human antibodies (**Figure 4.4**). As expected, the majority (>90%) of human MSCs bound to human MSC markers CD73, CD90 and CD105, with no contamination of endothelial cells, haematopoietic cells and leukocytes (shown by lack of CD34 and CD45 binding) (**Figure 4.6**)**.** Despite lack of binding to human antibodies, there have been multiple publications which demonstrated that changing media after 24-48 hours after direct plating of marrow leads to a pure MSC sample (29) (28) (31), with heavy haematopoietic and macrophage contamination following 72 hour media changes reported by Baddoo (2003) *et al.* (30). On the other hand, Schallmoser *et al.* (2008) reported that >95% of human BMMSCs expressed CD73, CD95 and CD105, with <2% expressing CD34, CD45, HLA-DR, CD14 and CD19 (159) when media changes were carried out at 72h after direct plating. Baddoo (2003) had used 10 % foetal calf serum in α-MEM while Schallmoser (2008) had used human platelet lysate therefore differences in MSC purity after a 72 hour media change could be attributed to the supplement used. Despite this limitation, primary MSC proliferation rates have been consistent at P3, P4 and P5, therefore cell contamination is highly unlikely.

4.4.2 – MSCs Favoured Glycolysis

Both primary and hTERT MSCs favoured glycolysis over oxidative phosphorylation, as the lactate: glucose ratio exceeded 2 (See **Figure 4.8b**). Heathman *et al.* (2016) reported lactate: glucose values ranging between 0.5 and 1.3 in his 5 patients (60). Schop *et al.* (2009) reported lactate: glucose ratios of 1.96 for human MSC and 1.86 for goats, much more in line with the reported results in this chapter (113). Nuschke *et al.* (2016) reported that P11 hTERT human BMMSCs favoured glycolysis: ATP production was abolished in the presence of glucose analogue 2-deoxy-D-glucose (2-DG) but not in the presence of oxidative phosphorylation (OXPHOS) inhibitors rotenone and Carbonyl cyanide-p-trifluoromethoxyphenylhydrazone (FCCP) (127).

Ko *et al.* (2012) reported that MSC umbilical cord growth is inhibited after exposure to reactive oxygen species (160). From this, it can be speculated that MSCs favour glycolysis to prevent ROS production, which would otherwise inhibit cell function. During preservation therefore, it is important to test that ROS does not rise in the preservation media, and that ROS increase, if any does not increase autophagy, a measure of cellular damage.

4.4.3 – MSCs did not Undergo Major Changes in Proliferation and Metabolism after 1 year of Cryopreservation

For primary bovine BMMSCs, cells frozen for 1 year exhibited a 15% decline in proliferation potential (**Figure 4.8a**) but no loss of metabolic activity (**Figure 4.8b**) compared to those frozen for 1 month. Bahsoun have conducted a systematic review of 20 studies on the effect of MSC cryopreservation in dimethyl sulfoxide; 17 reported no change, 2 reported loss of proliferation potential while 1 reported an increase in proliferation potential (161). P2 minipig BMMSCs have been stored in 90% FBS/10% DMSO for 11 weeks at -70°C, and reported a 50-80% decline in the number of post thaw population doublings and loss of osteogenic potential (162). Ginis *et al.* (2012) reported 0-2 fold increases in proliferation potential for primary human BMMSCs after 1-5 months cryopreservation in Cryostor, where they used DMSO concentrations of 5 and 10% (163). The % of caspase positive cells was roughly 15% for the cryopreserved group compared to 3% for fresh cells (163). The authors hypothesize that only "stronger cells" were left during the cryopreservation process (163).

4.4.4 – MSCs are Better Able to Withstand Room Temperature Conditions than Cold Storage

When cultured in DMEM/FBS **(Media B)**, MSC proliferation was slowed at 25^{o}C, while cell death occurred at 4^{o}C (**Figure 4.12A**). Both glucose consumption and lactate production fell with temperature; at 4^{o}C there was no clear change in glucose (**Figure 4.12B**), which may suggest that cells use glucose at room temperature, but not during cold storage. This may explain why MSCs in previously published literature have been able to survive for 7 days in Wisconsin Solution at 4^{o}C where glucose was not present (40). MSCs maintained their spindle morphology at 25^{o}C but failed to elongate at 4^{o}C, possibly due to complete inhibition of protein synthesis at cold temperatures. Previous authors have not studied the effect of temperature on MSC metabolism, proliferation or morphology. The results indicate that 25°C (room temperature) is more optimal for MSC survival compared to 4°C (cold storage), therefore room temperature will be selected for MSC preservation.

4.4.5 – MSC Metabolism Remained Constant during Culture, and was not Altered at 2.5 mM Glucose

Previous authors only made end point measurements of MSC metabolic activity, which does not reflect how the metabolism changes during the lag phase, and log phase of cell growth. This has been addressed by measuring proliferation, glucose consumption and lactate production at 12 hour intervals for a total of 72 hours.

Regardless of glucose concentration, both hTERT MSC glucose consumption and lactate production stayed fairly constant between seeding (12 hours) and 80% confluency (72 hours) (**Figure 4.10A and 4.10C**). This may suggest that the majority of ATP was used to drive processes not related to cell proliferation. It can be concluded that making 1 end point measurement accurately reflected cell metabolism during the entire passage.

During preservation at room temperature, glucose consumption was expected to be much lower than proliferating cells at 37°C, and therefore measurement of tiny changes from 5.5 mM may not be accurate. Therefore the effects of 2.5 mM and 1 mM glucose on proliferation and metabolism have been tested. When measuring at 12 hour intervals, proliferation and glucose consumption only fell at 1 mM media glucose, while lactate production fell at 2.5 mm glucose (**Figure 4.9 and Figure 4.10**). The Linweaver-burke plot (**Figure 4.11**) for glucose uptake gave a K_m value of 0.86 mM, which indicated that MSCs predominantly express glucose transporter 1 (GLUT-1), the only isoform that is ubiquitously distributed and has a glucose affinity (K_m) of 1-2 mM (164). It can be speculated that at 1 mM starting glucose, the glucose concentration fell too far below the K_m at the 60-72 hour timeframe, and glucose transport into cells was inhibited. Consequently, metabolism and proliferation was slowed. This shows that a starting glucose concentration of 2.5 mM is suitable for metabolic study of preserved cells at room temperature.

4.4.6 – MSCs Required Additional Extracellular Pyruvate and Cysteine

The GC-MS data suggested that hTERT MSCs consume extracellular pyruvate and cysteine (**Figure 4.8C**), despite both being synthesized from glucose and serine respectively. Eagle *et al.* (1958) reported that monkey kidney cells fail to proliferate after being deprived of non-essential amino acids (NEAAs), which may suggest that cells cultured *in vitro* lose their ability to synthesize these NEAAs (112). Higuera *et al.* (2009) reported that human MSCs produced alanine, cysteine, glycine, ornithine and proline, while other amino acids were consumed (138) in monolayer culture. They had used a lower seeding density of 100 cells/cm^2, used α-MEM (lower starting concentrations of amino acids and vitamins than DMEM), and cultured cells over 8 days as opposed to 3. Longer

culture periods and lower amino acid starting concentrations may produce larger and more measurable changes. Cysteine serves as an antioxidant by acting as the precursor for glutathione (165). At 21% pO_2, oxidative stress would be higher than MSCs in their native bone marrow environment (<7% pO_2) (166), and thus MSCs may consume more cysteine to clear out the ROS. Protease enzymes produced by MSCs may break down proteins in FBS and create amino acids, which may explain increases in essential amino acid concentration in the GC-MS data for this chapter. In the next chapter, the experiment has been repeated using a proprietary insulin supplement to keep cells alive without serum, thus preventing serum proteins from skewing GC-MS data.

4.5 – Conclusion

In conclusion both primary bovine and hTERT human BMMSCs have been characterised, and their metabolism, proliferation and morphology under normal culture conditions have been studied. This can then be used to determine how metabolism, proliferation and morphology changed during preservation in ITSE based media. The GC-MS data showed that MSCs required extracellular pyruvate and cysteine, though the presence of serum proteins may have skewed the data. As cell survival was superior at room temperature compared to cold storage, preservation studies of the final media have been carried out room temperature.

Chapter 5

Development of a Novel Preservation Media using hTERT Mesenchymal Stem Cells

5.1 – Introduction

During preservation it is important to circumvent the use of serum to prevent immune responses in patients (126). However, MSCs survive for only 4-5 days in media when deprived of serum or a serum free alternative (127). To achieve longer preservation times, the use of an insulin-transferrin-selenium-ethanolamine (ITSE) supplement at 1% concentration has been tested to determine if cell survival and function can be maintained over a 12 day period. DMEM served as the basal medium, which supplied the glucose, pyruvate, amino acids and vitamins necessary to keep cells viable. Media was first tested at incubator conditions for hTERT MSC monolayers. In the literature, insulin-transferrin-selenium (ITS) has previously been used to expand, in the absence of serum, bovine chondrocytes in agarose over a 35 day period (47), primary mouse granulosa cells over a 2 day period (167) and human and bovine corneal keratocytes (168). Ethanolamine was also included, as it acts as a phospholipid precursor, which is not present in DMEM basal medium (169).

Despite pyruvate synthesis from glycolysis, the gas chromatography and mass spectroscopy (GC-MS) data in **Chapter 4** demonstrated that human hTERT MSCs consumed most of the extracellular pyruvate over a 3 day period, suggesting that cells also require extracellular pyruvate. Therefore the effect of pyruvate reduction on hTERT MSC characteristics has been studied in ITSE based media; which determined whether the novel insulin based medium required pyruvate to maintain cell survival and function.

5.2 – Experimental Plan

The experimental techniques used are mentioned in **Chapter 3: Materials and Methods.** HTERT MSCs were seeded into well plates in DMEM basal medium alone or DMEM + ITSE, where cells were counted over a 12 day period (**Section 3.2.3**). The effect of pyruvate reduction (0.3 mM and 0 mM) in ITSE on cell proliferation and metabolism over a 3 day period was tested (**Section 3.1.5**), and whether loss of proliferation potential was due to absence of serum growth factors (basic fibroblast growth factor (bFGF), epidermal growth factor (EGF) and transforming growth factor – β1 (TGF-β1)) was investigated (**Section 3.1.5**). The differences in intracellular reactive oxygen species (ROS) production (**Section 3.2.1**) and autophagy (**Section 3.2.2**) between cells cultured in DMEM/FBS and ITSE based media was studied. For the final step, cell proliferation after 4, 8 and 12 days in ITSE based media at 37°C as monolayers was tested (**Section 3.1.7**). After finding that cells retained

proliferation potential after 12 days in ITSE, recovering cells were stained for lipids, calcium and glycosaminoglycans to assess differentiation (**Section 3.1.8.1**). The plan is outlined in **Figure 5.1.**

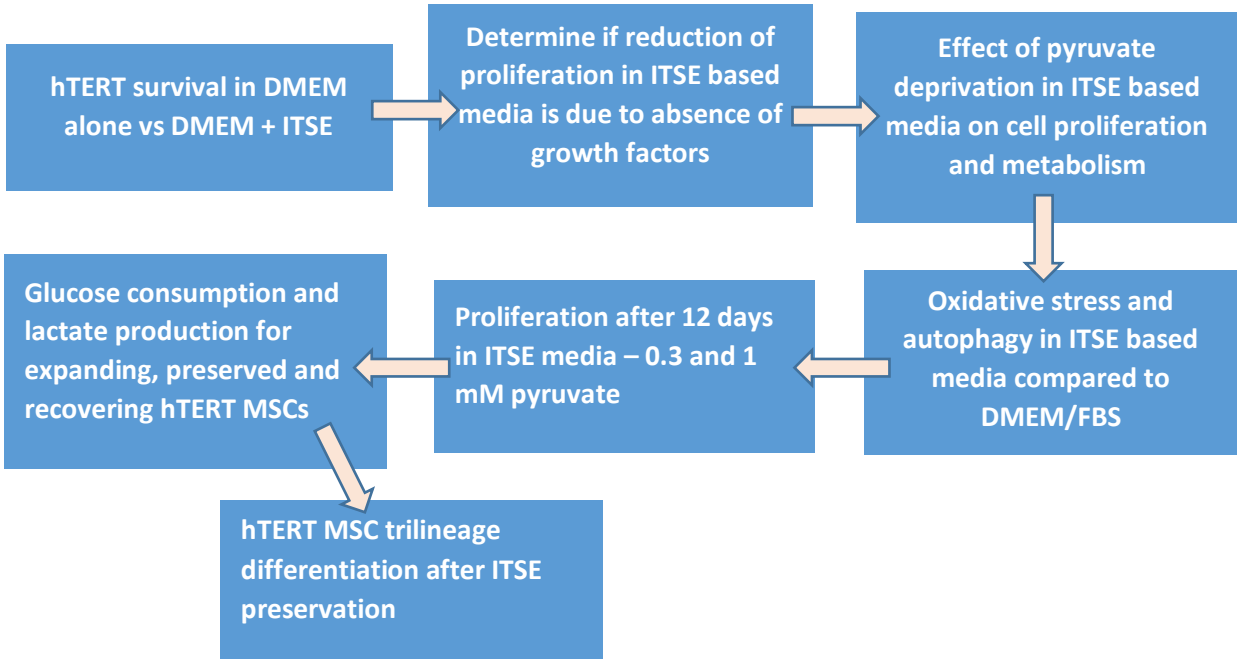

Figure 5.1: Experimental outline studying the proliferation, metabolism, ROS production, autophagy in hTERT MSCs in ITSE media, and subsequent study of metabolism, proliferation and trilineage differentiation potential for recovering cells.

All media, unless stated otherwise, contained 44 mM sodium bicarbonate. The media formulations used this chapter are listed in **Table 5.1**.

Table 5.1: Media formulations used for the development of an ITSE based medium.

Media Formulation	Experiment
DMEM, 5 mM glucose, no ITSE, no FBS **(Media A)**	Test whether ITSE supplement was required for MSC survival over a 12 day period
DMEM, 5 mM glucose, 1% ITSE **(Media B)**	Test whether ITSE supplement was required for MSC survival over a 12 day period
DMEM, 2.5 mM glucose, 1 mM pyruvate **(Media C1)**, 0.3 mM pyruvate **(Media C2)**, no pyruvate **(Media C3)** and 1% ITSE	Effect of pyruvate reduction on MSC proliferation, metabolism, oxidative stress, autophagy and recovery of proliferation following ITSE based medium preservation at 37°C.
DMEM, 2.5 mM glucose, 1 mM pyruvate, 5 µg/0.5 L bFGF, 5 µg/0.5 L EGF, 2.5 µg/0.5 L TGF-β1, 1% ITSE **(Media D)**	Determine if reduction of proliferation in ITSE based medium was due to absence of serum growth factors.
DMEM, 2.5 mM glucose, 10% FBS **(Media E)**	Media used to assess MSC recovery following preservation in ITSE based media. MSC proliferation and metabolism in expansion media.

5.3 – Results and Analysis

5.3.1 – Effect of 1% ITSE Supplementation on hTERT MSC Survival in DMEM Basal Medium over 12 Days

P15 hTERT MSCs were seeded at 7500 cells/cm^2 in 1 mL of blank DMEM with no serum or ITSE **(Media A)** or ITSE based medium **(Media B)**, and cell counts were performed at day 1, day 2 and 2 day intervals thereafter for a total of 12 days. For cells in blank DMEM, proliferation occurred up to day 6, and then a gradual drop in the number of attached cells was observed between day 6 and 12 ($r = -0.93$, $p = 0.07$, $n = 4$). Cells in ITSE based medium proliferated throughout the 12 days, and the r value between day 6 and 12 was (0.91, $p = 0.08$, $n = 4$). At day 12, there were significantly more attached cells relative to day 1 for the ITSE base media group compared to the blank DMEM group (4.29 ± 0.41 fold change for the ITSE group, 1.92 ± 0.58 fold change for the DMEM group, $p = 0.0008$, $n = 4$). The data is shown in **Figure 5.2**.

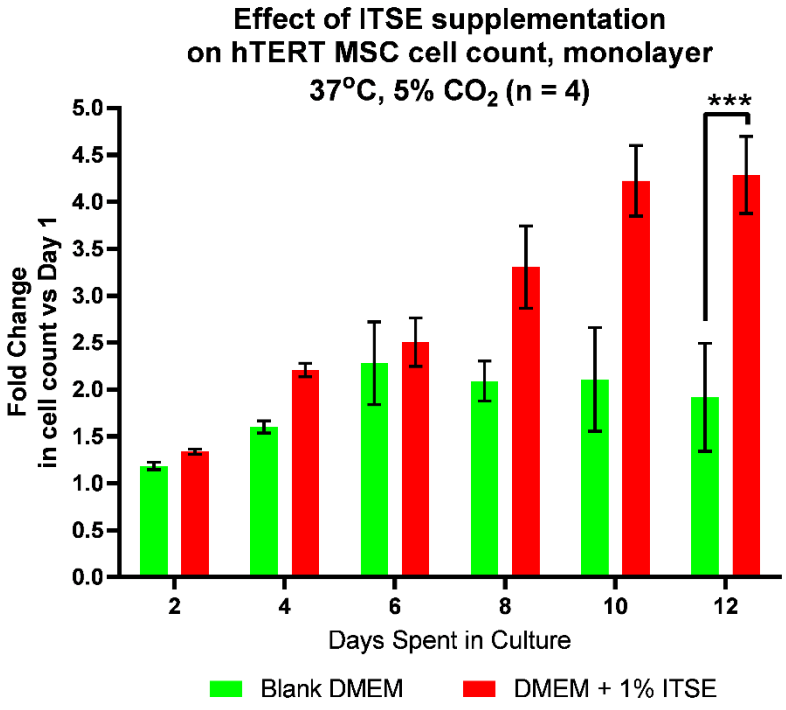

Figure 5.2: hTERT monolayer proliferation in blank DMEM (Media A) and ITSE based media (Media B) at incubator conditions without serum. When cultured in DMEM alone, cells proliferated up to day 6, and cells detached from day 6 to 12. When cultured in ITSE, cells proliferated continuously during the 12 days. Values displayed as mean ± SD, n = 4 for all groups, *** = $p \leq 0.001$ by way of Welch's t-test.

5.3.2 - Effect of Pyruvate Deprivation on hTERT MSC Proliferation and Metabolism in ITSE

In DMEM/FBS **(Media E)**, the doubling time for MSCs was 30 hours (Area under growth curve (AUC) = 146.79 ± 2.28 cell.hours, n = 8). In ITSE based medium with 1 mM pyruvate **(Media C1)**, MSCs took significantly longer to double (86 hours at in ITSE based medium, AUC = 95.27 ± 2.81 cell.hours, n = 4, p = < 0.0001 vs DMEM/FBS). When the ITSE medium was supplemented with growth factors **(Media D)**, doubling time was shortened to 49 hours (AUC = 108.15 ± 3.32 cell.hours, n = 4, p = 0.001 vs ITSE). When the pyruvate concentration was reduced from 1 mM to 0.3 mM in ITSE based medium **(Media C2)**, there was no significant difference in total cell proliferation (AUC drops from 95.27 ± 2.81 cell.hours to 93.56 ± 3.34 cell.hours, n = 4, p = 0.46). Compared to ITSE based media with 1 mM pyruvate, total cell proliferation fell when pyruvate was removed from the media **(Media C3)** (AUC drops to 77.87 ± 8.60 cell.hours, n = 4, p = 0.02 vs ITSE with 1 mM pyruvate).

In DMEM/FBS **(Media E)**, the total glucose consumption for MSCs was (1111.21 ± 89.83 pmoles/cell, n = 4) falling significantly in the presence of ITSE based medium with 0.3 mM pyruvate **(Media C2)** (585.59 ± 79.09 pmoles/cell, n = 4, p = 0.0001). When pyruvate was removed from the ITSE based media **(Media C3)** there was no significant change in glucose consumption (475.68 ± 86.47 pmoles/cell, n = 4, p = 0.11 vs ITSE with pyruvate). In DMEM/FBS **(Media E)**, the total lactate production for MSCs was (1962.28 ± 49.82 pmoles/cell, n = 4) falling significantly in the presence of ITSE based media with 0.3 mM pyruvate **(Media C2)** (1256.41 ± 44.30 pmoles/cell, n = 4, p < 0.0001 vs DMEM and FBS). When pyruvate was removed from the ITSE based media **(Media C3)** there was no further significant change in lactate production. (1389.65 ± 120.44 pmoles/cell, n = 4, p = 0.11 vs ITSE with pyruvate). Data is shown in **Figure 5.3** and **Figure 5.4.**

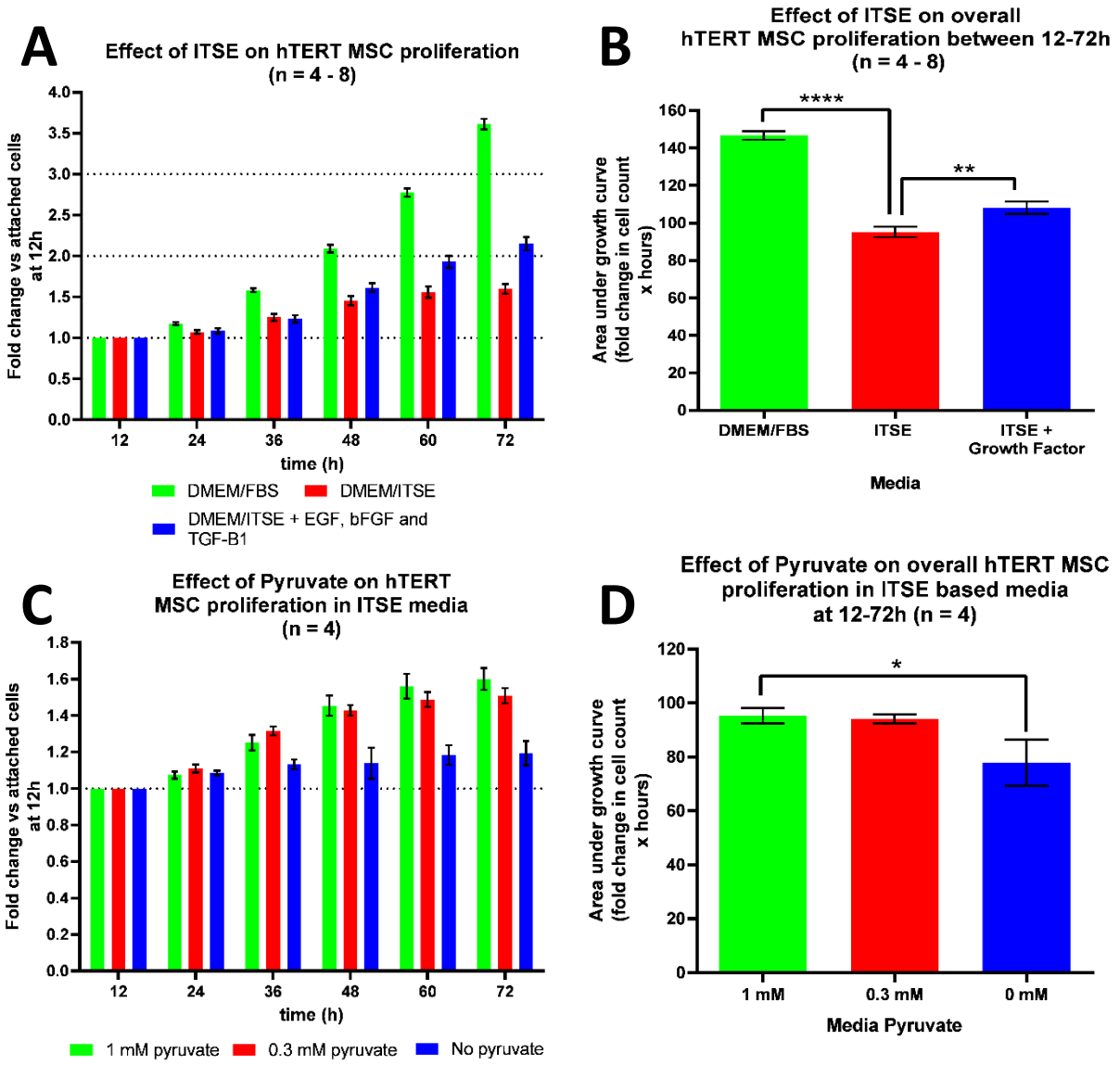

Figure 5.3: Effect FBS replacement with ITSE in DMEM and pyruvate reduction on hTERT MSC proliferation. (A) hTERT MSC fold change in cell count vs at 12h in DMEM/FBS **(Media E),** ITSE based media with 1 mM pyruvate **(Media C1)** and ITSE based media supplemented with EGF, bFGF and TGF-β1 **(Media D),** values are mean ± SD, n = 8 for MSCs in DMEM/FBS, n = 4 for all other groups. (B) Total area under growth curve from 12h-72h. Overall proliferation across 72 hours was slowed when FBS was replaced with ITSE, and partially restored when ITSE was supplemented with growth factors, values are mean ± SD, n = 8 for MSCs in DMEM/FBS, n = 4 for all other groups. (C) Effect of pyruvate reduction on the growth pattern of hTERT MSCs, values are mean ± SD, n = 4 for all groups. (D) Total proliferation was significantly reduced in ITSE based media with 0 mM pyruvate vs ITSE based media with 1 mM pyruvate, but not ITSE based media with 0.3 mM pyruvate, values are mean ± SD, n = 4 for all groups. * = $p \leq 0.05$, ** = $p \leq 0.01$, **** = $p \leq 0.0001$ by way of Welch's t-test.

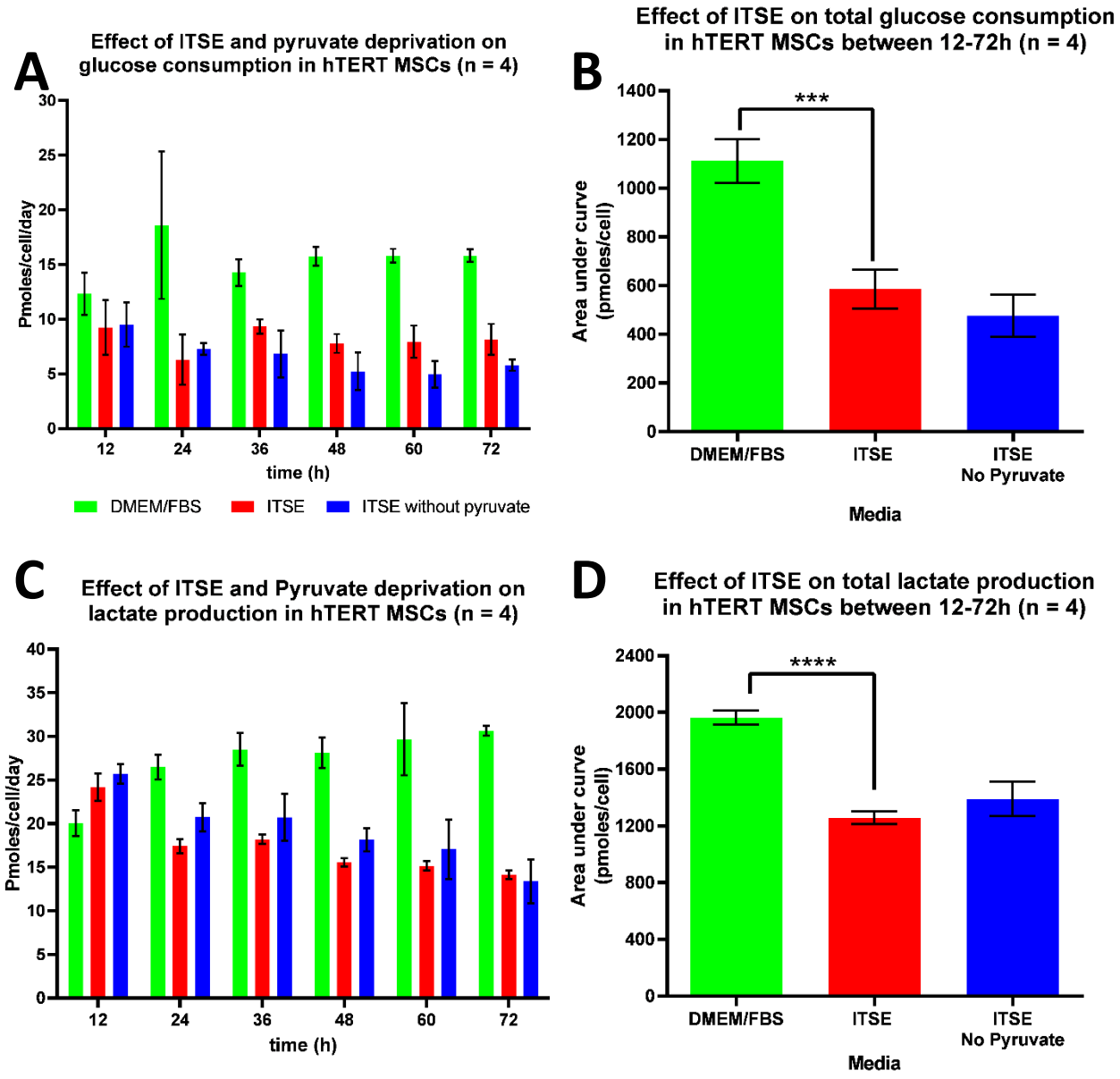

Figure 5.4: Effect of pyruvate reduction in ITSE based media on hTERT MSC metabolism: (A) Glucose consumption at 12 hour intervals. (B) Total Glucose consumption from attachment to 80% confluence was significantly lower in ITSE based medium **(Media C2)** vs DMEM/FBS **(Media E)**, but was not altered by pyruvate removal **(Media C3)**. (C) Lactate production at 12 hour intervals. Total lactate production from attachment to 80% confluence was significantly lower in ITSE based medium **(Media C2)** vs DMEM/FBS **(Media E)**, but was not altered by pyruvate removal **(Media C3)** (D). All values reported as mean ± SD, n = 4 for all groups, *** = $p \leq 0.001$, **** = $p \leq 0.0001$, all by Welch's t-test.

5.3.3 – Assessment of Cellular Damage in ITSE Based Medium

When MSCs were cultured in ITSE based media with 0.3 mM pyruvate but no N-acetyl-cysteine (NAC) **(Media C2)**, total ROS production from 2-6h was substantially and significantly higher than in DMEM/FBS **(Media E)** (AUC = 15.24 ± 1.68 in DMEM/FBS (**(Media E)** vs 198.31 ± 32.18 in ITSE based media **(Media C2)**, n = 4, p = 0.0014). There was no significant difference in ROS production in

ITSE based media when pyruvate was removed **(Media C3)** compared with ITSE based media with 0.3 mM pyruvate **(Media C2)** (AUC = 253.44 ± 58.18 in pyruvate free ITSE based media, n = 4, p = 0.16 vs ITSE based media with pyruvate). When ITSE based media **(Media C2)** was supplemented with NAC, ROS production was abolished (AUC = 19.38 ± 5.52, n = 5, p = 0.001 vs ITSE based media). Autophagosome production in DMEM/FBS and in ITSE based media was too low to accurately measure, as shown by huge error bars and lack of bright blue glowing cells (p = 0.24 when the 3 data sets were compared using the one-way ANOVA) . The data is shown in **Figure 5.5**.

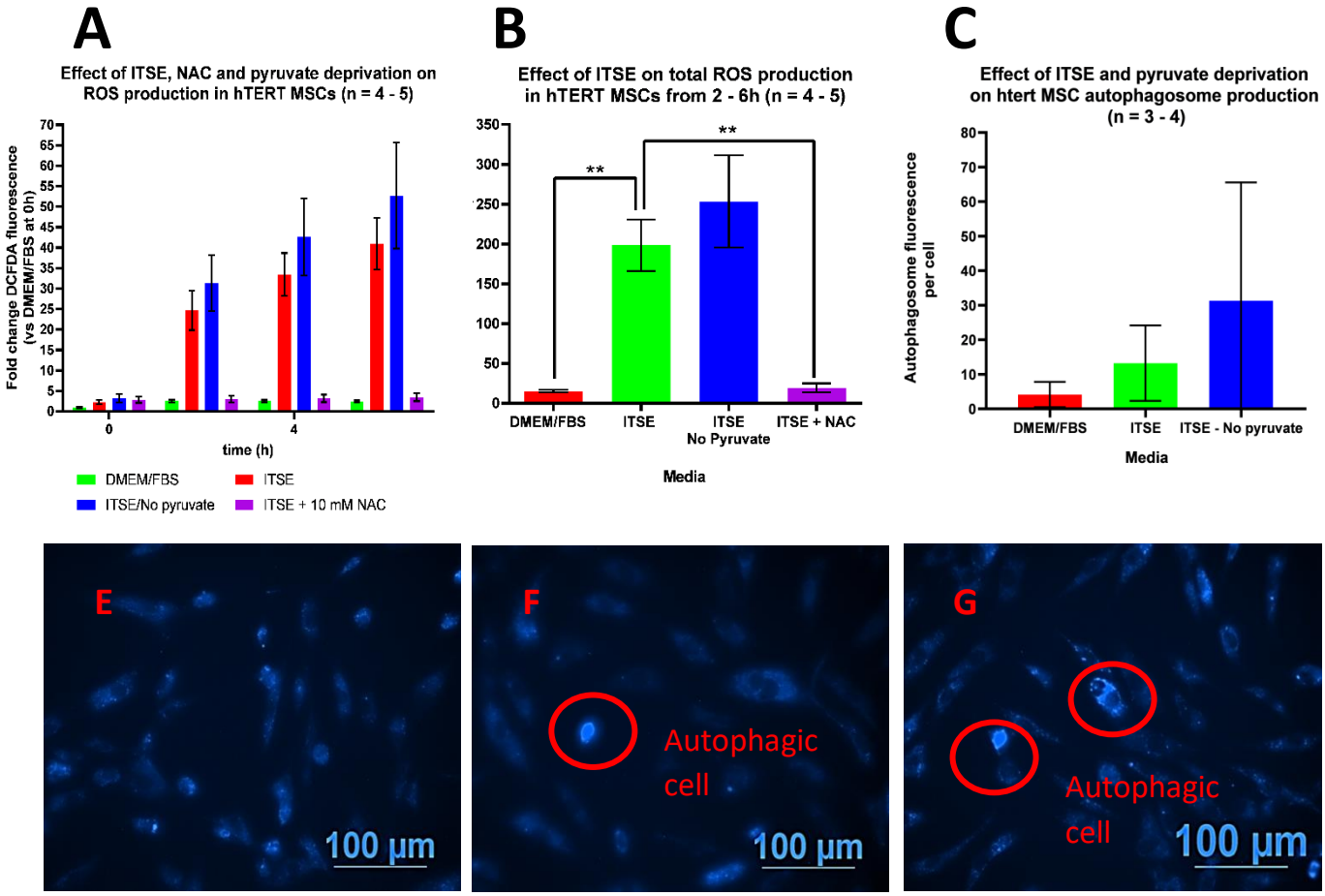

Figure 5.5: effect of ITSE based medium on cellular damage in hTERT MSCs. (A) Time course of ROS production in DMEM/FBS **(Media E)**, ITSE based media **(Media C2 and C3)** and ITSE based media + NAC, values are mean ± SD, n = 5 for cells in ITSE media + 10 mM NAC, n = 4 for all other groups. (B) Total ROS production in DMEM/FBS, ITSE based media and ITSE + NAC. ROS production was substantially and significantly higher in ITSE based media **(Media C2)** vs DMEM/FBS **(Media E)**, and is neutralised by 10 mM NAC, values are mean ± SD, n = 5 for cells in ITSE media + 10 mM NAC, n = 4 for all other groups. There were few cells undergoing autophagy in MSCs cultured in DMEM/FBS **(Media E)** (E), ITSE based media **(Media C2)** (F) and ITSE based media with no pyruvate **(Media C3)** (G) shown as few cells glowing bright blue, therefore autophagy levels for all 3 groups were too low to accurately measure (C), values are mean ± SD, n = 3 for MSCs in ITSE + No Pyruvate, n = 4 for all other groups. Performing a Brown-Forsythe ANOVA test on autophagosome fluorescence yielded no significant difference between the 3 media formulations (C). ** = $p \leq 0.01$, by Welch's t-test.

5.3.4 – Recovery of Proliferation, Metabolism and Morphology after Culture in ITSE Medium at 37^{o}C

When cultured in DMEM/FBS **(Media E)** without exposure to ITSE based medium, the doubling time of hTERT MSCs was 23 ± 0.4h, with a 2.07 ± 0.03 fold change in cell count between the 24-48h timeframe (n = 12). After 4 days culture in ITSE based medium **(Media C2)**, there was no clear difference in doubling time (22 ± 1h) when hTERT MSCs were transferred to DMEM/FBS **(Media E)** (n = 4). After 8 days exposure to ITSE based media **(Media C2)**, there was a small but statistically significant drop in proliferation when cells were transferred to DMEM/FBS **(Media E)** compared with cells that were never exposed to ITSE based media (1.84 ± 0.05 fold change, n = 4, p = 0.001 vs cells that were never exposed to ITSE based media). The drop in proliferative capacity became more profound after 12 days of culture in ITSE based medium, with PDT increasing to 36 ± 3h (1.58 ± 0.06 fold change, n = 4, p = 0.0002 vs cells that were never exposed to ITSE based media) when pyruvate concentration was at 0.3 mM **(Media C2)** and 31 ± 1h when pyruvate concentration was at 1 mM **(Media C1)** (1.72 ± 0.02 fold change, n = 4, p = 0.0002 vs cells that were never exposed to ITSE, p = 0.01 vs recovering cells after 12 days in 0.3 mM pyruvate). In the presence of ITSE based medium, the fold change in cell count over a 24 hour period was (1.06 ± 0.02, n = 4) at 0.3 mM pyruvate **(Media C2)** and (1.28 ± 0.02 fold increase, n = 8) for 1 mM pyruvate **(Media C1)**. Proliferation of MSCs in ITSE based medium was significantly faster in 1 mM compared to 0.3 mM pyruvate ($p < 0.05$). The data is shown in **Figure 5.6.**

When cultured in DMEM/FBS **(Media E)** without any exposure to ITSE based medium, the glucose consumption was 16.47 ± 0.13 pm/cell/day (n = 4). When cultured in ITSE based medium **(Media C2)**, glucose consumption dropped significantly (12.04 ± 0.94 pmoles/cell/day, n = 4, p = 0.0001 vs in DMEM/FBS) (**Figure 5.6**). When placed in DMEM/FBS after 4, 8 or 12 days culture in ITSE based medium **(Media C2)**, glucose consumption rose above pre ITSE levels (>20 pmoles/cell/day, n = 4, p = < 0.05 for all), as shown in **Figure 5.6**. In DMEM/FBS **(Media E)** without any exposure to ITSE based medium, lactate production was 34.29 ± 0.25 pmoles/cell/day. When cultured in ITSE medium **(Media C2)** lactate production dropped significantly (20.79 ± 1.34 pmoles/cell/day, n = 4, p = 0.0002). When placed back in 10% FBS after 4, 8 or 12 days culture in ITSE **(Media C2)**, lactate production rose above pre ITSE levels, as shown in **Figure 5.6**. **Figure 5.7** shows that MSCs adopted a wire morphology when cultured in ITSE, but regained their spindle morphology when placed back in DMEM/FBS **(Media E)**.

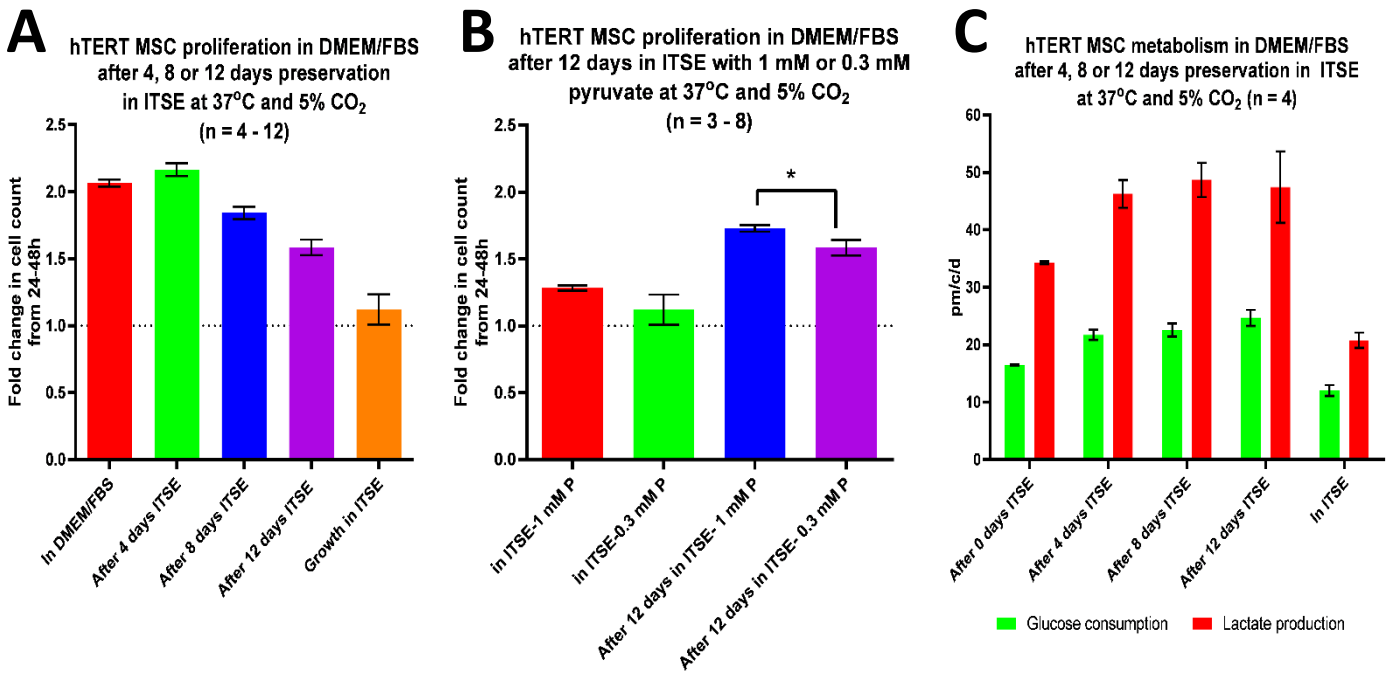

Figure 5.6: recovery of hTERT MSC proliferation and metabolism after preservation in ITSE based medium. (A) hTERT MSC proliferation in DMEM/FBS **(Media E)** over a 24 hour period after 0, 4, 8 or 12 days preservation in ITSE based media **(Media C1 or C2)** at 37°C . MSCs were able to proliferate after 12 days preservation in ITSE based media, but longer preservation times appeared to worsen recovery, values are mean ± SD, n = 12 for the DMEM/FBS group (red), n = 4 for all other groups. (B) After 12 days of preservation, MSC recovery was improved in ITSE based medium with 1 mM pyruvate as opposed to 0.3 mM pyruvate, values are mean ± SD, n = 3 for MSC recovery after 12 days in ITSE + 1 mM pyruvate (blue), n = 8 for MSC proliferation in ITSE with 1 mM pyryuvate (red), n = 4 for all other groups. (C) Specific glucose consumption and lactate production measurements for hTERT MSCs in DMEM/FBS after 0, 4, 8, or 12 days in ITSE based media, or during preservation in ITSE media. Cell metabolic activity was reduced in the presence of ITSE based medium, which was reversed upon transfer to DMEM/FBS - values are mean ± SD, n = 4 for all groups. * = $p \leq 0.05$ by way of Welch's t-test.

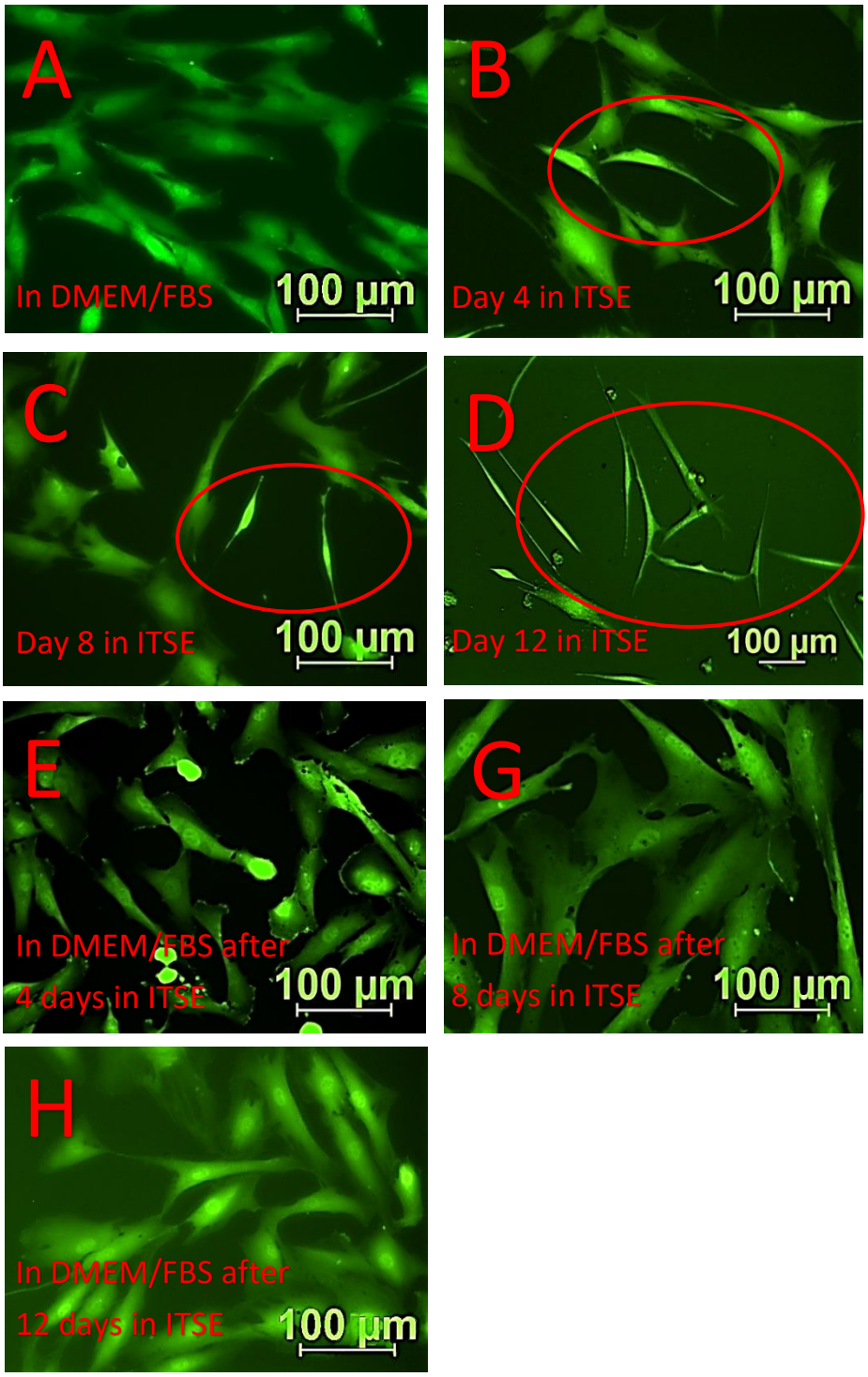

Figure 5.7: MSC morphology in ITSE based medium (Media C2) and upon recovery. MSCs without exposure to ITSE based medium cultured in DMEM/FBS **(Media E)** (A). MSCs adopted a wire like morphology after 4 days (B) and 8 days (C) in ITSE based medium, with all cells becoming wire like at day 12 (D). Regardless of time cultured in ITSE based medium, MSCs readopted their spindle shape when transferred to DMEM/FBS (E), but 8-12 days exposure caused MSCs to become large and flat upon recovery (G and H).MSCs that adopted a wire morphology have been highlighted with red circles for clarity.

5.3.5 – Effect of ITSE Based Media culture at 37^{o}C on Trilineage Differentiation Potential

Figure 5.8 demonstrates that cells tested positive for calcium, lipids and glycosaminoglycans when treated with differentiation media (**Media K1, K2 or K3 – Table 3.1**) and stained with Nile red, alizarin red or safranin O. MSCs were cultured for 12 days in ITSE based medium containing 1 mM pyruvate **(Media C1)**, as this concentration produced more even growth during preservation and improved recovery of proliferation compared to 0.3 mM. After 12 days, MSCs were transferred to 96 well plates for the study of osteogenesis and adipogenesis, or to 24 well plates for the study of chrondrogenesis. At day 7, day 14 and day 21, MSCs were stained with safranin-O, alizarin red or Nile red to measure the extent of chrondrogenesis, osteogenesis or adipogenesis respectively.

For the quantification of calcium, glycosaminoglycans and lipids, each value including baseline at day 7 was normalised as fold change vs mean for control cells in DMEM/FBS **(Media E)** at day 7. At day 14, the extent of chrondrogenesis was significantly higher for control MSCs in comparison to MSCs preserved in ITSE based medium (**Media C1**) (2.33 ± 0.17 fold change for control MSCs, 1.47 ± 0.13 fold change for ITSE preserved MSCs, n = 3-4, p = 0.0006). The extent of adipogenesis was significantly higher for preserved MSCs at day 14 in comparison to control MSCs (1.35 ± 0.21 fold change for control MSCs, 2.42 ± 0.78 for preserved MSCs, n = 6, p = 0.04). There was no significant difference in the extent of osteogenesis between control and preserved MSCs at day 14 of differentiation (9.31 ± 0.97 fold change for control MSCs vs 9.94 ± 1.49 fold change for preserved MSCs, n = 6, p = 0.4). The data is shown in **Figure 5.9.**

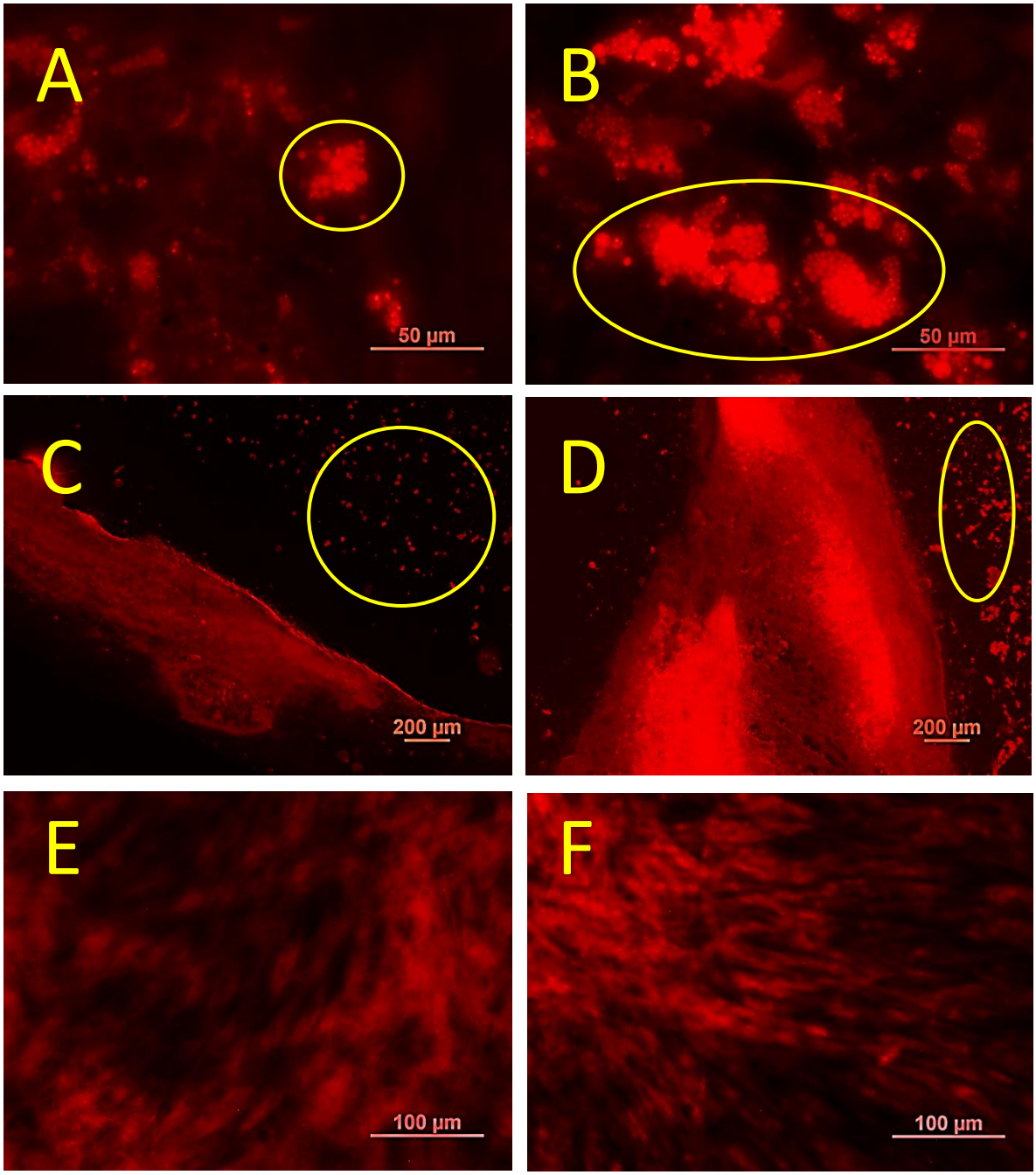

Figure 5.8: MSC differentiation at day 14. Both control MSCs and MSCs that were preserved in ITSE based medium **(Media C1)** for 12 days were stained with Nile red, alizarin red and safranin-o for adipocytes, osteocytes and chondrocytes respectively. Lipid droplets appeared upon Nile red staining of control MSCs (A) and ITSE preserved MSCs (B), while bone like formations and Ca^{2+} specks were shown for control (C) and ITSE preserved MSCs (D). Glycosaminoglycans were stained with safranin-O, showing as red blotches for both control MSCs (E) and ITSE preserved MSCs (F). Examples of lipid droplets (A, B) and calcium deposits (C,D) have been highlighted with yellow circles for greater clarity.

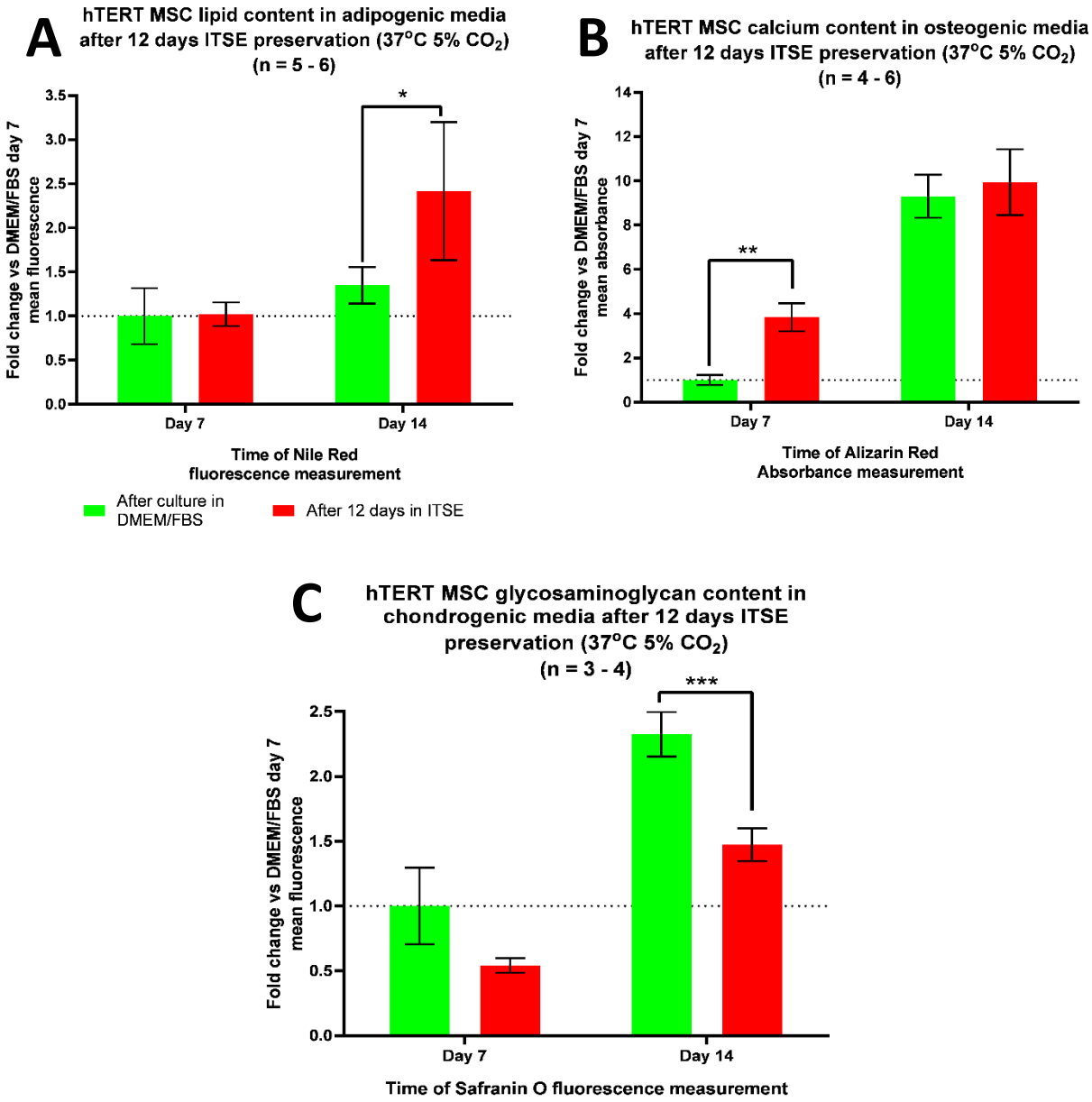

Figure 5.9: Effect of 12 days of culture in ITSE based medium on hTERT MSC differentiation potential. At day 14, recovering cells after 12 days of ITSE based media culture exhibited stronger adipogenic potential, values are mean ± SD, n = 5 for day 14 of differentiation after 12 days in ITSE media, n = 6 for all other groups (A), no difference in osteogenic potential, values are mean ± SD, n = 4 for day 7 of differentiation after 12 days in ITSE media, n = 6 for all other groups (B) and weaker chondrogenic potential compared to non-preserved cells, values are mean ± SD, n = 3 for day 7 and day 14 of differentiation after 12 days in ITSE, n = 4 for day 7 and day 14 of differentiation of non-preserved cells (C). * = $p \leq 0.05$, ** = $p \leq 0.01$, *** = $p \leq 0.001$, all by way of Welch's t-test.

5.3.6 – Amino Acid and Vitamin Requirements of hTERT MSC Monolayers Preserved in ITSE Based Media for 12 Days at 37°C and 5% CO_2

Analysis of spent media using GC-MS **(Figure 5.10)** demonstrated that hTERT MSCs consumed the following amino acids and vitamins:

- Isoeucine – 36% decrease vs control (fresh media)
- Leucine – 12% decrease vs control
- Lysine – 32% decrease vs control
- Threonine – 11% decrease vs control
- Tryptophan – 100% decrease vs control
- Valine – 8% decrease vs control
- Arginine – 37% decrease vs control
- Cysteine – 64% decrease vs control
- Aspartic acid – 37% decrease vs control
- Vitamin B5 – 55% decrease vs control
- Vitamin B8 – 24% decrease vs control
- Pyruvate – 94% decrease vs control

Other vitamins present in the medium (B1, B2, B3, B6 and B9), along with the amino acid histidine were not detected.

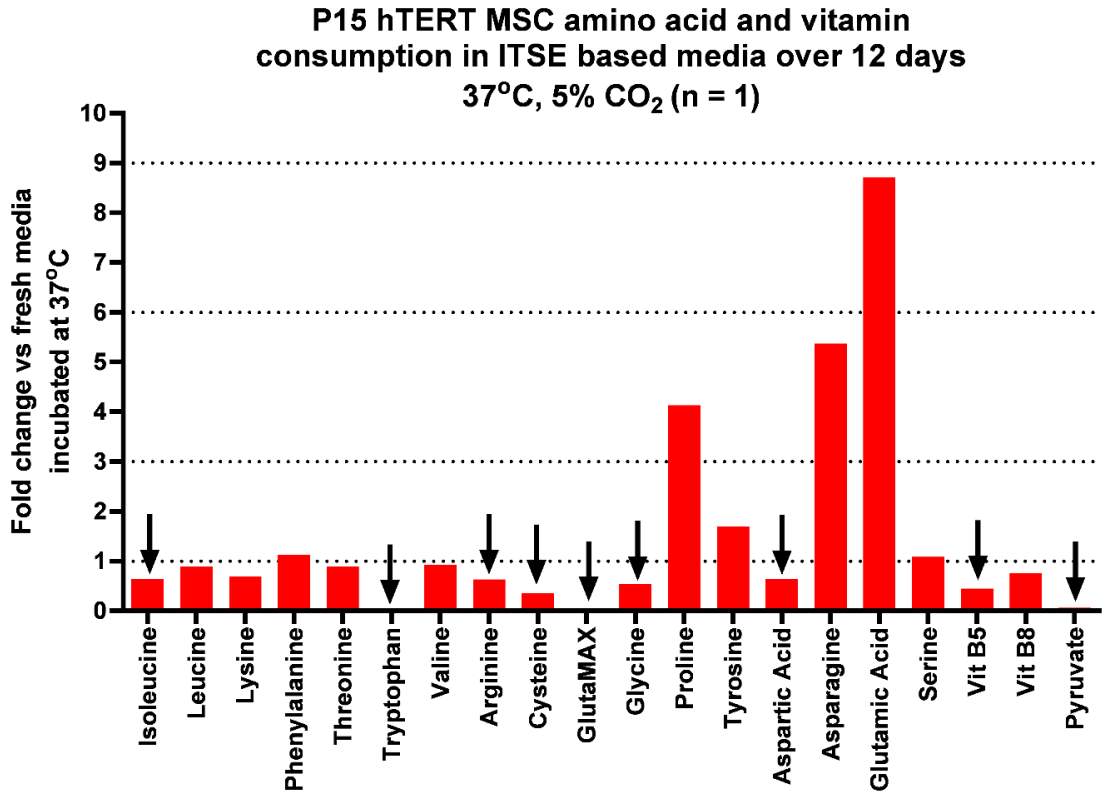

Figure 5.10: GC-MS data demonstrating hTERT MSC amino acid and vitamin consumption over a 12 day period in ITSE based media (Media B) at 37°C as monolayers. Cells produced proline, tyrosine, asparagine and glutamic acid, while no changes were seen phenylalanine and serine concentrations after 12 days. n = 1 for all groups. Decreases vs media without cells (also incubated at the same time and temperature) deemed as substantial (≥ 34% decrease vs fresh media) have been marked with arrows.

5.4 – Discussion

5.4.1 – ITSE Supplementation to DMEM Basal Medium Prevented Cell Death at 37°C over a 12 Day Period

The results demonstrated that supplementation of DMEM basal medium with ITSE prevented cell death of hTERT MSCs after day 6, and instead allowed continued cell proliferation from day 6-12 (**Figure 5.2**). This suggests that ITSE is the backbone in maintaining MSC survival in the absence of serum in DMEM. Therefore a combination of DMEM + 1% ITSE was used as the basis for further media optimisation.

5.4.2 – ITSE Media Reversibly Inhibited Cell Proliferation and Metabolism Without Loss of Proliferation or Differentiation Potential

During preservation, proliferation and metabolism must be reduced to prevent replication scenesence and metabolic waste buildup respectively. MSC proliferation was inhibited when FBS was replaced by ITSE in DMEM medium, and was partially restored by the presence of growth factors EGF, bFGF and TGF-β1 (**Figure 5.3A** and **5.3B**). Therefore, MSC growth inhibition in ITSE could be due to the absence of growth factors found in FBS. Cell growth was not fully restored, possibly due to inadequate concentrations of growth factors tested, and possible other factors such as ascorbic acid and fat soluble vitamins. Jung *et al.* (2012) reported similar results, where MSCs required bFGF and TGF-β1, along with other factors such as ascorbic acid, hydrocortisone and fetuin for expansion (133). Bhatti *et al.* (2017) reported that vitamin E protected rat MSCs against H_2O_2 induced apoptosis (170). As the aim of the project is cell preservation, other factors required for cell expansion are not needed for the finalised media.

Despite pyurvate synthesis from glycolysis, MSCs still required extracellular pyruvate, regardless of expansion in DMEM/FBS (**Figure 4.8c**) and preservation in ITSE based media (**Figure 5.10**). Proliferation over a 72 hour period was significantly reduced in ITSE based media with no pyruvate vs ITSE based media with 1 mM pyruvate (**Figure 5.3C** and **5.3D**), but no reduction in proliferation occurred in ITSE based media with 0.3 mM pyruvate. This demonstrated that extracellular pyruvate may be required for cell proliferation. No prior studies involving the effect of media pyruvate on MSCs have been conducted in the literature. Babich *et al.* (2009) reported that pyruvate protects HF-1 Fibroblasts against polyphenol induced cell death and ROS production (171).

Within the ranges of 0-5 mM pyruvate, Nath have demonstrated a concentration dependent decrease in calf thymus DNA degradation by H_2O_2 (172). Therefore pyruvate concentration should not be altered in the ITSE based media from its default concentration of 1 mM, as it may be needed to protect cells from oxidative stress.

Replacement of FBS with ITSE in DMEM lead to a reduction in glucose consumption and lactate production (**Figure 5.4A-D**). This could be a consequence of reduced growth and therefore reduced protein synthesis, leading to a reduction in the ATP requirement by MSCs. There was no clear trend in glucose consumption or lactate production for cells in ITSE based media or in DMEM/FBS when measured at 12 hour intervals for 72 hours (**Figure 5.4A** and **5.4C**), and removing pyruvate caused no further change in metabolic activity (**Figure 5.4B** and **5.4D**).

MSCs retained their proliferation potential (**Figure 5.6A-B**), differentiation potential (**Figure 5.8** and **Figure 5.9**) and regained their metabolic activity (**Figure 5.6C**) when placed in DMEM/FBS **(Media E)** after 12 days of ITSE based media exposure. However, recovery of proliferation worsened with increasing ITSE exposure times (**Figure 5.6A**), and MSCs in ITSE based media exhibited enhanced adipogenic potential (**Figure 5.9A**) and reduced chondrogenic potential when transferred to DMEM/FBS **(Media E)** after 12 days (**Figure 5.9C**). It is important to find out whether these changes were due to lack of serum growth factors, albumin or fat soluble vitamins normally present in serum. (**Figure 5.7**) shows that MSCs adopted a wire like morphology when cultured in ITSE based medium **(Media C2)**, and readopted their spindle morphology when transferred to DMEM/FBS **(Media E)**.

During preservation, cells must survive and remain functional, where proliferation and differentiation is maintained after preservation. As MSCs exert their therapeutic effect via the release of growth factors and anti-inflammatory mediators (See **Table 1.1**), complete restoration of proliferation and differentiation potential is not required, only partial. Since MSCs in ITSE based medium for 12 days did not detach, while maintaining growth the trilineage differentiation after preservation, no further media optimisations to maintain cell function are needed. When human adipose derived MSCs were encapsulated in alginate at 15°C for 3 days, complete restoration of proliferation and trilineage differentiation potential and cells maintained a spindle morphology during recovery (44), in agreement with other authors who preserved MSCs for 7 days in spheroids (48), 7 days in wisconsin solution (40) or 3 days in trehalose (173). Differences in post preservation recovery between this study and the published literature could be attributed to longer times used

(12 days for this project vs ≤7 days in the literature) or preservation methods used. Overall, the results show that MSCs maintain some degree of proliferation and differentiation potential after exposure to ITSE based media, making it a suitable candidate to adapt to work at atmospheric conditions.

5.4.3 – hTERT MSCs still Required Non-essential Amino Acids, Pyruvate and B Vitamins during Preservation in ITSE Based Media (Media B) at 37°C as Monolayers

GC-MS analysis of spent media after 12 days of preservation in ITSE based media (**Media B**) demonstrated that hTERT MSCs still required non-essential amino acids such as Arginine, Cysteine, Glycine and Aspartic Acid (**Figure 5.10**). Cells still proliferated in ITSE based media (**Media B**), and thus may have required amino acids as building blocks for proteins. hTERT MSCs at 37°C rather than primary cells or room temperature experiments were selected for GC-MS to guarantee that metabolite changes were large enough to accurately measure. In the published literature, amino acid and vitamin consumption for MSCs during preservation has not been studied. Eagle *et al.* (1958) reported that monkey kidney cells were unable to proliferate without non-essential amino acids (NEAA), which may suggest that NEAA synthesis is lost during *in vitro* culture (112).

Tryptophan and glutaMAX were depleted during preservation, though the time when this occurred is unclear. As present it is not clear whether tryptophan and glutaMAX depletion resulted in the wire like morphological changes shown in **Figure 5.7D** due to the inability of the cell to make the necessary proteins**.** GlutaMAX instead of glutamine had been used for preservation, as it does not degrade to form ammonia at warm temperatures (174). However, glutamine and alanine changes cannot be accurately assessed, as any glutamine/alanine consumption by MSCs would be masked by increases in media glutamine/alanine from GlutaMAX breakdown. MSCs produce Indolamine 2,3-dioxygenase (IDO), which converts tryptophan into kynurenine, where the tryptophan depletion inhibits T-cell proliferation (175). When the finalised preservation media has been developed, it is inportant to ensure that tryptophan is not depleted during preservation, though this is unlikely at room temperature where metabolism is expected to be much slower.

Vitamin B5 is required for the synthesis of Coenzyme A for cell metabolism (176). As cells consumed glucose and produced lactate in ITSE based media (**Media B**) (See **Figure 5.6C**) it was expected that Vitamin B5 consumption occurred. Cells produced proline, tyrosine, asparagine and glutamic acid, and did not utilise phenylalanine or serine, which suggests that they are not required

in the finalised preservation medium. The boiling points of some vitamins (B1, B2, B3, B6 and B9) may have been too high, and therefore cannot be quantified by GC-MS. Histidine could not be detected either, as their retention times were similar to glucose derivatives in the spent media sample. Metabolomics on preserved MSCs in ITSE based medium have not been carried out in the published literature. Despite this, a comprehensive view of MSC metabolite requirements in ITSE based media has been determined by GC-MS in this project. This in turn allows the correct formulation of vitamins and amino acids in the finalised ITSE based media for clinical use.

Media components that were depleted to a large extent, such as tryptophan, cysteine, GlutaMAX, vitamin B5 and pyruvate may need to be supplemented for the finalised version of the ITSE media, while phenylalanine, proline, tyrosine, asparagine and glutamic acid can be removed from the final media formulation.

5.4.4 – Higher Levels of ROS Production for cells in ITSE Based Medium did not Increase the amount of Damaged Cellular Components

Cells cultured in ITSE based media exhibited higher levels of oxidative stress compared to those cultured in DMEM/FBS **(Media E)** (**Figure 5.5A-B**), but no clear difference in autophagy (**Figure 5.5C**). The lack of bright blue cells after autophagosome staining demonstrated that the majority of cells in DMEM/FBS **(Media E)** and in ITSE based media **(Media C2 and C3)** do not have damaged cell components, thus autophagy levels in all groups are too low to measure (**Figure 5.5E-G**). This shows that higher ROS production for MSCs cultured in ITSE based media was not sufficient to cause cellular damage. In the literature, MSC cell death occur at H_2O_2 concentrations of 250 µM or higher (177), though the total ROS concentration cannot be determined by DCFDA. Perhaps ROS production in ITSE based media did not exceed 250 µM since cell death did not occur over a 12 day period (**Figure 5.2**). Pyruvate exclusion had no clear effect on oxidative stress or autophagy (**Figure 5.5A-C**), while neutralisation of ROS was achieved by 10 mM NAC **(Figure 5.5A and B).** The NAC result confirmed that fluoresence increases were due to ROS. Tauffenberger have demonstrated with SH-SY5Y cells that has 6 hours pretreatment with 20 mM pyruvate or 20 mM lactate, or culture in 20 mM pyruvate, significantly reduced cell death after exposure to 150 µM H_2O_2 (178). As MSCs retained survival, proliferation and differentiation potential in ITSE based media despite elevated ROS, media pyruvate does not need to be modified and ROS inhibitor NAC does not need to be added.

5.4.5 – Although Pyruvate Reduction Halted Cell Proliferation more Effectively, Recovery was Optimal in ITSE Based Media with 1 mM Pyruvate

Figure 5.6A and **5.6B** demonstrated that pyruvate reduction from 1 mM to 0.3 mM in ITSE based media lead to worse recovery of MSC proliferation upon transfer to DMEM/FBS. This was unlikely to be a a reduction of pyruvate mediated ROS production, as the results demonstrated that pyruvate removal did not alter ROS production in ITSE based media (**Figure 5.5**). In triple negative breast cancer cells, supplementation of media with pyruvate has been shown to stabilise hypoxia inductible factor 1α (HIF-1α) in a dose dependent manner (179), causing the cells to act as if they were in hypoxic conditions. As MSCs favour hypoxic conditions, this may explain why proliferation recovery was greater in ITSE based media with 1 mM pyruvate vs 0.3 mM. This further reinforces the need to keep the default concentration of 1 mM pyruvate in the preservation media.

5.5 – Conclusion

The data showed that cell growth and metabolism can be slowed for preservation by making simple media modifications, without permanent loss of proliferation, morphology and differentiation potential upon recovery. This was achieved by replacement of FBS with ITSE. Although cells in ITSE based media exhibit more oxidative stress, the extent of cellular damage was not altered and cells regained their proliferation and differentiation potential when transferred to expansion media. This shows that ITSE is a suitable alternative to maintain cell survival in the absence of serum, and therefore ITSE based media serves as a suitable candidate for adaptation of cell preservation at atmospheric conditions.

Chapter 6

Atmospheric Preservation of hTERT and Primary MSCs in ITSE Medium and Alginate Beads

6.1 - Introduction

Cell culture is normally carried out at 37^{o}C and 5% CO_2 to maximise proliferation and maintain physiological pH levels respectively. DMEM culture media is supplemented with 44 mm $NaHCO_3$ to maintain the correct pH levels at 5% CO_2. At atmospheric CO_2 levels (0.04%), the media would turn alkaline if 44 mm $NaHCO_3$ is used. Maintaining a refrigerator, incubator or gas cylinders during cell storage would be expensive and inconvenient, so it is important to adapt the preservation medium keep cells viable at atmospheric conditions (20^{o}C and 0.04% CO_2), if possible. Various phosphate, MOPS and HEPES based buffer formulations will be tested to determine if cell attachment and elongation occurs at atmospheric conditions prior to preservation studies.

Previous authors have not tested MSC preservation times beyond 7 days; for those who encapsulated MSCs in alginate, the longest time ever tested was 5 days. Once the optimal buffer was found, both hTERT and primary bovine MSCs were preserved in ITSE based media + novel buffer in monolayers for a total of 12 days at atmospheric conditions, measuring cell survival and pH at regular intervals. Glucose consumption and lactate production over the 12 day period, along with cell recovery after preservation were also be studied.

Up to billions of MSCs may be needed for therapy, so the preservation of hundreds of thousands of cells/mL of media may be needed. MSC survival has been compared in suspension and in alginate beads to determine whether alginate encapsulation is required to keep cells viable and functional. This chapter has determined whether the cause of cell death at atmospheric conditions during 3D preservation was due to pH acidity from lactate accumulation, lack of nutrient access for core cells in the 3D structure, or lack of serum components.

Previous authors have not studied specific glucose consumption and lactate production per cell during room temperature preservation, this has been addressed by culturing both primary and hTERT cells for 6 days in 3D at different densities, and harvesting the media by day 6 for glucose and lactate production measurements.

The data produced has determined whether a preservation media consisting of a DMEM base in addition to an insulin supplement (ITSE based media) can be used to keep MSCs for long periods of time in 3D at atmospheric conditions. It has revealed the effect of density and bead size on cell survival, and also cell glucose requirements. This will ultimately allow the estimation of the optimal preservation density.

6.2 – Experimental Plan

The experimental techniques used are mentioned in **Chapter 3: Materials and Methods.** In summary, hTERT human MSCs were cultured in 2D in a range of buffer formulations in ITSE based media, where the buffer was considered as successful if cell elongation occurred at day 2 and all cells remained attached (**Section 3.4.1**). Both hTERT (17,500 cells/cm^2) and primary bovine MSCs (25,000 cells/cm^2) were preserved as monolayers in ITSE based medium with 1 mm glucose and 0.9 mm bicarbonate, to study metabolism, survival, morphology and recovery after 12 days at atmospheric conditions (**Section 3.4.2.1**). Preservation experiments were repeated for hTERT MSCs for 12 days in suspension above agarose (**Section 3.4.3.2**) and in 4 mm alginate beads (**Section 3.4.3.4**). As the survival was higher in the alginate group, this was repeated for primary P3 bovine MSCs. Since cells in 4 mm beads at a density of 750k/0.5 mL of alginate (49k cells/bead) did not remain functional for more than 4 days, experiments were repeated with smaller handmade beads, ranging from 2-2.7 mm and reducing the cell density to 600k cells/0.5 mL alginate (12k cells/bead) (**Section 3.4.3.5**). For the 4 mm bead at 750k cells/0.5 mL alginate, 400 µL alginate was added to 1.5 mL ITSE per well (400k cells per mL of media). For the 2-2.7 mm beads at 600k cells/0.5 mL alginate, 300 µL alginate was added to 1 mL ITSE per well (360k cells per mL of media). To assess recovery, cells were released from alginate (**Section 3.4.3.6**) and cultured in DMEM/FBS, where proliferation (**Section 3.4.3.6**) and trilineage differentiation potential (**Section 3.1.8**) were assessed. To assess glucose consumption and lactate production, cells were seeded in 4 mm alginate beads at 60k and 15k cells/well and the pH, survival, glucose consumption and lactate production was measured at day 6 (**Section 3.4.3.8**). The experimental plan is illustrated using a flow chart in **Figure 6.1.**

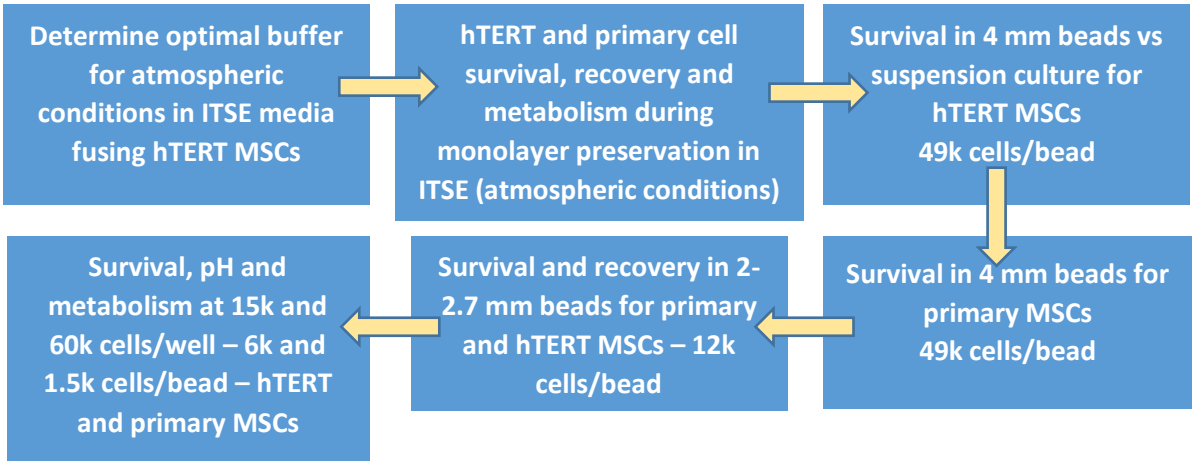

Figure 6.1: Experimental outline for the preservation of MSCs in ITSE media at atmospheric conditions (20°C and 0.04% CO_2) as 2D monolayers and 3D structures.

All media, unless stated otherwise, contained 1 mM pyruvate. The media formulations used this chapter are listed in Table 6.1.

Table 6.1: Media formulations used for preservation at atmospheric conditions

Media Formulation	Experiment
DMEM, 2.5 mM glucose, 10% FBS, 44 mM bicarbonate **(Media A)**	Study MSC morphology under normal culture conditions
DMEM, 5 mM glucose, 10% FBS, 44 mM bicarbonate **(Media B)**	Assess recovery of MSC proliferation after alginate preservation
DMEM, 1% ITSE, 0.9 mM bicarbonate, 5 mM **(Media C1)**, 2.5 mM **(Media C2)** or 1 mM **(Media C3)** of glucose	Media C1 - cell preservation for 12 days in suspension or in alginate beads at atmospheric conditions Media C2 – Preservation of cells in monolayer in flasks for 12 days to assess pH and recovery Media C3 – Metabolism for cells preserved in monolayer and in alginate beads at atmospheric conditions, viability for monolayer preservation at atmospheric conditions

6.3 – Results and Analysis

6.3.1 – Determine the Optimal pH Buffer for MSCs under Atmospheric Conditions

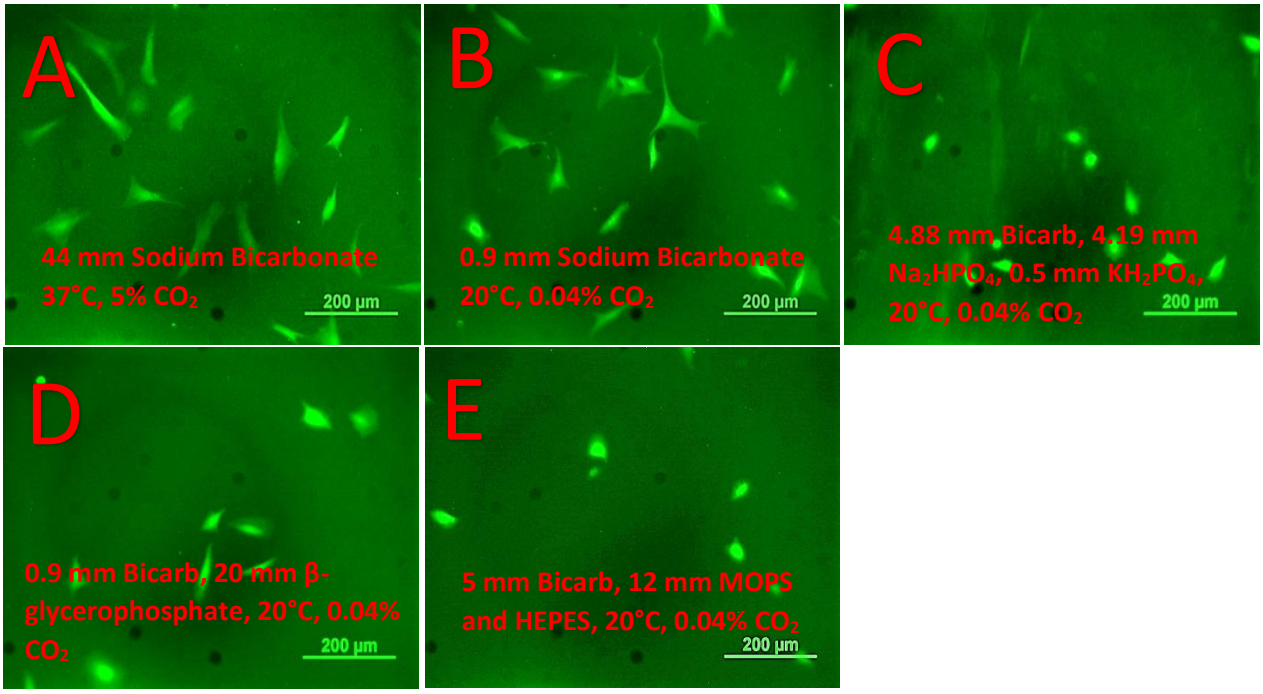

Figure 6.2: Effect of buffers to replace 44 mM bicarbonate in ITSE based medium (Media A) on MSC attachment and elongation at day 2. Under standard culture conditions (37°C, 5% CO_2) in 44 mm bicarbonate (A) and at atmospheric conditions (20°C, 0.04% CO_2) in 0.9 mm bicarbonate (B), P15 hTERT human MSCs attached and fully elongated at day 2. MSCs failed to elongate when ITSE medium was buffered with HEPES/MOPS (E) or with Na_2HPO_4/K_2HPO_4 (C). Partial elongation occurred in glycerophosphate (D).

6.3.2 – Monolayer Preservation of MSCs under Atmospheric Conditions in ITSE Based Media

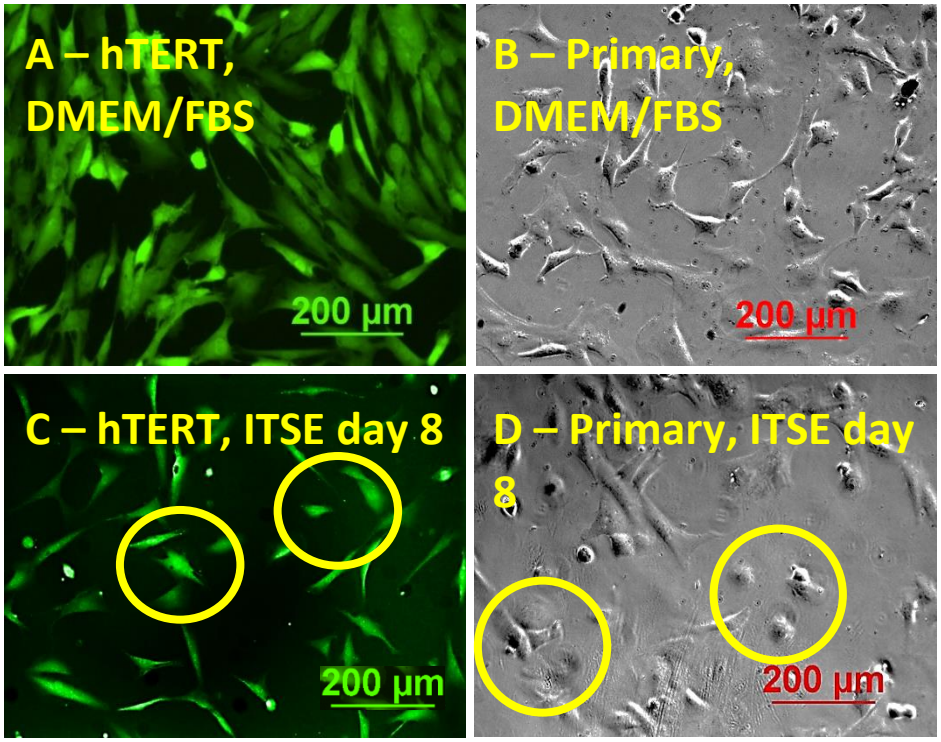

Figure 6.3: Effect of monolayer preservation at atmospheric conditions on hTERT and primary MSC morphology. When expanded in DMEM/FBS **(Media A)** at 37°C and 5% CO2, both hTERT (A) and primary (B) MSCs adopted a spindle morphology. Monolayer preservation of hTERT (C) or primary (D) MSCs in ITSE based media **(Media C2)** caused them to adopt a smaller irregular morphology, with some cells failing to elongate (yellow circles).

In flasks **(Media C2)**, media pH remained within acceptable levels (7-7.5, n = 1) during the 12 days of atmospheric preservation in ITSE based media (7.0 at day 4, 7.16 at day 8 and 7.33 at day 12 for hTERT MSCs. For primary MSCs: 7.11 at day 4, 7.01 at day 8 and 7.44 at day 12 (**Figure 6.4B**). At day 12, there was no significant difference in survival between hTERT and primary MSCs in ITSE based medium **(Media C3)** (71.85 ± 1.82 % survival for hTERT MSCs, 68.74 ± 2.24 % for primary MSCs, n = 4, p = 0.08). Immortal cells experienced a gradual decline in cell survival from day 2-12, while primary MSCs experienced a sharp decline in cell survival up to day 6, with no clear change thereafter (**Figure 6.4A**). When hTERT MSCs were transferred from ITSE based medium to DMEM/FBS **(Media B)** to assess recovery, MSC death occurred in the opening 72 hours, before lagging during the 72-96h timeframe. Exponential growth occurred after 96h (**Figure 6.4C**). Primary MSCs remained anchored to the flask upon trypsinisation. Recovering hTERT MSCs displayed inconsistent morphologies, with a mixture of large and flat cells, while others failed to elongate (**Figure 6.5B**). Over the 12 day period in ITSE based medium in 24 well plates **(Media C3)** at room temperature, the average glucose consumption and lactate production for hTERT MSCs was 0.36 ± 0.02 pmoles/cell/day and 0.75 ± 0.01 pmoles/cell/day respectively, n = 4. For primary MSCs, the

average glucose consumption and lactate production was 0.10 ± 0.02 pmoles/cell/day and 0.46 ± 0.02 pmoles/cell/day respectively, n = 4. The data is shown in **Figure 6.4D**.

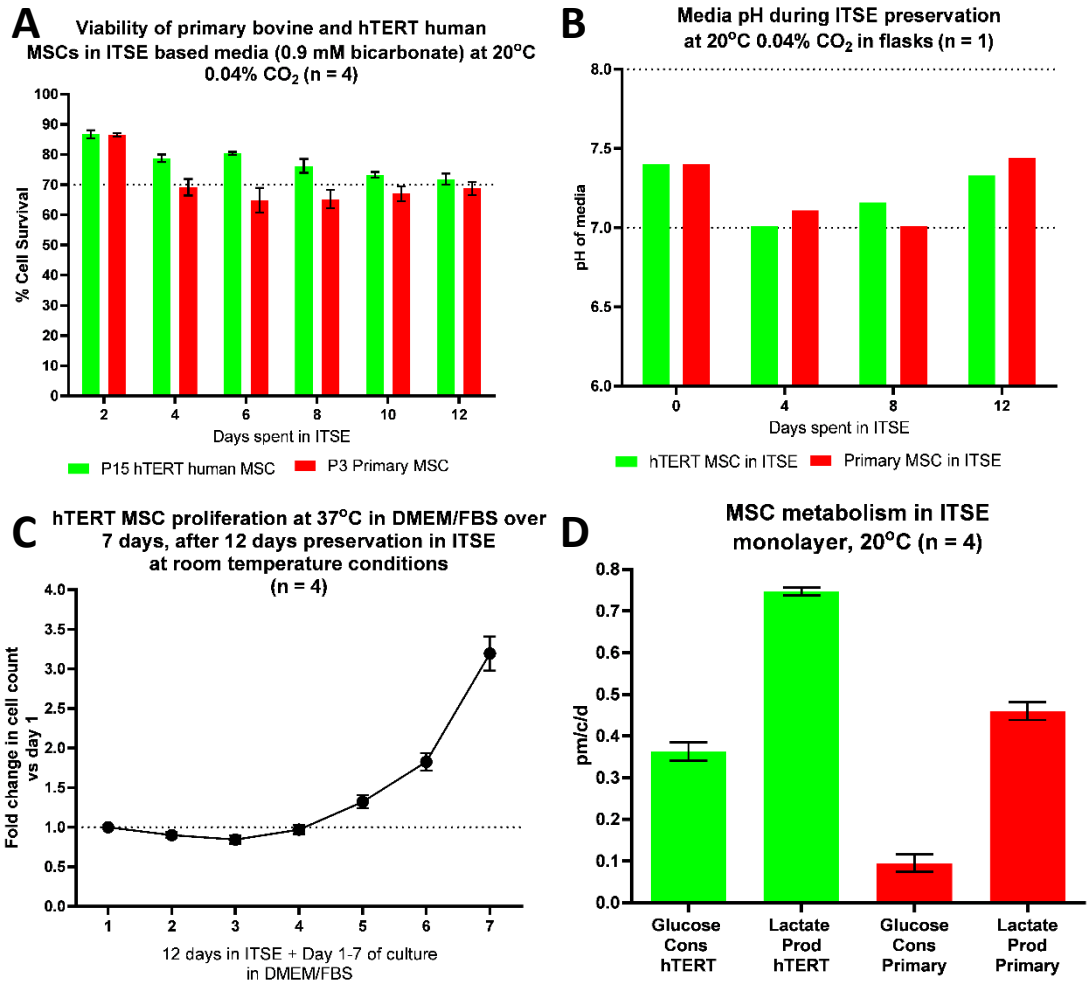

Figure 6.4: Survival, metabolism, recovery and morphology of hTERT and primary MSCs when preserved in ITSE based media as monolayers at atmospheric conditions. (A) Both hTERT and primary MSCs reached a survival rate of approx. 70% at day 12 in ITSE based media. (B) When cultured in T75 flasks with 10 mL of ITSE based media, pH remained within physiological levels (7-8). (C) hTERT MSCS retained proliferation potential after 12 days in ITSE based media preservation at room temperature. (D) Glucose consumption and lactate production of MSCs in ITSE based media. All values shown as mean ± SD, n = 1 for media pH measurements, n = 4 for all other groups.

Recovering hTERT MSCs displayed inconsistent morphologies, with a mixture of large and flat cells, while others failed to elongate. As shown in **Figure 6.5**.

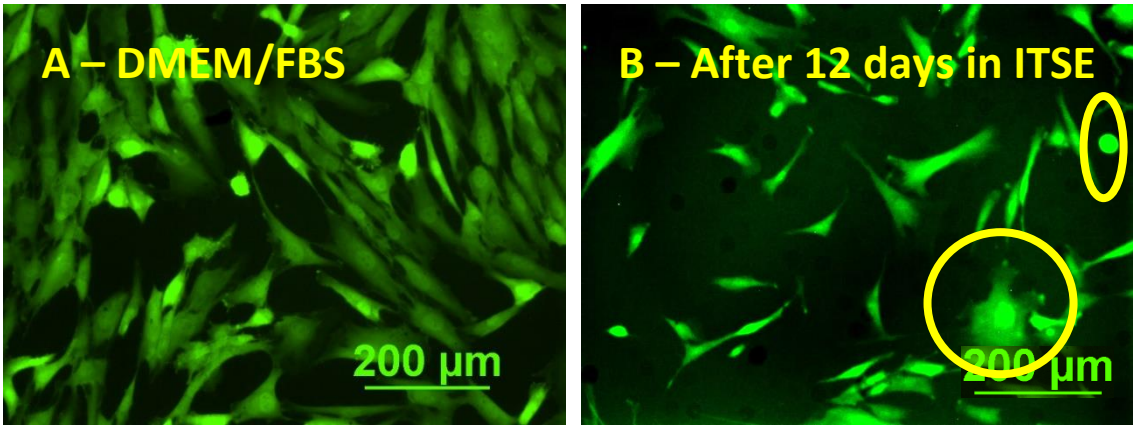

Figure 6.5: Morphology of hTERT MSCs during recovery in DMEM/FBS (Media B) after 12 days of preservation in ITSE media at atmospheric conditions. Non-preserved MSCs in DMEM/FBS **(Media A)** and incubator conditions have a uniform spindle morphology (A). After 12 days preservation as a monolayer in ITSE based media **(Media C2)** at atmospheric conditions followed by 7 days of culture in DMEM/FBS **(Media B)**, cells develop an irregular and varied morphology (B).

6.3.3 – Preservation of MSCs in 4 mm Alginate Beads at High Density (49k cells/bead)

For hTERT MSCs in ITSE based medium **(Media C1)**, the percentage of viable cells was significantly higher at day 12 when encapsulated with 4 mm beads compared to suspension cells (**Figure 6.8A**); 56.43 ± 8.44 % survival rate for the alginate group vs 14.09 ± 12.46% for the agarose group, n = 4, p = 0.002). Survival rates were significantly lower at day 12 for primary MSCs compared to hTERT MSCs when encapsulated in 4 mm beads at day 12, 56.43 ± 8.44 % survival rate for hTERT MSCs, 34.92 ± 5.52 % survival rate for primary MSCs, n = 4, p = 0.007) (**Figure 6.8B**). When preserved for 8 days or longer in 4 mm beads, both hTERT and primary MSCs failed to proliferate within a 96 hour timeframe when released and cultured in DMEM/FBS **(Media B)** (1.01 ± 0.10 fold increase for hTERT MSCs vs 1.14 ± 0.14 fold increase for primary MSCs). After 4 days preservation in 4 mm beads, hTERT MSCs proliferated more rapidly compared to primary MSCs (3.09 ± 0.19 fold increase for hTERT MSCs at 96h of recovery vs 1.83 ± 0.19 fold increase for primary MSCs, n = 4, $p < 0.05$) – this is shown in **Figure 6.8C**. **Figure 6.8D** shows that syringe pump generated beads have been perfectly round and consistent in size (4 mm). After 4 days of preservation in 4 mm alginate beads, hTERT MSCs displayed a flat and large morphology during recovery, while the morphology of primary MSCs could not be determined, as shown in **Figure 6.6.** When a beads containing primary MSCs were taken after day 12 and stained with propidium iodide, a substantial number of late apoptotic cells were observed as fluorescent red spots (**Figure 6.7**).

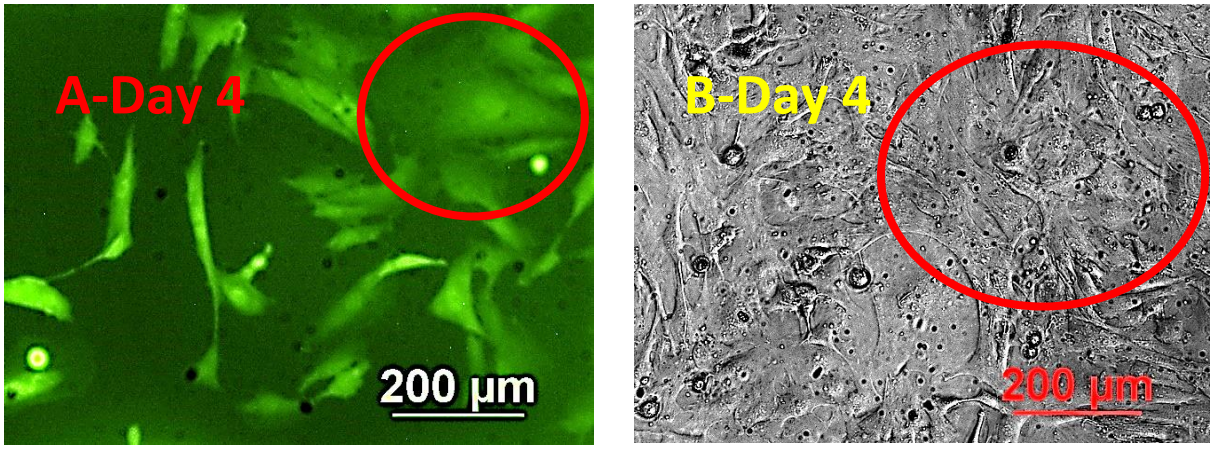

Figure 6.6: hTERT and primary MSC morphology at day 4 after 4 days of alginate preservation in 4 mm beads in ITSE based media (Media C1). At day 4 of recovery in DMEM/FBS **(Media B)**, hTERT MSCs adopted a flat morphology (A), while the cell membranes of primary MSCs could not be clearly seen due to morphological alterations and will ultimately require CellBrite® membrane staining (B).

For hTERT human MSCs, the percentage of live cells attached in DMEM/FBS **(Media B)** after preservation in 4 mm alginate beads and ITSE based media were 68.23 ± 3.68% after 4 days, 44.55 ± 1.89% after 8 days and 13.70 ± 2.85% at day 12. For primary bovine MSCs, cell attachment rates in DMEM/FBS **(Media B)** after preservation in alginate beads was 78.55 ± 4.16% after 4 days and 38.95 ± 1.45 % after 8 days. Since bovine cells did not proliferate after 8 days in 4 mm beads, recovery was not studied beyond this point.

6.3.4 – Preservation of P3-4 Primary Bovine MSCs in 2-2.7 mm Alginate Beads at High Density (12k cells/bead)

The percentage of viable cells in ITSE based media **(Media C1)** was significantly higher at day 8 when encapsulated with 2- 2.7 mm beads (12k cells/bead) compared to 4 mm beads (49k cells/bead) (**Figure 6.9A**); 83.52 ± 10.63 % survival rate, n = 6 for the 2-2.7 mm vs 40.18 ± 6.18 %, n = 4 for 4 mm group, p = <0.0001). However, 2-2.7 mm beads failed to keep survival rates above the target (70%) at day 12. When preserved for 12 days in ITSE based media **(Media C1)** supplemented with 10% FBS, cell survival rates were significantly greater compared to cells preserved in serum free ITSE based media **(Media C1)** (84.39 ± 2.48 % for ITSE + 10% FBS vs 42.24 ± 6.91 % for serum free ITSE, n = 3, p = 0.004) (**Figure 6.9B**).

After 4 days preservation in ITSE based media, primary bovine MSCs proliferated more rapidly in the 2-2.7 mm bead group compared to primary MSCs in the 4 mm bead group upon transfer to DMEM/FBS **(Media B)** (3.82 ± 0.34 fold increase at 96h for 2-2.7 mm beads vs 1.83 ± 0.19 fold increase for 4 mm beads, n = 4, p < 0.05) – this is shown in (**Figure 6.9C – yellow and red**). Rapid

exponential growth for primary MSCs was still observed after 8 days preservation in 2.7 mm beads (3.44 ± 0.28 fold change at day 4, n = 8) (**Figure 6.9C – purple**). After 12 days of preservation, proliferation rate of recovering cells was significantly faster for the ITSE/FBS group compared to ITSE/serum free group (3.39 ± 0.24 fold increase at day 4 for the ITSE/FBS group vs 1.90 ± 0.15 fold increase for the serum free ITSE group, n = 4, p = 0.0001) (**Figure 6.9C – green and orange**). After 4 or 8 days of preservation of serum free ITSE based media, some recovering cells adopt a flat morphology, while others adopt the spindle morphology (**Figure 6.10A and 6.10B**). After 12 days preservation in serum free ITSE based media, some cells fail to elongate (**Figure 6.10C**), while after 12 days in ITSE/FBS, all cells elongate but develop a flat morphology (**Figure 6.10D**).

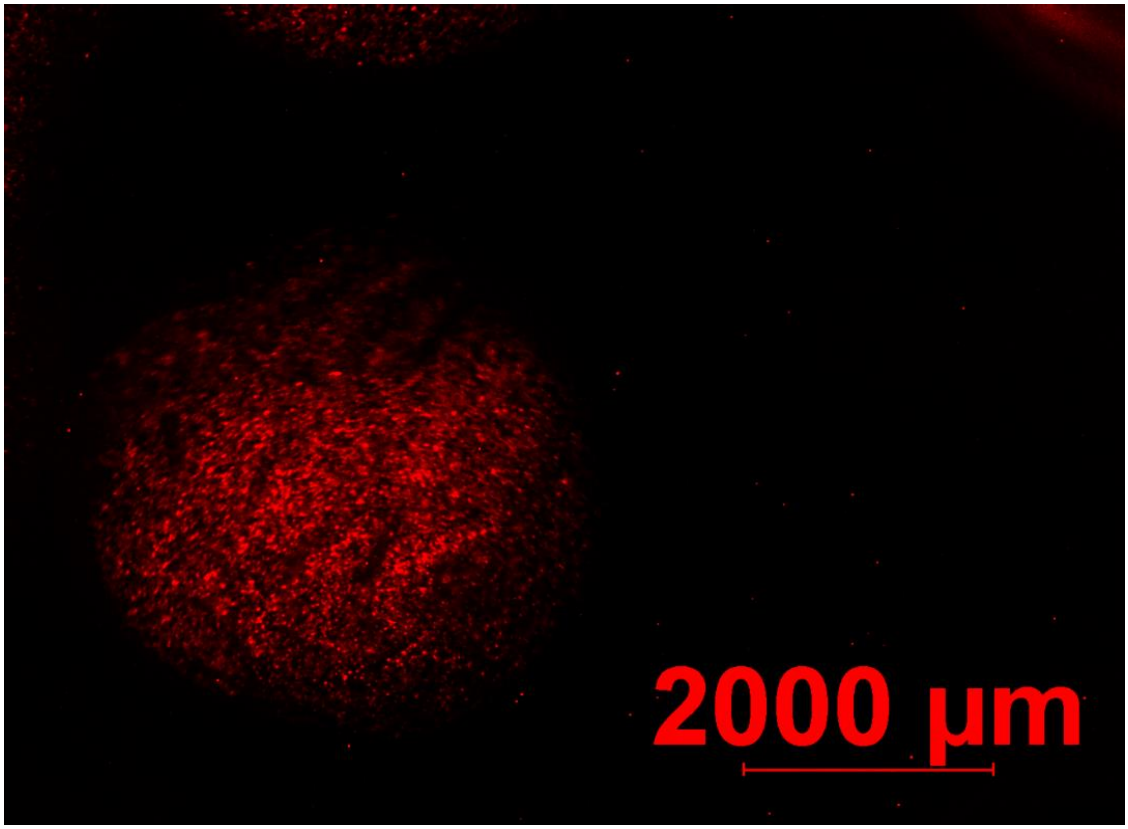

Figure 6.7: Propidium iodide staining of P3 primary bovine MSCs in a 4 mm alginate bead after day 12. The location of late apoptotic cells are shown as fluorescent red spots in the alginate bead, where propidium iodide has penetrated the cell membrane and bound to DNA. Additional staining with acridine orange and Annexin V is required to determine the location of live and early apoptotic cells respectively, along with a superimposed phase contrast counterpart to show the edge of the bead.

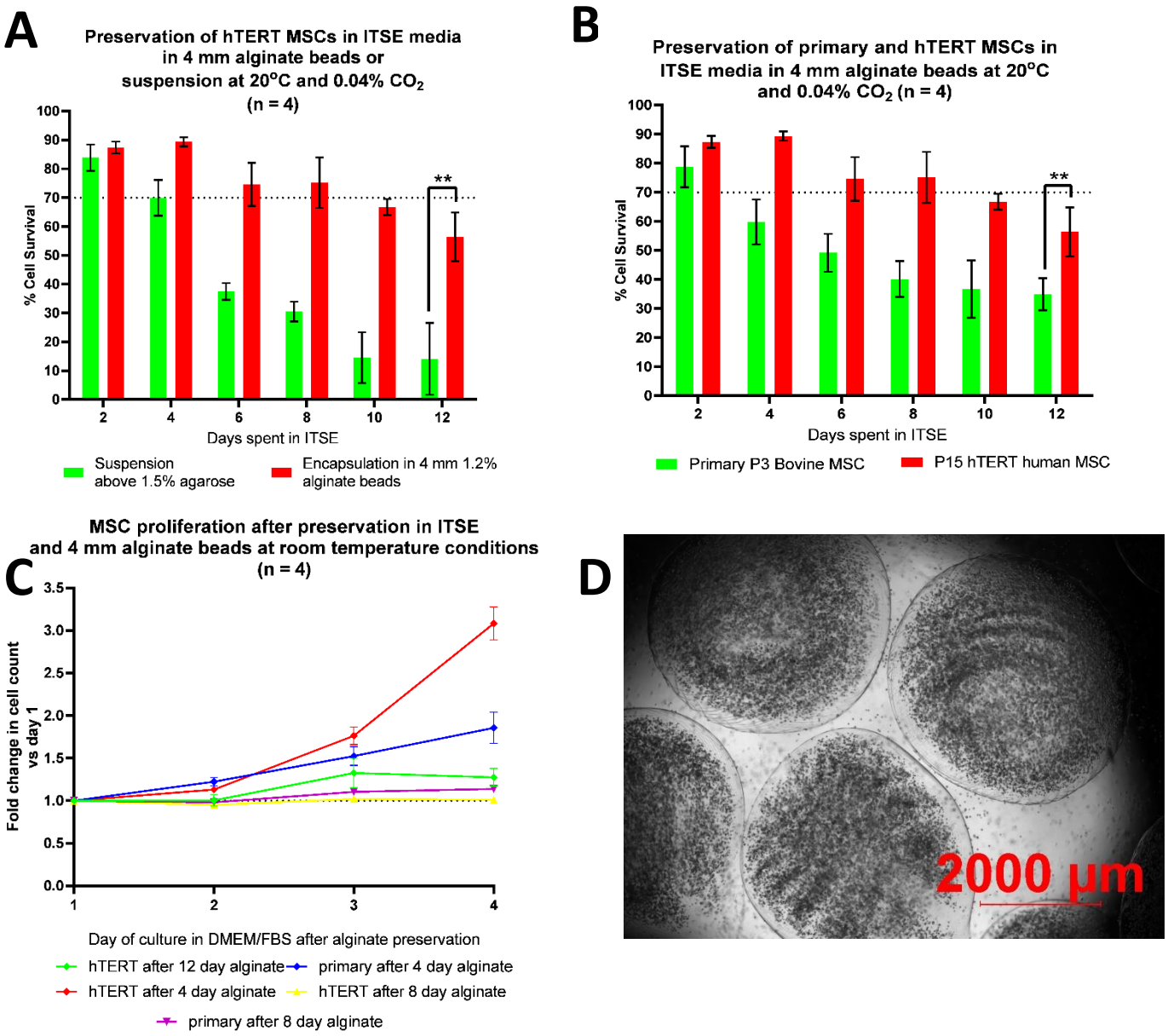

Figure 6.8: preservation of primary and hTERT MSCs in 3D in alginate beads or in suspension in ITSE based media (Media C1). Alginate encapsulation improved survival of hTERT MSCs preserved in ITSE based media at atmospheric conditions (A). In alginate beads at 4 mm, hTERT MSCs exhibited higher survival rates than primary MSCs (B). hTERT and primary MSCs regained proliferation potential after 4 days preservation in alginate when transferred to DMEM/FBS **(Media B)**, but not at 8 days or longer (C). hTERT MSCs encapsulated in 4 mm alginate beads at day 4 in ITSE based media (D). Values shown as mean ± SD, n = 4 for all groups, ** = $p \leq 0.01$ by way of Welch's t-test.

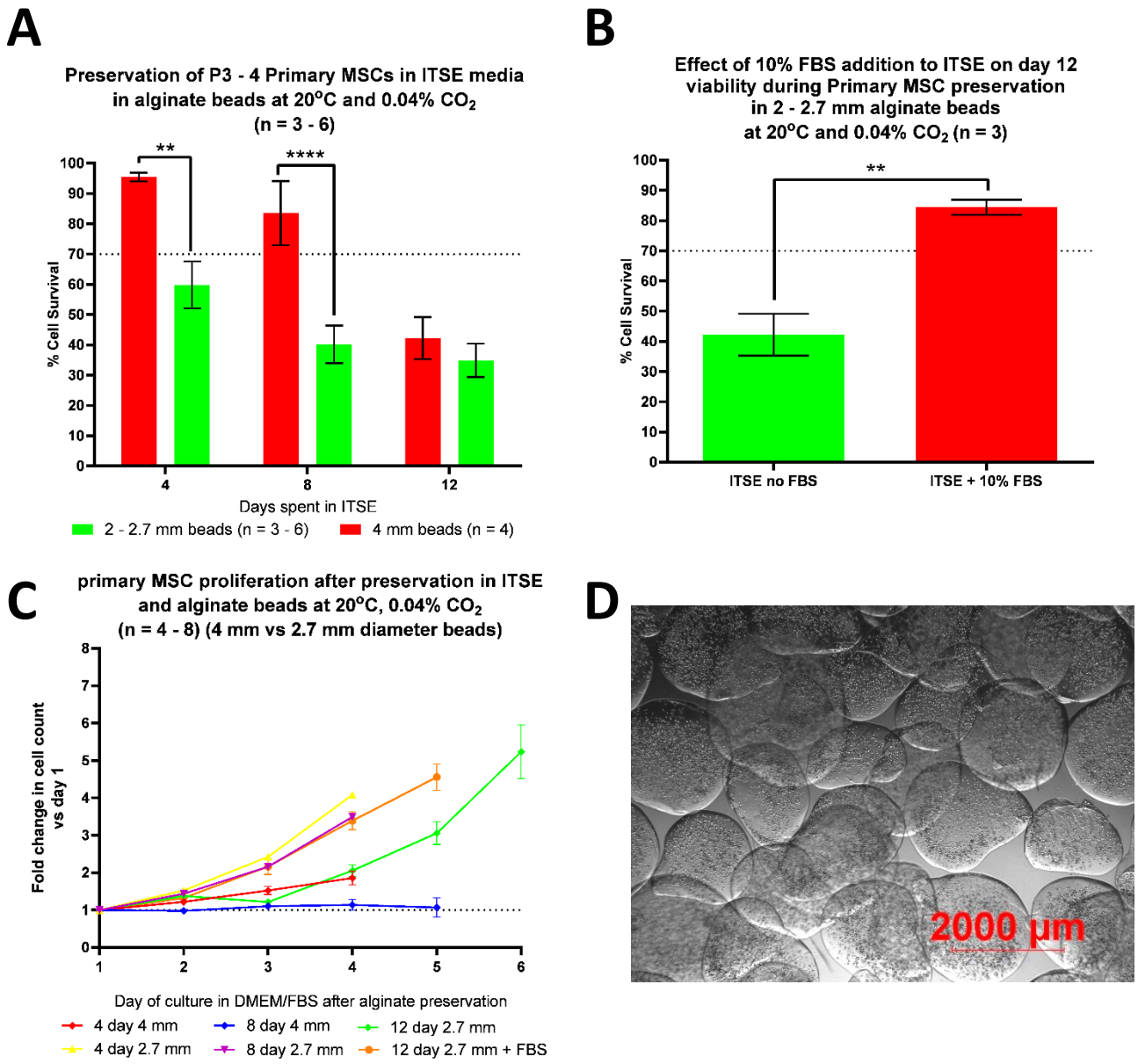

Figure 6.9: Effect of bead diameter and FBS addition to ITSE based media (Media C1) on survival and recovery of primary P3 bovine MSCs. (A) Reducing bead size from 4 mm to 2-2.7 mm improved survival for primary MSCs, values are mean ± SD, n = 4 for cells in 4 mm beads, n = 3 for cells in 2-2.7 mm beads at day 4 and day 12, n = 6 for cells in 2-2.7 mm beads at day 8. (B) For cells to remain viable for 12 days, FBS was required in ITSE based media, values are mean ± SD, n = 3 for all groups. (C) Recovery was improved and cells remained functional for longer in 2-2.7 mm beads values are mean ± SD for all groups, values are mean ± SD, n = 8 for proliferation in DMEM/FBS after 8 days in ITSE and 2-2.7 mm beads (purple), n = 4 for all other groups. (D) Creating beads manually lead to variation in size and caused them to adopt a droplet rather round shape, most of which were between 2-2.7 mm in size. ** = $p \leq 0.01$, **** = $p \leq 0.0001$, all by way of Welch's t-test.

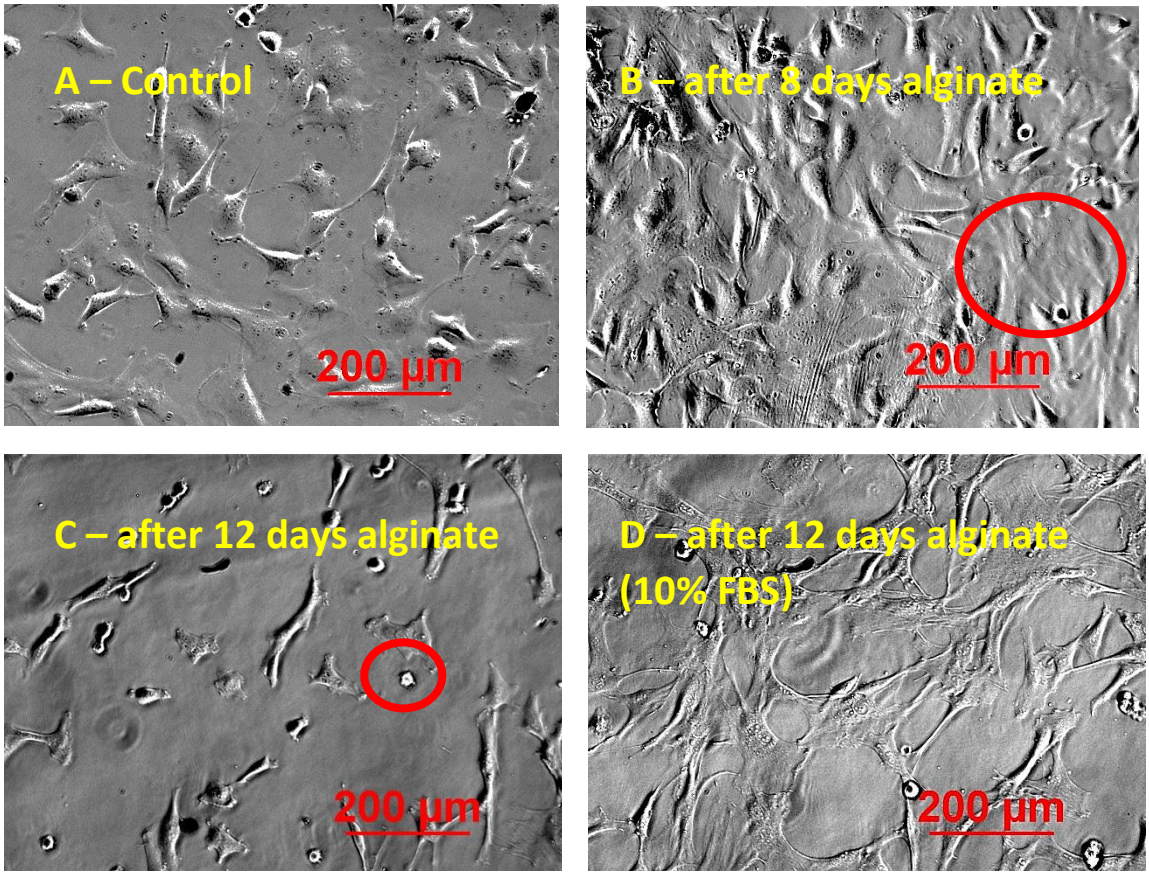

Figure 6.10: Morphology of recovering P3-4 primary bovine MSCs in DMEM/FBS (Media B) at the 96 h timeframe after preservation in 2-2.7 mm alginate beads and ITSE based media (Media C1). Non preserved cells cultured in DMEM/FBS **(Media A)** adopt a spindle morphology (A). Recovering cells preserved for 8 days (B) in serum free ITSE based media developed a mixed morphology, with some adopting a spindle morphology, while others adopting a flat morphology (as shown by red circles). After 12 days preservation in serum free ITSE based media (C), some cells developed a spindle like morphology while others failed to elongate (as shown by red circles). When preserved for 12 days in ITSE based media with 10% FBS, all recovering cells developed a flat morphology (D).

6.3.5 – Preservation of P15 hTERT Human MSCs in 2-2.7 mm Alginate Beads at High Density (12k cells/bead)

At day 4 of alginate preservation, there was no significant difference in survival rates between hTERT MSCs preserved in ITSE based media **(Media C1)** in 4 mm beads vs 2-2.7 mm beads (89.36 ± 1.59% survival in 4 mm beads (n = 4) vs 88.84 ± 5.60% survival in 2-2.7 mm beads (n = 8), p = 0.81) nor at day 8 (75.21 ± 8.76% survival rates in 4 mm beads (n= 4) vs 70.93 ± 18.60% in 2-2.7 mm beads (n = 8), p = 0.60) or at day 12 (56.42 ± 8.44% for 4 mm beads, 47.49 ± 11.67% for 2-2.7 mm beads (n = 4, p = 0.27). The data is shown in **Figure 6.11A.**

After 4 days preservation in alginate and ITSE based media **(Media C1)**, hTERT MSCs in 2.7 mm beads adopted a faster growth pattern upon recovery in DMEM/FBS compared to hTERT MSCs in 4 mm beads. At day 5 of recovery in DMEM/FBS **(Media A)**, the fold change in cell count was 4.31

± 0.35 for 4 mm beads vs 5.19 ± 0.59 fold change for 2-2.7 mm beads (n = 4, p = 0.02) (**Figure 6.11B**, red and purple). For the 2-2.7 mm beads, cells lagged for 3 additional days before proliferating, exhibiting a 1.85 ± 0.19 fold change at day 5 (n = 4), while the 4 mm bead group exhibited no proliferation over a 5 day period (1.08 ± 0.24 fold change by day 5 (n = 4)). (**Figure 6.11B**, green and blue). After 12 days preservation, regardless of bead size, hTERT MSCs failed to proliferate during recovery (at day 5 a 1.05 ± 0.06 fold change for the 2-2.7 mm group vs 1.08 ± 0.14 fold change for the 4 mm group was observed, n = 4) (**Figure 6.11B**, yellow and orange). The pH of fresh ITSE based media was 7.63. For hTERT MSCs in 2.7 mm alginate beads, pH fell to 6.43 at day 8, and 6.19 at day 12 (n = 1), where media from 3 wells were combined prior to pH measurement. For the primary bovine MSCs the pH was 7.14 at day 8, n = 1.

For hTERT human MSCs, the percentage of live cells attached in DMEM/FBS **(Media B)** after preservation in 2-2.7 mm alginate beads (n = 4 for all) were 51.68 ± 6.06 % after 4 days, 47.90 ± 1.02% after 8 days and 33.29 ± 8.08% at day 12. For primary bovine MSCs, cell attachment rates in DMEM/FBS **(Media B)** after preservation in alginate beads was 84.98 ± 7.45% after 4 days (n = 4) and 64.98 ± 8.78 % after 8 days (n = 8) and 62.01 ± 10.03% after 12 days (n = 4). When the ITSE based medium was supplemented with 10% FBS for primary bovine cells, cell attachment rate after 12 days in 2-2.7 alginate beads was 76.41 ± 4.90% upon transfer to DMEM/FBS (p = 0.0001).

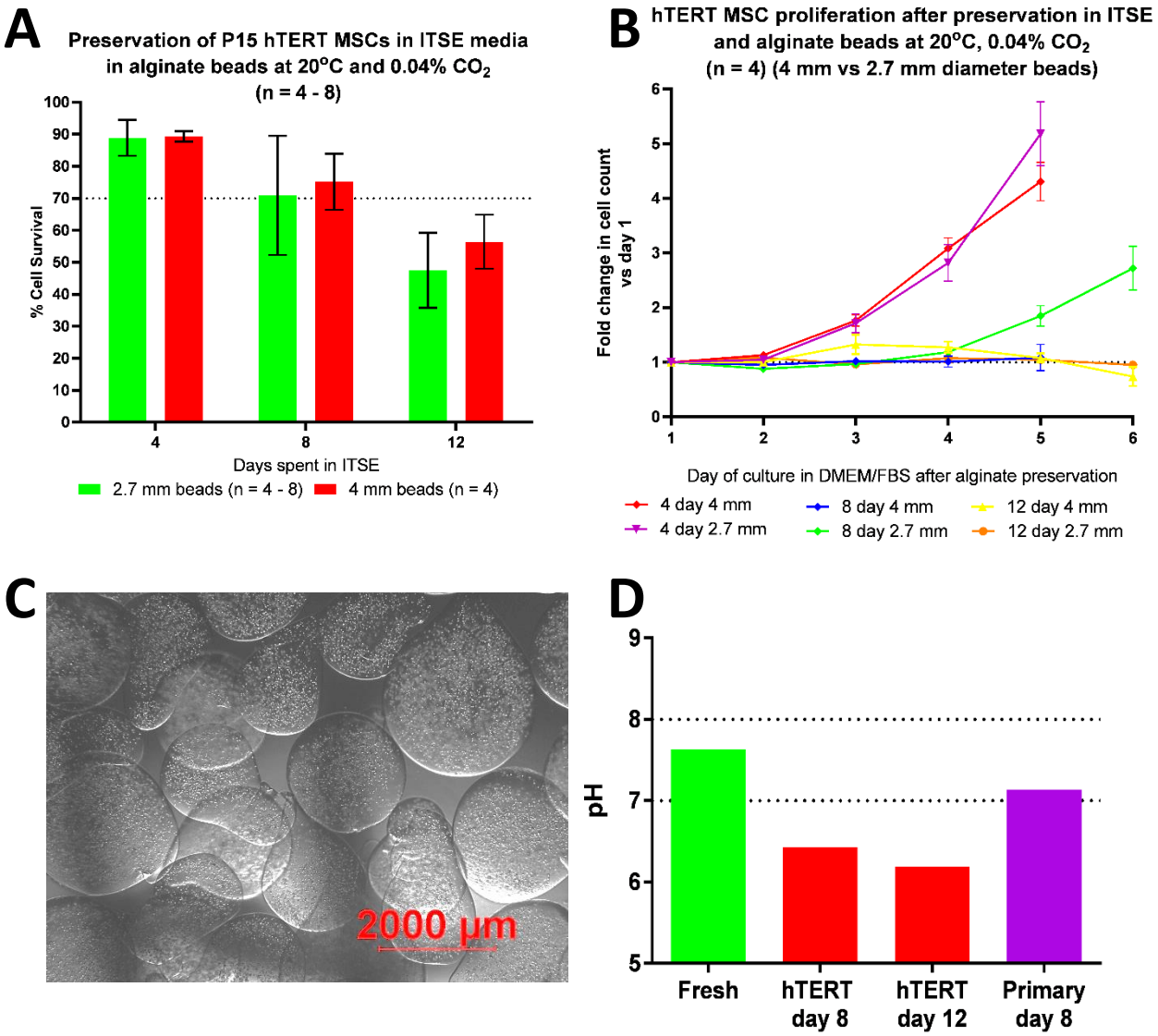

Figure 6.11: Effect of bead diameter and FBS addition on survival and recovery of hTERT P15 human MSCs in ITSE based media (Media C1). (A) Reducing bead size from 4 mm to 2-2.7 mm did not improve cell survival, values are mean ± SD, n = 8 for cell survival in 2-2.7 mm beads at day 4 and day 8, n = 4 for all other groups. (B) Recovery after preservation was improved and cells remained functional for longer in 2-2.7 mm beads, values are mean ± SD, n = 4 for all groups. (C) Creating beads manually lead to variation in size and causes them to adopt a droplet rather round shape, most of which are between 2-2.7 mm in size. (D) For hTERT MSCs in 2-2.7 mm beads, pH became acidic at day 8, but not for primary bovine MSCs, n = 1 for all groups.

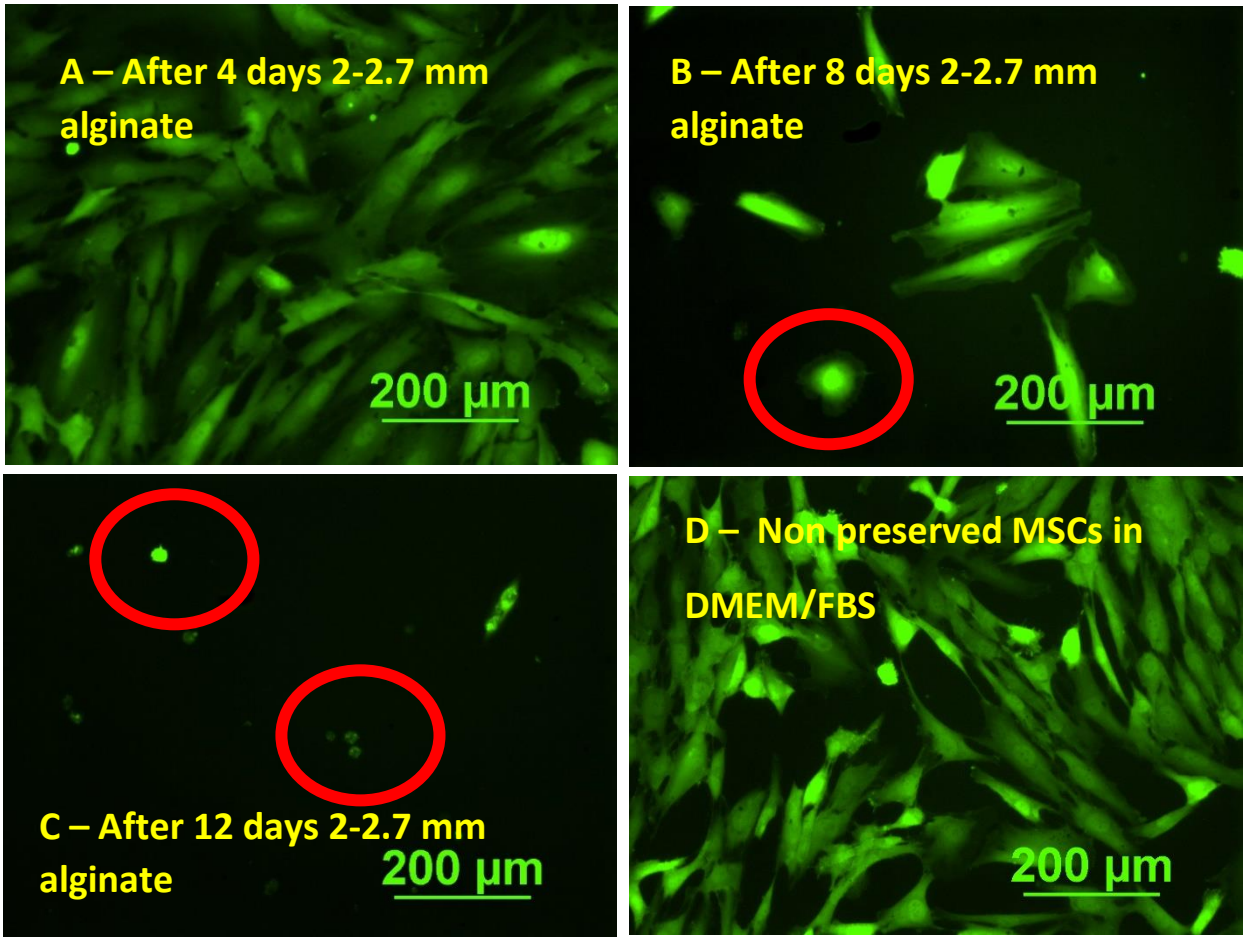

Figure 6.12: P15 hTERT MSC morphology in DMEM/FBS (Media B) at 96h (4 and 8 days preservation) or at 144h (12 days preservation) following preservation in 2-2.7mm alginate beads. After 4 days in alginate, cells readopted a spindle morphology when cultured in DMEM/FBS **(Media B)** (A). After 8 days preservation, some cells failed to elongate, as shown by red circles (B). Recovering cells failed to proliferate or elongate after 12 days preservation, with attached cell debris visible (C). Non preserved cells adopted a spindle morphology **(Media A)** (D).

P15 hTERT MSCs were released from 2-2.7 mm alginate beads and visualised at 100x magnification under the green filter of a fluorescent microscope. Recovering cells failed to elongate with prolonged preservation times, as shown in **Figure 6.12**. For non-preserved cells, both hTERT (**Figure 6.13 G-I**) and primary (**Figure 6.13 A-C**) demonstrated trilineage differentiation following treatment with differentiation media (**Media K1, K2 or K3 – Table 3.1**). After release from 2-2.7 mm alginate beads after 8 days, both hTERT (**Figure 6.13 J-L**) and primary (**Figure 6.13 D-F**) demonstrated trilineage differentiation. For recovering bovine MSCs in osteogenic media, cell death occurred after week 1, while for all other groups cells survived in differentiation media (**Media K1, K2 or K3 – Table 3.1**) for the entire 2 weeks.

6.3.6 – Trilineage Differentiation Potential of MSCs Following 8 days Preservation in ITSE and 2-2.7 mm Alginate Beads

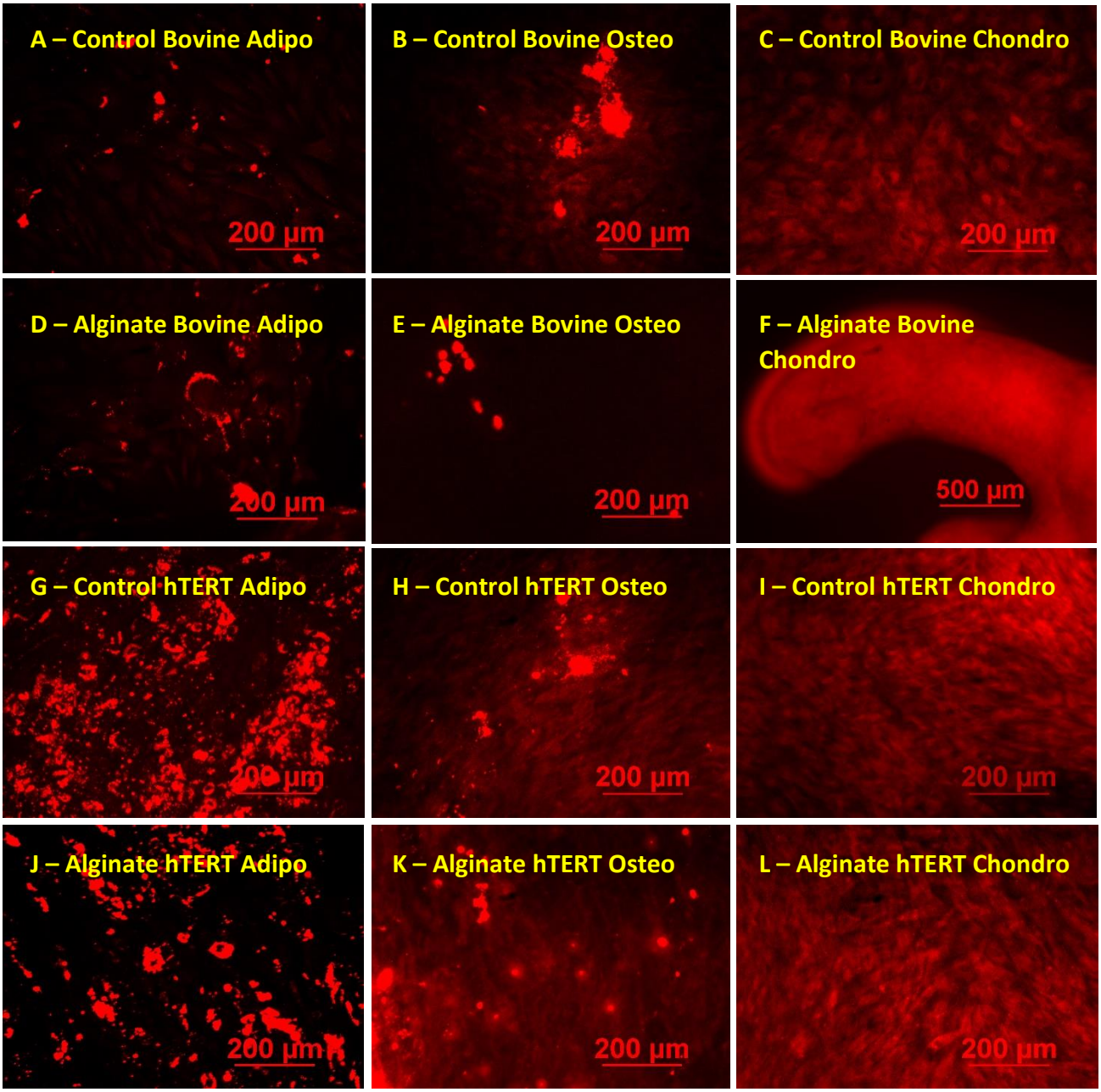

Figure 6.13: Lipid, calcium and glycosaminoglycan staining following 8 days preservation and 1-2 weeks treatment with differentiation media (Media K1, K2 or K3 – Table 3.1) for MSCs. For primary P4 bovine MSCs preserved in 8 days alginate and treated with osteogenic media, cell death occurred during week 2 of differentiation, so the photo at week 1 was used (E). For the alginate primary bovine MSCs group, chondrocytes formed a nugget during day 7 (F).

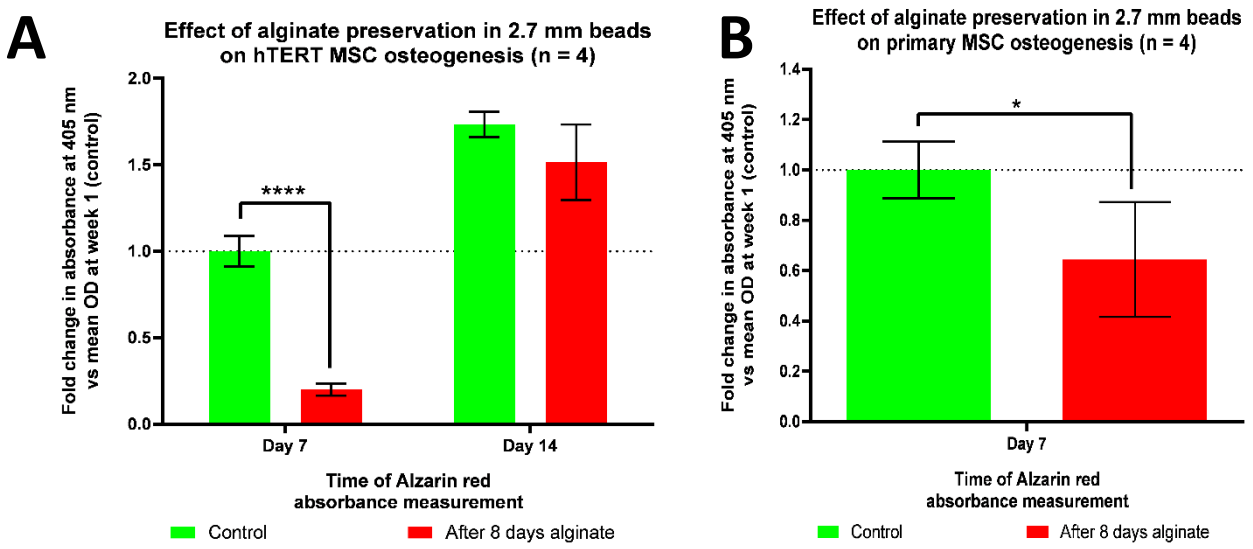

Figure 6.14: Effect of alginate preservation on osteogenic potential in MSCs. For both hTERT and primary MSCs, alginate preserved cells displayed lower osteogenic potential compared to non-preserved cells when treated with osteogenic media. All values are mean ± SD, n = 4 for all groups, * = $p \leq 0.05$, **** = $p \leq 0.0001$, all by way of Welch's t-test.

After treatment with osteogenic media, hTERT MSCs that have been preserved for 8 days in alginate displayed lower amounts of calcium compared to non-preserved hTERT MSCs, where each data point was reported as fold change vs the average absorbance/cell at week 1 for non-preserved MSCs. The amount of calcium for non-preserved hTERT MSCs was 1.00 ± 0.09 OD/cell at day 7 and 1.74 ± 0.07 at day 14. For hTERT MSCs after 8 days in 2.7 mm alginate beads, the average OD/cell at week 1 was 0.20 ± 0.04 OD/cell ($p < 0.0001$ vs control) and 1.51 ± 0.22 OD/cell at week 2 ($p = 0.14$ vs control), (n = 4 for all groups). At day 7, primary bovine MSCs displayed lower amounts of calcium formation per cell after preservation; 1.00 ± 0.11 OD/cell for control, 0.65 ± 0.23 OD/cell for the alginate group, n = 4, $p = 0.04$. At day 14, OD/cell for the control group for bovine MSCs increased to 2.84 ± 1.66 OD/cell (n = 4), while for the alginate group the amount of calcium was 1.70 OD/cell (n = 1), where cell death occurred for the remaining 3 wells. The data is shown in **Figure 6.14.**

6.3.7 – Effect of Cell Density on MSC Survival, pH and Metabolism in Alginate Beads at Atmospheric Conditions in ITSE Based Medium (Media C3)

For hTERT MSCs seeded in 4 mm beads and ITSE based medium **(Media C3**), there was no significant difference in survival rates between 6k cells or 1.5k cells/bead (60k and 15k cells per mL media), or compared to the previous experiment where cells were seeded at 750k cells/0.5 mL (49k cells/bead, 400k cells per mL) (74.60 ± 7.54 % survival rates at 49k cells/bead, 78.98 ± 4.99 % survival rates for 6k cells/bead ($p = 0.38$ vs 49k cells/bead) and 66.78 ± 13.45% survival rates for 1.5k cells/bead ($p = 0.17$ vs 6k cells/bead) n = 4 for all). At 60k cells per well for hTERT MSCs, the glucose consumption was 0.67 ± 0.11 pmoles/cell/day while the lactate production was 1.58

pmoles/cell/day, n = 4). For all other groups, there was no clear change in glucose or lactate levels compared to fresh media, indicating that changes were too small to be detected by the ARCHITECT bio analyser.

For primary MSCs seeded in 4 mm beads and ITSE based media **(Media C3)**, survival rates were significantly higher at 6k cells/bead compared to 49k cells/bead (49.20 ± 6.52 % survival rate at 49k cells/bead, 81.14 ± 3.10 % survival rate for 6k cells/bead (p = 0.0006, n = 4). At 1.5k cells/bead, the survival rate was 74.48 ± 1.57 %, (p = 0.02 vs 6k cells/bead, n = 4). For all groups, there was minimal change in pH at day 6 (7.02 ± 0.08 for hTERT 60k cells/mL, 7.09 ± 0.08 for hTERT 15k cells/mL, 7.13 ± 0.07 for primary 60k cells/mL and 7.12 ± 0.13 for primary 15k cells/mL). For the negative control, which consisted of empty beads in media, the pH was 7.22, compared to 7.63 at day 0 (n = 1). The data is shown in **Figure 6.15.**

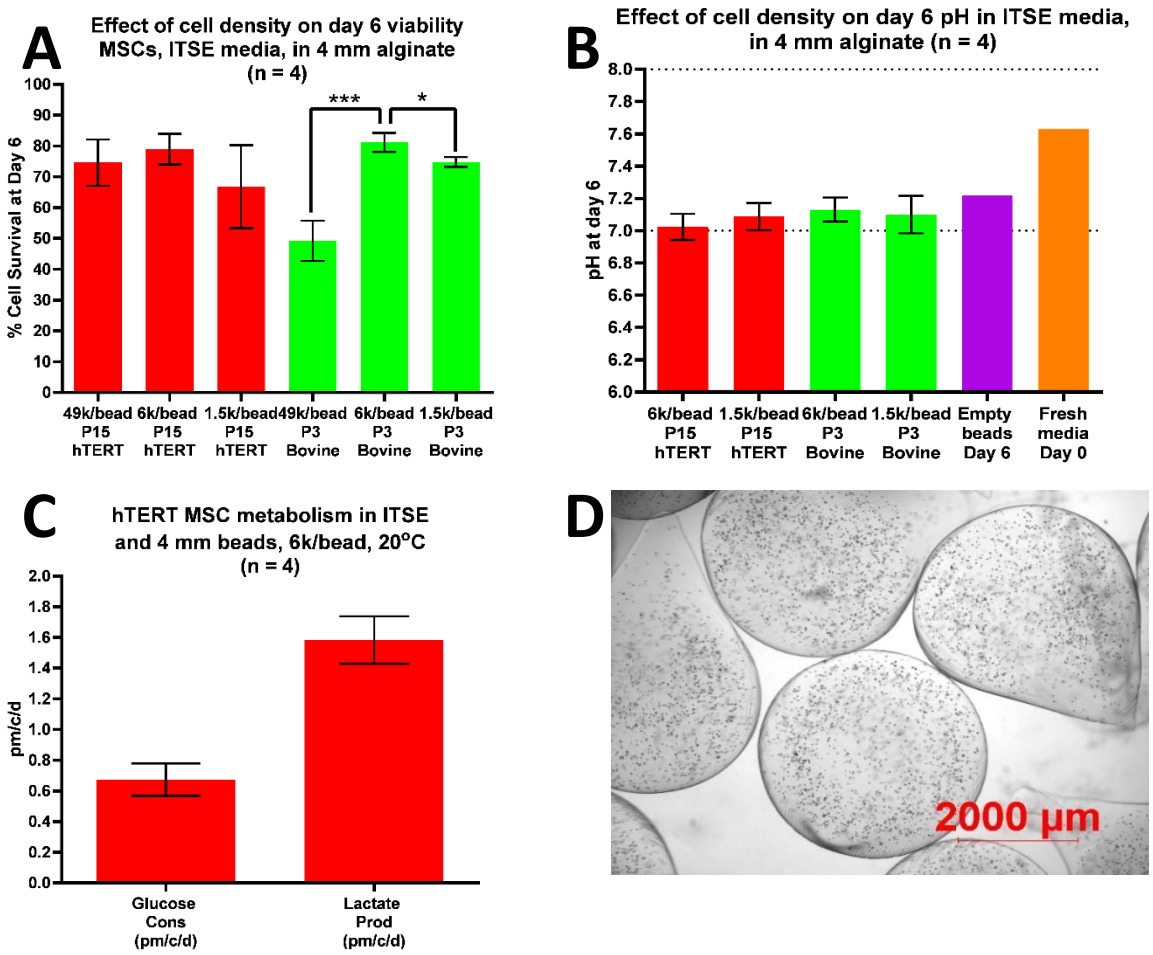

Figure 6.15: Effect of bead density on MSC survival, media pH and metabolic activity. (A) Decreasing cell density from 6k/bead to 1.5k/bead (60k/mL media to 15k cells/mL media) does not improve survival for either hTERT or primary bovine MSCs, values are mean ± SD, n = 4 for all groups. (B) At 15k or 60k cells/mL media, lactate production has minimal effect on pH, since the majority of pH drops is due to background reactions with atmospheric CO_2, values are mean ± SD, n = 1 for fresh media from fridge at day 0 (orange), n = 1 for media without cells incubated for 6 days at room temperature (purple), n = 4 for all other groups. (C) At 6k cells/bead, hTERT MSCs still consume glucose and favor glycolysis values are mean ± SD, n = 4 for all values. (D) Primary bovine MSCs, seeded at 6k cells per 4 mm bead. * = $p \leq 0.05$, *** = $p \leq 0.001$ all by way of Welch's t-test.

6.3.8 – Study of Alginate Bead Size Distribution

For 4 mm beads created by the SY pump, the mean bead size was 4.13 ± 0.38 mm (n = 13, interquartile range 3.89-4.23 m, or 8.26 % of the mean), while the range of bead size was between 3.71 mm and 4.86 mm. For 2-2.7 mm beads created by hand, the mean bead size was 2.34 ± 0.38 mm (n = 55, interquartile range 2.06-2.57 mm, or 21.82% of the mean), while the range of bead size was between 1.71 and 3.43 mm. The data is illustrated in **Figure 6.16**.

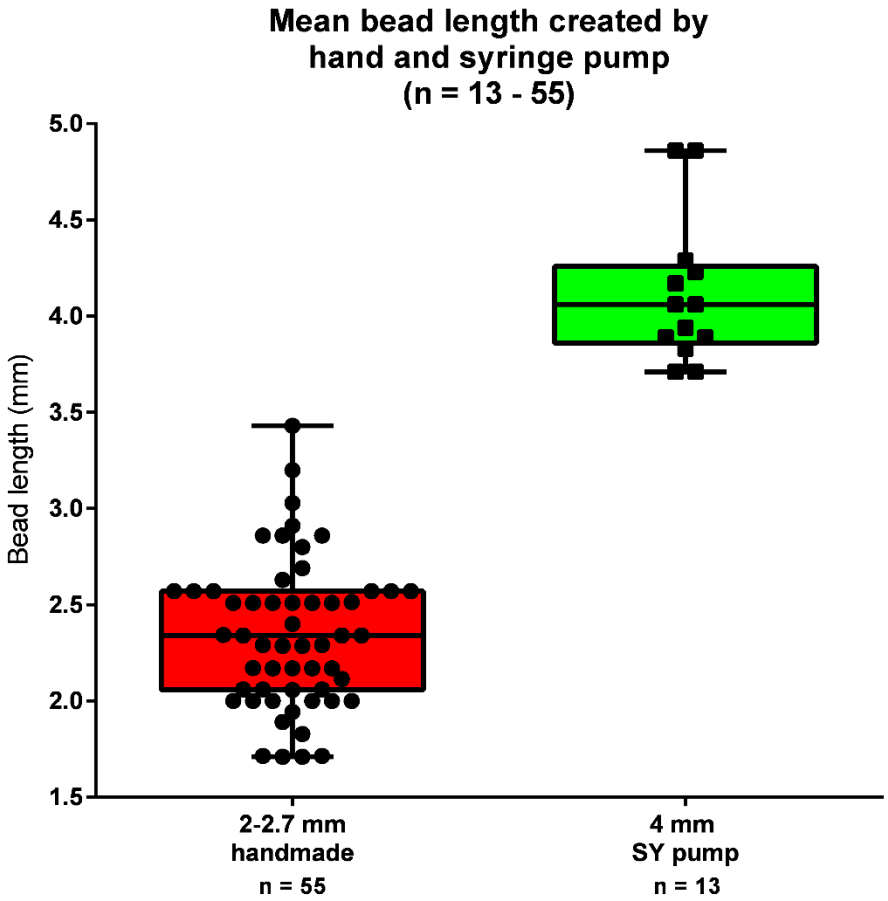

Figure 6.16: Bead size distribution for both 4 mm beads made by the SY pump and 2-2.7 mm beads created by hand. Box and whisker plots demonstrate the range (upper and lower tails) and interquartile range (upper and lower boundary of box) of bead sizes for both groups. The exact size of each bead measured is displayed as a black spot on the box and whisker plot. n = 13 for 4 mm beads, while n = 55 for 2-2.7 mm beads.

6.4 – Discussion

6.4.1 – For Monolayer Preservation at Atmospheric Conditions, 0.9 mM Bicarbonate is a Suitable pH Buffer.

The only suitable buffer for ITSE based preservation at atmospheric conditions was 0.9 mm bicarbonate, as it allowed elongation by day 2 with no cell detachment. In the presence of HEPES, MOPS and phosphate based buffers, hTERT MSCs failed to elongate in ITSE based media (see **Figure 6.2**). Currently the exact mechanism behind lack of cell attachment and elongation in non-bicarbonate buffers is not known, but it could be a result of increased sensitivity to non-bicarbonate pH buffers at atmospheric conditions compared 37°C. Effect of buffers in serum free media at atmospheric conditions on MSC morphology and attachment has not been studied in the literature.

Ultimately, the finalised preservation media will rely solely on low bicarbonate to maintain physiological pH.

6.4.2 – ITSE Based Media Maintained MSC Monolayer Survival for 12 days. hTERT MSC Proliferation Potential was Retained, but Primary MSCs Became Anchored to the Flask

HTERT MSCs were able to proliferate when transferred to DMEM/FBS after 12 days preservation (See **Figure 6.4C**) as a monolayer under atmospheric conditions in ITSE based medium, although the lag phase was prolonged by a further 3 days compared to MSCs preserved in ITSE based medium at 37^{o}C. During preservation as monolayers at room temperature, hTERT MSCs exhibited a shrunken and irregular morphology (See **Figure 6.5B**). As ionic pump activity has been shown to be halved in rabbit kidney microsomes at 25°C compared to 37°C (180) in the literature, MSC morphology alterations at room temperature be a result of ionic imbalance and subsequent osmotic stress on cell membranes. When placed in DMEM/FBS after 12 days room temperature in ITSE based media, cells may therefore need additional time to repair damaged components before proliferating.

Primary MSCs however, remained attached to the flask after trypsin treatment following 12 days preservation in ITSE based media. The mechanism behind this finding has not yet been determined, and previous authors have not preserved MSCs as monolayers at room temperature, only 3D structures, and have only used human cells. It is therefore unclear if the difference in recovery between human hTERT and bovine primary MSCs in this study was due to species differences in response to atmospheric preservation. The mechanism behind why primary bovine MSCs remained anchored was not further investigated, as the problem was resolved by alginate encapsulation of primary MSCs, where cells exhibited >70% survival and retained function after 8 days preservation.

Over the 12 days of monolayer preservation at room temperature in ITSE based medium, hTERT MSCs consumed roughly 3x more glucose per cell than primary MSCs, and produced 2x more lactate (See **Figure 6.4D**). MSC metabolism at room temperature has not been studied in the literature. Nevertheless, the results show that glucose must still be included in the ITSE based media during room temperature preservation.

6.4.3 – For Long Term Survival of MSCs in 3D in ITSE Based Medium, Alginate Encapsulation was Required and the Density must be Around 12k cells/bead

HTERT MSCs died rapidly when cultured in suspension in ITSE based media by agarose coating of well plates **(Figure 6.8A)**. When hTERT MSCs were encapsulated with 4 mm alginate beads

(Figure 6.8A) at a density of 750,000 cells per 0.5 mL of 1.2% alginate, survival of hTERT MSCs improved compared to suspension culture, and survival did not fall below target (70%) until day 10. Swioklo *et al.* (2016) and Damala *et al.* (2019) reported an improvement in survival when MSCs were preserved in alginate at room temperature compare to suspension cells, though they tried a 3-5 day period (43, 44). Although the exact mechanism has not yet been determined in this study or the published literature, previous authors have proposed mechanisms for the cytoprotective effect of alginate. Rat hepatocytes have been encapsulated in alginate for up to 48 hours at either 4°C or in cryopreservation, and showed improved survival, increased glutathione-S-transferase activity and reduced caspase-3 activity compared to suspension cells (181). During cold storage, cell membranes experience osmotic stress and increased vulnerability to mechanical stress as a result (181). The authors proposed that alginate stabilised the cell membrane during storage, and also prevented caspase-3 activity by integrin ligation (181). Overall these findings demonstrate that MSCs must be encapsulated in alginate during storage in ITSE medium in 3D at atmospheric conditions to maintain cell survival.

Tarusin *et al.* (2015) reported that MSCs preserved at cold temperature in Wisconsin solution for 7 days yielded no improvement during alginate encapsulation (40). As alginate has been proposed to stabilise cell membranes due to osmotic stress at room temperature and cold storage, encapsulation of alginate may provide no extra benefit in Wisconsin solution, due to osmoprotectants (raffinose, lactobionic acid) already present in the formulation (182). It is therefore important to investigate whether alginate is still required to maintain cell survival when raffinose and lactobionic acid is added to ITSE media.

When primary MSCs were encapsulated in 4 mm beads (49k cells/bead) and stained with propidium iodide for over 12 days, most of the bead contained late apoptotic cells (shown as bright red by PI staining) **(Figure 6.7)**. Ultimately, co-staining with Annexin V and acridine orange are required to determine the location of early apoptotic and live cells respectively.

Drastic improvements in survival were observed for primary MSCs (**Figure 6.9A**) and recovery for both primary and hTERT MSCs (**Figure 6.9C** and **Figure 6.11B**) when the bead size was switched from 4 mm to hand made beads ranging from 2-2.7 mm (12k cells/bead). **Figure 6.10B** demonstrates that after 8 days preservation in 2-2.7 mm alginate beads, primary MSCs adopted a flat morphology upon recovery, which could be attributed to osmotic stress during preservation.

The alginate bead diameter required given a certain number of cells/bead have been calculated below. However this may not account for swelling or shrinking during preservation at room temperature.

1. The upper limit of diameter for a single MSC is 30 µm, equivalent to 0.03 mm (183).
2. Radius = 0.5 x diameter, therefore radius of MSC is 0.015 mm
3. Total volume of N number of cells = 4/3 x π x r^3 x number of cells. For 49,000 cells per bead, the total volume from the cells is 0.6926 mm^3. For 12,000 cells, the total volume from the cells is 0.1696 mm^3.
4. The packing efficiency for multiple small spheres in 1 large sphere is 0.64, to account for spaces between spheres. The reciprocal would be 1.5625, as the volume of a number of spheres are being calculated, rather than the number of small spheres in 1 large one.
5. The volume, to account for spaces between spheres would therefore be volume of all cells x reciprocal of packing efficiency (1.5625). For 49,000 cells, the volume would be 1.0822 mm^3 . For 12,000 cells, the volume would be 0.2650 mm^3.
6. Radius = (0.75 x volume/ π) ^ 0.33333. For 49,000 cells, the radius of the 3D structure would be 0.6369 mm. For 12,000 cells, the radius of the 3D structure would be 0.3985 mm.
7. To hold 49,000 cells, the minimum diameter of the alginate bead needs to be 1.27 mm. To hold 12,000 cells, the minimum diameter needs to be 0.80 mm.

Both primary and hTERT MSCs stained positive for calcium, lipids and glycosaminoglycans following 8 days of alginate preservation and subsequent treatment with differentiation media (**Media K1, K2 or K3 – Table 3.1**) (**Figure 6.13**), which indicated that cells retained differentiation potential. Death of osteocytes occurred during week 2 of differentiation for primary MSCs among alginate preserved cells only. This could be due to prolonged exposure to room temperature, coupled with lack of bFGF, albumin and Vitamin E in ITSE media which in turn pushed cells towards apoptosis. In the literature, MSC preservation has not been achieved in the absence of both serum and refrigeration. Small changes in lipid content in 96 well plates with oil red O could not be measured; alternative methods, such as the use of ELISA to measure OCT-4 expression in both preserved and control MSCs could be employed. After 3 days preservation of MSCs in alginate, Swioklo *et al.* (2016) reported no significant difference in osteogenic potential (alizarin red) and adipogenesis (oil red O) between preserved and non-preserved cells at week 5 of recovery after preservation (44), while Jiang *et al.* (2017) reported no loss of differentiation potential for MSCs cultured for 7 days as spheroids in α-MEM/FBS (57). Maintenance of differentiation potential in the literature could therefore be attributed to shorter preservation times or presence of serum.

Despite partial loss of osteogenic potential after 8 days room temperature 3D preservation in ITSE based media (**Figure 6.14**), MSCs still retained some level of differentiation potential which indicates that cells remain functional during storage in ITSE media at atmospheric conditions.

6.4.4 – When MSCs Were Preserved in 3D, Acidic pH and Serum absence may have Acted as Limiting Factors and Prevented Cell Survival Beyond Day 8

hTERT human MSCs exhibited slower proliferation in DMEM/FBS after 8 days of alginate preservation in 2-2.7 mm beads and ITSE media (**Figure 6.11B**) compared to primary MSCs in the same bead size **(Figure 6.9C). Figure 6.11D** demonstrates that at day 8, media pH for primary MSCs remained within physiological levels (7-8) while the pH of hTERT MSCs became acidic (pH 6.4). This could be due to higher lactate production by hTERT MSCs compared to their primary counterparts as demonstrated in **Chapter 4.** For the hTERT MSCs, cells failed to elongate (**Figure 6.12C**) when transferred to DMEM/FBS after 12 days in 2-2.7 mm alginate beads, likely a result of prolonged exposure to acidic pH. For rabbit MSCs, Chen *et al.* (2009) reported that acidifying media pH using lactate from 7.2-7.4 to 6.8-7.0 reduced specific glucose consumption and lactate production by 48% and 95% respectively (184). At pH 6.4, in addition to absence of growth factors, metabolic alterations could be further exacerbated, rendering cells unable to take in glucose or expel lactate. The results and literature demonstrate that during storage, pH must be kept close to 7.4 to keep cells viable and functional.

Overall, the data showed that MSCs can be kept viable and functional for 8 days when preserved in alginate in ITSE based media, though further extension of preservation times to 12 days required foetal bovine serum (FBS), as **Figure 6.9B** and **6.9C** demonstrated significantly higher survival at day 12 and faster proliferation upon recovery when primary P3 bovine MSCs were preserved in ITSE based media + 10% FBS instead of ITSE based media alone. Vitamin E, which is present in FBS but not in basal media or the insulin supplement, has been shown in the literature to prevent apoptosis of H_2O_2 treated rat MSCs in a concentration dependent manner (12% for control, 45% for 100 µM H_2O_2, 30% for 50 µM Vitamin E, falling to 15% for 100 µM vitamin E) (170).

Nawrocka *et al.* (2017) reported that basic fibroblast growth factor (bFGF) at 5 and 10 ng/ mL prevented the apoptosis and senescence of MSCs from type 2 diabetic patients (9), where they observed the downregulation of pro-apoptotic genes p21 and p53 by 6% and a 30% reduction in ROS production compared to untreated cells (185). The presence of albumin in serum scavenges free radicals produced by cells (186). To extend preservation times to 12 days, there is a need to determine the optimum concentrations of vitamin E and bFGF as additions to ITSE based media.

6.4.5 – Media Glucose was still Required During Preservation at Atmospheric Conditions in Alginate

To ensure that pH acidity does not become a limiting factor when studying MSC metabolism in alginate, both primary and hTERT MSCs were preserved in 4 mm alginate beads at densities of 60k cells/mL and 15k cells/mL in ITSE based media. The only group that produced measurable changes in glucose and lactate media concentrations was the 60k hTERT MSC group. In the literature, glucose consumption during alginate preservation has not been studied. The data therefore demonstrates that glucose present in ITSE based media must not be removed, and preservation densities in a clinical setting should not be too high to prevent accumulation of metabolic waste. Currently it is not known if localised lactate accumulation in alginate beads reduces cell survival. A novel lactate fluorophore in the published literature has been developed using the protein TTHA0766 from *Thermus thermophiles*, which has been employed to track lactate in the brain of *Drosophila melanogaster* larvae (187), which could be adapted to track lactate movement for preserved MSCs in alginate beads.

6.4.6 – Other Experimental Limitations and Proposed Solutions

The literature demonstrates enormous patient to patient variation in MSC metabolism, proliferation rate and immunomodulatory activity (60). Primary bovine cells were chosen for preservation experiments as the calves are relatively young (3 weeks), healthy and did not require paper work nor any costs. This ensured that if preservation was unsuccessful, the cause would be the preservation technique itself rather than the cell source being faulty. This study, nor previous authors have investigated the effect of age, source (adipose vs umbilical cord vs bone marrow) or species on MSC survival during preservation. This can be addressed by comparing cell survival between bone marrow, umbilical cord and adipose MSCs from the same calf, and between 3 week old calves and 10 year old cattle. As there is considerable variation in cell metabolism and proliferation rates between the same species (60) it may be challenging to study how cell survival differs between MSCs of different species. Ultimately, the ITSE based media may need to be tailored for the storage of MSCs from elderly or diabetic patients.

Proliferation and differentiation were chosen to assess cell function following alginate preservation as the techniques are cheap and straight forward. However, it is the growth factors and signalling molecules released by MSCs that exert their therapeutic effect. For example, it was MSC release of SCDF-1α that reduced symptom severity in 6-OHDA mice models of Parkinson's disease (71) in the literature, while most MSCs injected travelled to the pulmonary tissue at day 2. Therefore, live cells that lost proliferation and differentiation potential may still be useful for

therapy. Jiang *et al.* (2017) reported human MSC survival for 7 days as spheroids at room temperature in α-MEM/FBS (25°C), and preserved cells retained their ability to prevent weight loss in mice colitis models (48). As MSCs were preserved in 3D at room temperature for 8 days in this study, there is a good chance that therapeutic potential is maintained.

A significant portion of cells marked as live by trypan blue failed to attach in DMEM/FBS following alginate preservation, which may suggest that cells have lost attachment proteins during alginate preservation, or perhaps there were additional cells that were moving towards apoptosis. Swioklo *et al.* (2016) reported that the CD166 adhesion marker expression for human MSCs falls from 76% to 20% after 72 hours of preservation in alginate discs at 15°C and a serum free expansion medium (44). To assess whether cells that failed to attach are still viable, they can be cultured at 37° C to test if glucose consumption and lactate production occurred.

In the literature, MSCs were able to survive for a fair amount of time in cold storage in glucose free isotonic solutions (buffered trehalose solution for 3 days (173), Wisconsin solution for 7 days (40)), and it was initially uncertain if cells still required glucose when not in a proliferative state. The results in this study show that MSCs still consume glucose and produce lactate in alginate beads at room temperature (**Figure 6.15C**), even when cells are not in a proliferative state during preservation. Previous authors have not studied specific glucose consumption during preservation, either at room temperature or cold storage. The shift towards acidity over the 6 day period was mainly due to background reactions between the 0.9 mm buffer and atmospheric CO_2 as opposed to lactate production, as there was little difference in pH between spent media and fresh media with empty beads at day 6 (**Figure 6.9B**), where pH ranged from 7-7.2 for all groups. Previous authors have not developed pH buffers for preservation at atmospheric conditions, nor have they determined whether pH change was a limiting factor during preservation. These limitations have been addressed, and the results demonstrate that only a reduction in bicarbonate concentration is needed to maintain media pH during MSC storage, without the requirement of other chemicals such as MOPS, HEPES or phosphate buffers previously used for cell expansion.

6.5 – Conclusion

In conclusion, the results demonstrated that ITSE based media was able to keep 3D MSC structures viable and functional for long periods of time (8 days) at atmospheric conditions without the need of serum, cryopreservation or refrigeration, and addition of serum further extended preservation times (to 12 days). pH acidity, lack of serum components, along with apoptosis of cells at the bead core may act as a limiting factors during 3D preservation. The results also showed that

cells still underwent low levels of glycolysis under atmospheric conditions in ITSE based media. Further optimisation of growth factor and vitamin E concentrations are still needed to extend the duration of cell survival.

Chapter 7

Generalised Discussion

7.1 - Introduction

The purpose of this thesis was to determine if a combination of Dulbecco's Modified Eagle's Medium (DMEM) and insulin based supplement (ITSE) could be used to keep bone marrow mesenchymal stem cells (BMMSCs) viable and functional for long periods of time in the absence of cryopreservation, refrigeration and serum to aid their clinical applications.

Primary bovine and human immortal (hTERT) mesenchymal stem cells (MSCs) were expanded and characterised by flow cytometry to assess stem cell marker expression. Proliferation, glucose consumption and lactate production in monolayers were studied at 37°C. For hTERT MSCs, the effect of room temperature (25°C) and cold storage (4°C) on metabolic activity was investigated, and GC-MS was employed to determine the amino acid and vitamin requirements.

The project investigated whether an ITSE supplement could be added to DMEM basal medium to maintain cell survival, looking at the differences in metabolism, proliferation potential, morphology, oxidative stress, autophagy and differentiation potential vs cells cultured in DMEM/FBS. In the final step, the preservation media was adapted to maintain cell survival at atmospheric conditions in 3D, by modifying the bicarbonate concentration and encapsulating cells in alginate.

7.2 – Contributions to the Field of MSC Preservation

Previous cell preservation methods in the literature have not tested for beyond 7 days, and required expensive materials such as trehalose and mucin based hydrogels, or were kept in media with serum. The authors did not measure specific glucose consumption or lactate production during preservation, nor did they investigate how amino acid consumption changes for preserved cells. This has been addressed by using a cheap and chemically defined insulin supplement and basal medium to keep cells viable and functional at atmospheric conditions, and specific glucose consumption and lactate production in alginate was measured, which allowed the estimation of the cell preservation density. Amino acid requirements have been determined with GC-MS, which has only been done with expanding cells in serum in the literature.

The results showed that supplementation of DMEM basal medium with 1% ITSE prevented gradual hTERT MSC cell death between day 6 and 12, where it allowed continuous proliferation

instead. Despite exhibiting higher levels of oxidative stress than cells in DMEM/FBS, hTERT MSCs cultured in ITSE based media did not exhibit higher levels of autophagy. When cultured for 12 days in ITSE based media, hTERT MSCs retained their proliferation and differentiation potential. Overall, the results demonstrated that a chemically defined preservation media can be made using common cell culture ingredients.

At atmospheric conditions, the results showed that reducing the bicarbonate concentration to 0.9 mM maintains pH within the ranges of 7-8 for hTERT human and primary bovine MSC monolayers over 12 days. In 3D, cells only survived long term (8 days) and maintained proliferation and trilineage differentiation when encapsulated in alginate, and when the number of cells/bead was 12,000 or less (600k cells/0.5 mL of alginate, 2-2.7 mm bead diameter). At day 8 and beyond, lack of serum and pH acidity was a limiting factor, where the survival of bovine MSCs was high only when ITSE medium was supplemented with 10% serum. For hTERT MSCs, cells underwent low levels of glycolysis in alginate beads in ITSE at atmospheric conditions. Further optimisations that could be made to the preservation media have been identified.

Current challenges in MSC preservation were summarised in **Table 1.2**; MSCs cannot survive long term (>5 days) without serum, buffer formulations for standard culture conditions (5% CO_2) are unsuitable for atmospheric CO_2 (0.04%) and metabolic requirements during preservation have not been studied. This project has demonstrated that human hTERT and primary bovine MSC survival and function can be maintained for 12 days in 2D at 37 °C and 8 days in 3D at 20°C using 1% ITSE supplement in the absence of serum, where 0.9 mM bicarbonate prevented deviations in media pH at atmospheric CO_2. GC-MS measurement of media samples for hTERT MSCs in ITSE based media at 37°C demonstrated that proline, tyrosine, asparagine, glutamic acid, phenylalanine or serine were not consumed over a 12 day period, while glucose requirements per cell per day has been determined during room temperature preservation, both as 2D monolayers and in alginate beads. The findings would in turn allow the reformulation of DMEM basal media to exclude non-essential components, and allow the clinician to determine the preservation density/mL of media given a target preservation time once the glucose consumption rate of the patient's MSCs have been determined. Additionally, preservation of MSCs in ITSE based media has eliminated the need for freezing or refrigeration for short term storage.

7.3 – Thesis Limitations and Future Work to Address Them

Surface marker expression of bovine primary MSCs could not be assessed with human antibodies due to structural incompatibility, and therefore the exact percentage of primary MSCs could not be determined. Despite this, proliferation rates at P3, P4 and P5 were consistent, so contamination by other cell types is highly unlikely. In addition, cells demonstrated trilineage differentiation into adipocytes, osteocytes and chondrocytes, which confirms the presence of MSCs. A search on Biocompare.com did not yield any antibodies for CD73, CD90 or CD105 of bovine origin. Given more time, flow cytometry experiments will be repeated with antibodies of other species to see if there is any cross reactivity. If any cross reactivity were to occur, cells would bind CD73, CD90 and CD105, but not CD34 and CD45.

Although MSCs cultured in ITSE displayed higher levels of intracellular ROS production compared to cells in DMEM/FBS, it is important to determine whether it was due to insufficient amounts of selenium in the ITSE, lack of ascorbic acid normally present in serum or absence of albumin. This can be addressed by supplementing the media with different concentrations and combinations of selenium, albumin and ascorbic acid, and measuring ROS production afterwards. The finalised media would therefore contain the combination that minimises ROS production without loss of cell survival or function. Preservation of hTERT MSC monolayers in ITSE based media at 37°C yielded significant increases in adipogenic and decreases in chondrogenic potential. Transcriptome analysis may be needed as future work to determine the exact genetic changes caused by ITSE based medium, with focus on genes associated with differentiation.

For GC-MS, B vitamins other than B5 and B8 could not be detected, possibly because their boiling points are too high. This can be addressed by repeating metabolomics studies using liquid chromatography and mass spectroscopy (LC-MS).

As the microscope on bright field setting was not able to produce colour images, the red filter was used instead to visualise oil red o, Nile red, safranin-O and alizarin red. Although this confirms the presence of calcium, lipid and glycosaminoglycans in differentiated MSCs by displaying them as red patches, further improved images could be created on a bright field setting, highlighting both the staining and chondrocytes/adipocytes/osteocytes.

When the initial seeding density for alginate preservation was 360,000 cells/mL of ITSE, where cells were encapsulated in 2-2.7 mm diameter beads at 12k cells/bead, pH became acidic at day 8 (from 7.6 to 6.4) for hTERT MSCs. However, 0.4 of the loss in pH was due to atmospheric CO_2. Given more time, alternative concentrations of bicarbonate will be tested, firstly without cells to ensure pH is maintained. At the appropriate bicarbonate concentration, minimal deviation from pH 7.4 during would be expected over a 12 day period.

Following alginate preservation at 12k/bead in 2-2.7 mm diameter beads and at 49k/bead in 4 mm diameter beads, not all live cells retained attachment once plated in DMEM/FBS. A considerable amount of cell debris was observed, which may show that additional apoptotic cells were breaking apart during preservation. Therefore, only intact dead cells are accounted for by trypan blue counting. In 3D, recovering MSCs displayed slower proliferation potential, and weaker osteogenic potential compared to non-preserved cells, along with a flatter morphology. This could be a result of osmotic pressure at room temperature, which may have also contributed to cell death. Osmotic stress could be alleviated by the addition of raffinose and lactobionic acid to ITSE based media. The Fluvolt membrane potential dye (Thermofisher, UK) could be used to determine the effect of preservation on membrane potential, where significant deviations as a result of reduced Na^+/K^+-ATPase and other ionic pump activity is expected.

During preservation of hTERT MSC monolayers in ITSE based media at room temperature, hTERT MSCs exhibited a gradual decline in the % of viable cells from seeding to day 12, while primary MSCs exhibited a sharp decline up to day 6, followed by no clear trend from day 6 to 12 (See **Figure 6.4A**). As primary cells could not be detached by trypsin, some dead cells may have remained permanently anchored. For future work, primary MSC monolayers should be stained with 7-Aminoactinomycin D to check whether the number of attached cells is equivalent to the number of viable cells. If apoptotic cells remained adherent, an increase in the number of red fluorescent cells would be seen in the well plate.

Preservation could be scaled up for future experiments, with 300k cells in 5 mL of ITSE in a 6 well plate – where each replicate consists of 3 wells. It would be more accurate to count larger number of cells in suspension.

Recovering MSCs after preservation at room temperature developed flat and irregular morphologies. It is important to measure the differences in membrane potential between MSCs in DMEM/FBS at 37°C and MSCs preserved in ITSE based media at room temperature to see if ion

balance is altered. This will determine if permanent morphology changes were a result of osmotic stress due to room temperature inhibition of Na^+/K^+-ATPase.

During alginate preservation, the results demonstrated that bovine MSC cell death after day 8 was due to lack of serum components, most likely basic fibroblast growth factor (bFGF), vitamin E and albumin. To address this, multiple concentrations and combinations of each would be tested and the formulation that maximises cell survival would be selected. Once the buffer has been further optimised, and the appropriate amounts of serum components and osmoprotectants have been determined, cell preservation experiments will be repeated with 4 mm beads, this time with only 6k cells/bead and 60k cells/mL ITSE based media in total. Cells would likely remain viable and functional for the entire 12 days, without any debris formation. pH is likely to remain within 7-8 once the buffer has been further optimised. For hTERT MSCs preserved at 15k cells/mL and primary bovine MSCs preserved at 60k or 15k cells/mL, there was no clear glucose consumption over a 6 day period. Scintillation counting of radiolabelled glucose may be a more accurate method to assess metabolism, where glucose consumption and lactate production would be expected for both primary and hTERT MSCS regardless of preservation density.

Empty ITSE based media (0.9 mM bicarbonate) pH dropped from 7.6 to 7.2 over 6 days. Higher bicarbonate concentrations may be required to prevent pH drops, or cell preservation could be repeated in an airtight container.

This study only involved very young calves (3 weeks) or immortal human MSCs. The finalised media needs to be tested on a range of different patient groups, ensuring coverage of different ethnicities, ages and illnesses. Enormous patient to patient variation in the duration for survival, and post preservation recovery is expected, with worse results from elderly and diabetic patients.

Transcriptome analysis could be employed to get a comprehensive view of genetic changes after 12 days of preservation. As weaker osteogenic potential was observed following alginate preservation, lower expression of stemness marker OCT-4 would be expected. Downregulation of glucose transporter enzymes, enzymes associated with glycolysis and the monocarboxylate (MCT) transporter would likely be reported since metabolic activity is lower at room temperature.

Due to time and budget limitations, amino acid consumption was only studied for hTERT MSCs cultured at 37°C as a monolayer. Given more time, GC-MS will be repeated with both primary and hTERT cells after 12 days in alginate at room temperature to determine how amino acid requirements change at atmospheric conditions. A dramatic reduction in amino acid consumption is

expected, since cells do not proliferate at atmospheric conditions, and thus do not need the amino acids to make new proteins.

Alginate beads containing MSCs need to be co-stained with Annexin V and acridine orange to mark the location of early apoptotic and live cells respectively, and fluorescent images should be superimposed on a phase contrast counterpart to indicate the edge of the bead.

When the preservation media has been finalised and tested, an appropriate storage device will be developed to allow large scale preservation of MSCs in ITSE based media. The preservation density would remain at 6k cells/bead and 60k cells/mL. An example would be a polypropylene bottle containing 60 million cells in 1 litre of ITSE media.

7.4 – Concluding Remarks

Drawbacks on mesenchymal stem cell preservation have been addressed. The novel medium, once finalised, will eliminate the requirement of cryopreservation, refrigerators and serum, using a media that can be formulated using common and chemically defined cell culture ingredients.

The central hypothesis proposed that supplementation of DMEM basal media with ITSE will improve cell survival compared to cells cultured in basal DMEM, and keep MSCs functional in the absence of cryopreservation, refrigeration or serum. Only 8 days preservation time has been achieved under these conditions instead of the target of 12 days, and alginate encapsulation was needed to maintain cell survival in 3D at room temperature. The media can be further optimised by addition of growth factors, albumin, fat soluble vitamins and osmoprotectants, and the perseveration density needs to be reduced in order to achieve the desired preservation time of 12 days.

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