Hypothermic preservation of cellular products for advanced therapies.

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Abstract.

Cell and gene therapies (CGT) are showing positive results in managing a range of healthcare challenges, and one of the exciting examples is the successful treatment of relapsed acute lymphoblastic leukaemia using adoptive transfer of chimeric antigen receptor (CAR) T cells. Many of the processes in CGT product preparation and administration require optimisation, with the preservation of the therapeutic products being identified as a main challenge. Cryopreservation is the current practice, but it leads to losses in viability and functionality.

This research focussed on development of a novel process, hypothermic preservation, for short-term preservation (days rather than months) between manufacture and application to the patient at the clinic to avoid cryopreservation during transport. This study adopted Design of Experiments (DoE) as a method to rigorously establish a suitable base preservation medium using a fractional factorial design to minimise the number of trials performed, and assessing the responses by viable cell counts and measurement of metabolic activity at the end of the preservation period. Components with negative effects were identified and discarded, which significantly improved the preservation outcome. Optimisation of the medium composition by central composite DoE methods found that antioxidants played a role in the metabolic response of T cells after hypothermic preservation but were unable to maintain the cell functions themselves. Pre-treating T cells with interventions to inhibit cell proliferation forcibly reduced their metabolic activity before exposure to hypothermia and maintained complete functions after recovery. An investigation into cell sedimentation found that gelatin could avoid cell sedimentation but effectively paused T cell activity, which did not recover after preservation. The Ficoll® PM70 additive only slightly reduced the settling velocity but stimulated T cell activity on recovery. The experimental results indicated, however, that sedimentation during preservation did not negatively affect T cell function. A mass transfer computational model showed that oxygenation by conventional cell culture methods maintained suitable oxygen concentrations during preservation. A final study investigated the limitation on the duration of the proposed preservation methods.

Overall, it was concluded that T cells could be preserved up to 3 days using hypothermic preservation at 4^{o}C if their proliferation is paused before the procedure.

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Abbreviations and acronyms.

Abbreviation	Full name	Abbreviation	Full name
A1A, A2A	Adenosine receptors	ADP	Adenosine diphosphate
AICAR	Aminoimidazole carboxamide ribonucleotide	AICD	Activation induced cell death
ALL	Acute lymphoblastic leukaemia	AMP	Adenosine monophosphate
AMPK	AMP-activated protein kinase	APC	Allophycocyanin
ATOC	Alpha-tocopherol	ATP	Adenosine triphosphate
BES	N,N-bis(2-hydroxyethyl)-2-aminoethanesulfonic acid	BSO	Buthionine sulfoximine
CAR	Chimeric antigen receptor	CCD	Central composite design
CD	Cluster of differentiation	CGT	Cell and gene therapy
CIK	Cytokine induced killer cell	COG	cost of goods
CPDA	Citrate, phosphate, dextrose, adenine medium	CWID	Cytokine-withdrawal induced cell death

DCF	2′, 7′-Dichlorofluorescein diacetate	DNA	Deoxyribonucleic acid
DoE	Design of experiments	FBS	Foetal bovine serum
FDA	U.S. Food and drug administration	FEP	Fluorinated ethylene polypropylene
FFD	Fractional factorial design	Ficoll	Ficoll® PM70
FITC	Fluorescin isothiocyonate	HES	Hydroxyethyl starch
HOPE	Hypothermic oxygenated machine perfusion	HSP	Heat shock protein
HTK	Histidine-tryptophan-ketoglutarate	HTS	Hypothermosol® FRS medium
IFN-γ	Interferon gamma	IL-2	Interleukin 2
iRNA	silencing ribonucleic acid	IS	Immunological synapse
ME_2SO	Dimethyl sulfoxide	MHC	Major histocompatibility complex
mTOR	Mammalian target of rapamycin	mUW	Modified University of Wisconsin preservation solution
Na^+-K^+ ATPase	Sodium-potassium adenosine triphosphatase	NAC	N-acetyl-cysteine
NFAT	Nuclear factor of activated T-cells	NKG	Natural killer group
NMP	Normothermic machine perfusion	OFAT	One factor at a time
OKT-3	CD3 antibody	PBMC	Peripheral blood mononuclear cell
PD-1	Programmed cell death protein one	PE	Phycoerythrin
Pe	Peclet number	PEG	Polyethylene glycol
PFC	Perfluorocarbon	pKa	Acid dissociation constant
PMA	Phorbol 12-myristate 13-acetate	QbD	Quality by design
Re	Reynolds number	RNA	Ribonucleic acid
ROS	Reactive oxygen species	RSM	Response surface model
SAGM	Sodium, adenine, glucose, mannitol medium	SCS	Static cold storage
SUL-109	6-hydroxyl-2,5,7,8-tetramethylchroman-2-yl)(4-(2-hydroxyethyl)piperazin-1-yl)methanone	TCR	T cell receptor
TIL	Tumour infiltrating lymphocyte	Tregs	Regulatory T cells
Trolox	6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid	TUNEL	Terminal deoxynucleotidyl transferase dUTP nick end labelling
UW	University of Wisconsin preservation solution	VCC	Viable cell count

Symbols.

Symbol	Meaning (unit)	Symbol	Meaning
C	Concentration ($mol.m^{-3}$)	D	Diffusion coefficient ($m^2.s^{-1}$)
J	Flux ($mol.m^{-2}.s^{-1}$)	mmols	millimoles
N	number of biological replicates	Q_{in}	Heat input to the system (joule)
R_{cell}	Cellular consumption of oxygen ($mols.cell^{-1}.s^{-1}$)	t	Time (s)
T_c	Temperature of cold reservoir (K)	T_h	Temperature of hot reservoir (K)
u_f	Velocity ($m.s^{-1}$)	W_{in}	Work input to the system (joule)
y	Distance (m)	$\Delta\psi$	Mitochondrial membrane potential

1 Introduction.

1.1 Background.

Cell and gene therapies (CGT) are showing promise in overcoming healthcare challenges. Stem cells are one type that show suitability for a vast range of medical conditions [1], and the adoptive transfer of T cells is showing good results in treating malignancies and immunological disorders [2]. Some of these CGTs have gained regulatory approval and have been launched as commercial products, including; Kymriah, Yescarta, and Provenge [3]. These therapies can have a large overall cost to producing each dose, therefore, new strategies for the processing of CGTs are required [4]. To establish where this cost can be reduced, cost of goods (COG) studies have investigated the impact of centralised and decentralised manufacture on the overall cost of cell therapies [5,6]. It is suggested from these studies that, the more centralised manufacture becomes, the more important the correct technology for transportation becomes. Preservation is often regarded as a peripheral activity in CGT processing, but has been suggested to have a key role in the supply chain, particularly as it is a process that can impact the aforementioned transportation [7–9]. It is essential that a preservation strategy for a biological therapy product that involves transportation in the process chain must achieve two goals. Firstly, preservation must maintain the qualities of the CGT product developed in the processing that precedes it [10]. Secondly, preservation must use simple technology that facilitates the transportation of the product to, and the subsequent handling in, a healthcare facility prepared mainly for the care of critically ill patients.

Reasonable success has been achieved in cryopreserving T cells in research laboratory experiments [11,12]. This success has led to the use of cryopreservation, with specialist logistic chains and equipment for cryogenic temperatures, as the current low-scale method for preserving CGTs during transport [4,13]. However, cryopreservation is reported to

require further improvement through generating a better understanding of the molecular biology involved in the process to allow long-term cell recovery by avoiding preservation induced cell death [14,15]. It has also been suggested that the complex supply chain that cryopreservation uses, particularly the ultra-cold temperatures, will need replacement by CGT formulations and technologies that allow stability of the product at 2-8^{o}C and room temperatures [10]. Cryopreservation can maintain the qualities of a CGT product thereby achieving the first requirement of a preservation strategy. However, cryopreservation is an energy intensive method for preserving cells over short durations and requires complex equipment to achieve both mobility to distribute CGT products and the subsequent handling of the product at a healthcare facility. Therefore, cryopreservation does not achieve the second requirement of simplicity for a preservation strategy.

While cryopreservation is currently an acceptable, but limited, method for CGT preservation, it may eventually become an economic burden, and alternative methods to replace cryopreservation need to be identified to increase technology readiness so that they can be applied when required by CGT product manufacturers. Simple hypothermic conditions are utilised to reduce cell activity during storage of both blood and organs for therapeutic uses. This thesis investigates the methods required to maintain CGT quality during a preservation strategy at hypothermic temperatures. If quality of a CGT product were maintained during hypothermia, it would enable a simple, short-term preservation strategy for transportation of CGTs and storage at a healthcare facility.

1.2 Scope.

This thesis examines the practical methods to achieve low temperature preservation of CGT products. It does so by focussing on one example that appears to have eluded investigation of low temperature biology, which is the preservation of T cells following activation to achieve the biological state applied in the end-use of adoptive transfer to treat cancer. The specific example of the biological state of T cells used in this study was cytokine induced

killer (CIK) cells. All the T cells used in this study were cultured in-vitro in a method closely matching a published protocol for the generation of CIK cells that were tested in-vivo to treat solid tumours [16]. The exact protocol used to culture the cells throughout is detailed in Section 3.2.1.

It is known that the supply of fresh CGT products at 37^{o}C costs more than cryopreservation and can result in product wastage [17], suggesting that this temperature is not suitable for preservation and transportation. Cryopreservation at -80^{o}C and below is beyond the requirement for short term preservation, and the range of -60^{o}C to -15^{o}C are the temperatures where cryopreserved cells suffer the most resulting damage [18]. Excluding these temperatures leaves the option of supercooled or hypothermic temperatures. A supercooled temperature is a temperature below the freezing point of a fluid that occurs with cooling before solidification. Supercooled temperatures are proving useful in organ preservation, but still require the addition of cryoprotective agents to facilitate success [19]. These limitations suggest that a more suitable preservation condition must be found in the temperature range above 0^{o}C to less than 37^{o}C. Stability at 2-8^{o}C or ambient conditions would avoid the biochemical activity that would continue if cells were transported at 37^{o}C [20]. Therefore, hypothermia was investigated at two easily implementable temperatures. The first option was refrigeration over the range of 2 to 8^{o}C, for which a representative, controlled 4^{o}C was used throughout the data presented in this thesis. The second option was a controlled ambient temperature of 22^{o}C. The following studies evaluated membrane integrity; metabolic activity, both during hypothermia and subsequently after recovery; energy state of the cells on recovery; and the onset of apoptosis. These conditions combined represent the majority of tests performed in existing preservation literature and have been correlated to a number of different mechanisms relating to cell biology during hypothermic preservation. The studies presented within this thesis also improve on the majority of existing biological preservation literature by assessing and achieving a therapeutically active CGT after

preservation. Therapeutic activity was assessed by performing in-vitro cytotoxicity assays in addition to the previously noted tests.

One challenge of generalising the specific study of T cells used in CGTs detailed in this thesis is that requirements for a successful hypothermic preservation method could be cell-type specific [21]. This challenge may be compounded further due to uncertainty in the cell types required for a "blockbuster" therapy [17]. To overcome this challenge of hypothermic preservation specialism, quality by design (QbD) methods were investigated to evaluate their suitability for optimising the practical aspects of preservation strategies to an individual CGT type. The use of QbD would readily allow re-optimisation of this work to any CGT that requires non-frozen preservation. The literature review establishes that the preservation of tissue engineered therapies can be achieved for long durations with simple methods, which may not urgently require further investigation. However, QbD methods may help to facilitate transfer of concepts developed for the example of T cells.

1.3 Aims and objectives.

Cryopreservation is effective at recovering all pre-preservation cytotoxic activity of T cells [11,22,23]. However, the same cryopreservation studies reported that this cytotoxic activity was accompanied by a decline in other biological aspects of T cells, which suggests that these aspects may not be important to consider in overall activity of a preservation method. A combination of these two concepts led to the primary definition of achieving successful preservation in this thesis: T cells preserved with hypothermia must display an indistinguishable difference in cytotoxic action against a target cell line before and after preservation. This definition was further justified by literature review that showed T cells have limited mechanisms for achieving cytotoxicity over target cells. This suggests that, if there was no difference in cytotoxicity recorded, the T cells were functioning in the expected way.

A preservation duration of 3 days was targeted because it would allow the transportation of the CGT product to take place [24]. Achieving this duration would then be sufficient to replace cryogenic temperatures for the duration of transportation and allow healthcare facilities some flexibility by storing a product that does not require complex cryogenic equipment. An extension of this objective to 7-10 days would be ideal because it would then allow the quality control aspects of T cells to take place during the hypothermic preservation [8]. If the objective of maintaining T cell cytotoxicity over the longer duration cannot be achieved, then it may be sufficient to maintain membrane integrity to allow future studies to take place for identification of the biochemical changes that are affecting the preserved cells. Membrane integrity itself is another vital requirement for CGT products [10].

As a final aim, it would be desirable for the T cells to remain stable on recovery after hypothermic preservation because this would indicate longer term persistence of the cells after infusion to the patient. This could be evaluated by the ability of preserved T cells to avoid necrotic cell death for a period of 24 to 48 hours after recovery to 37^{o}C, which would indicate that the preserved T cells remain free from preservation-induced, delayed-onset cell death [14]. If the cells survive this period intact, they should be recoverable longer term, but this may be complicated by the nature of T cells in an immune response. The role of T cells in an immune response is discussed in the literature review.

1.4 Structure of thesis.

Chapter 2 presents a review of existing literature to understand the methods that may be required to preserve T cells for a CGT. In this review, the biology and the complex immune response mechanisms specific to the T cell are discussed. In addition, the specific challenges in the current methods of the CGT processing chain presents are identified. The review establishes how three examples of live cell products are preserved for therapeutic uses. Finally, the review establishes the current understanding of low temperature cell biology,

which mainly examines studies that investigate isolated cells as a model for tissue and organ preservation.

There was a diverse range of knowledge available from existing studies, which was tested in combination by Design of Experiments (DoE) methodology in Chapter 3 to generate a base preservation medium for the subsequent studies. This study investigated a widely used preservation medium, University of Wisconsin solution. This medium was found to have components that displayed negative effects on T cell response, which served as justification for the later studies to develop novel methods to preserve T cells.

Chapter 4 uses DoE response surface models (RSM) to investigate the requirement for compounds that stimulate the activity of T cells with the application of hypothermia. This chapter also used one-factor-at-a-time (OFAT) methods to identify the requirement of antioxidant compounds to increase metabolic activity on recovery.

Chapter 5 describes a study that resulted from the lack of ability to either maintain or stimulate metabolic activity recovered as discovered from the results in Chapter 3 and Chapter 4. Chapter 5 therefore shows that inhibition of T cell activity before exposure to hypothermia would be required.

Chapters 6 and 7 assess processes that could occur during preservation that might negatively affect the preservation. Chapter 6 investigates the requirement to suspend cells during preservation, comparing; liquid medium control; a method that reduces settling velocity; and a method that avoids settling by complete encapsulation. Chapter 7 uses computational modelling of mass transfer to understand if oxygen diffusion and consumption could lead to development of severely reduced concentrations that may affect a T cell response. A long-term hypothermic preservation study was conducted to establish if the methods developed had any limitations when applied for longer than the initial successful 3 days.

Chapter 8 presents overall conclusions drawn from all five of these studies.

2 Literature Review.

2.1 Introduction.

Cancer is a major cause of death and is expected to be the largest global barrier to increasing life expectancy in the 21st Century. Established treatments have some curative effects with mortality at about 53% across all cancers [25]. Successful cell and gene therapies (CGT) that can be very effective at targeting and killing tumour cells may be suitable for treating the disease in a large numbers of patients [26]. CGT could reduce mortality rates. There were 85 CGT clinical trials in the UK in 2018, an increase over the previous year. 41% of these trials used T cells [27]. This suggests this cell type could be one of the prominent cell types in therapies seeking further approvals in the near future, but this will be dependent on the trials to demonstrate their suitability. T cells are used in CGT to take advantage of their role in the immune system. The use of adoptive transfer of CGT consisting of a range of T cell populations is demonstrating a lot of promise in treating various malignancies. The range can be categorised on the basis of: already possessing desirable tumour killing aspects, such as tumour infiltrating lymphocytes (TIL); by genetic modification of the T cells to achieve targeting capabilities, for example chimeric antigen receptor (CAR) T cells; or by in-vitro culturing with cytokines, such as cytokine induced killer cells (CIK). These three examples are known to induce lytic capacity against target malignant cell types [16,28,29]. There are many challenges to developing the adoptive transfer of T cells into a successful cancer treatment. There are biological challenges that must be overcome first to prove the applicability of the therapy [30,31]. There are also engineering challenges such as culturing cells on a large scale to obtain enough cells for treatment [32]. Finally, there is the challenge of preserving the living cells in the therapy until they are applied to the patient [33].

2.2 Cell and gene therapies.

Cell and gene therapies (CGT) cover a range of medical activities that use live cells as a method to provide immunomodulation, protect existing cells, or use those live cells as a

method to deliver a genetic alteration, and is a rapidly growing field [34]. The cells used can be autologous, taken from and used in the same patient, or allogeneic, where cells are taken from a different donor to the patient, both of which create challenges for delivering the live cells [8]. The majority of trials currently show a 2 to 1 ratio in favour of autologous products [27]. Stem cells have been used as either autologous or allogeneic therapies [1], whereas the favoured method for the currently popular T cell is that of autologous adoptive transfer [13,35]. However, there is some evidence to support allogeneic use [36]. The flow chart in Figure 2-1 depicts a typical process flow of an autologous therapy developed from information in existing literature discussed in this review. The following review focuses on the methods that T cells can be used for to treat malignancies.

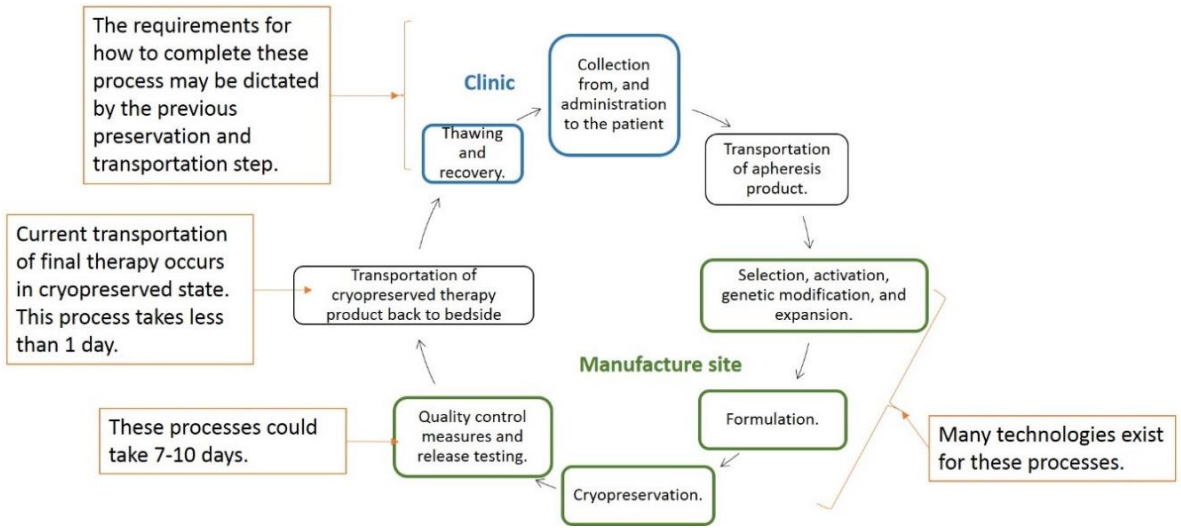

Figure 2-1 The chain of cell therapy processing for an autologous therapy. The starting material is collected from, and returned to, the same patient. In an autologous setting, the start and end-point would be different people, but would likely still require the shipment of live cell products between a clinic and manufacture site.

2.2.1 Chimeric antigen receptor T cells.

Genetic engineering of the adoptive population of T cells with chimeric antigen receptors (CAR) is demonstrating high response rates against B cell malignancies, particularly Acute Lymphoblastic Leukaemia, (ALL) [2,29,37]. CAR T cells are genetically engineered by transfection with a surface receptor that is capable of targeting tumour cells, for example, T cells transfected with CD19 specific CAR can induce remissions in B cell malignancy. The main anti-tumour mechanism is believed to be through release of granules that contain

perforin and granzyme; blocking of the Fas ligand or perforin formation both reduced the killing capacity of CAR T cells [38]. CAR T cells are reported as releasing inflammatory cytokines, Interleukin 2 (IL-2) and interferon gamma (IFN-γ), after CD19+ K562 cells have activated the CAR [39]. This cytokine release may be one contributor to the cytotoxic side-effect of CAR T CGT, termed cytokine release syndrome [29]. Also, ALL is just one type of numerous, different malignancies, and research is being completed on achieving functionality of the adoptive populations to work on solid tumours [30,40]. Although the CD19 CAR T cell has been shown to be cytotoxic against ALL, the other requirements for the T cell subset have yet to be decided. It has been shown that CD4+ CAR T cells share CD8+ CAR T cell cytotoxicity but, due to their CD4+ nature, they were more persistent in-vivo and prevented long term relapse [41,42]. In summary, there are some limitations to the use of CAR T as a clinical therapy. However, there is still great promise shown by this process of adoptive transfer of T cells and, therefore, work on improving the production chain must be conducted to improve scale-up or scale-out of the therapy [4,13].

2.2.2 Adoptive transfer of tumour infiltrating lymphocytes.

Another example of adoptive transfer of immune cells to treat cancer are tumour infiltrating lymphocytes (TIL). TIL are excised from near to a tumour site, expanded in-vitro, and then adoptively transferred back to the patient [43]. However, initial trials produced complications related to cytokine release [28,44]. Lymphodepletion protocols improved results, but challenges remain with the “youth” of the cell linked to CD28 expression [45]. Antigen specific T cells for treatment of cancer have also been explored. For this treatment a specific population from lymphocytes is identified, then activated by the T cell receptors and cytokines to expand cell number to achieve a therapeutically active cell type, and then infused back to the patient [46]. T cells manipulated by simple protocols can also be used as effective treatments.

2.2.3 Cytokine induced killer T cells.

There are also a number of other types of T cells that can be used in adoptive transfer to treat cancer. Cytokine Induced Killer cells (CIK), which are a CD3+CD56+ subset of T cells, achieve cytotoxic effects against lymphoma models and leukemic blasts [47,48]. CIK cells have also been tested against myelomas with some efficacy reported and investigations continue in to the use of this cell type in treatment of lung cancer [49]. Furthermore, research has recently been completed on the efficacy of the therapeutic product such as interaction between CIKs and suppressor cells [50]. The CIK cell type is another potentially useful therapy. CIK cells can be differentiated from Peripheral Blood Mononuclear Cells (PBMC) populations by IFN-γ and CD3 antibody (OKT-3), and then maintained with the addition of IL-2 with in-vitro culture for 21 days [16]. Activation with CD3 in combination with CD28 is also possible, and increases the expression of the natural killer group (NKG) 2D mRNA compared to that of CD3 alone [51,52]. In small trials, CIK cells are often formulated into an infusion buffer and applied to the patient within a period of hours [16,53]. In other trials, cryopreservation was used during in-vitro processing with no reported negative effects on function [54]. The CIK population can be simply achieved by culturing a PBMCs starting population with readily available antibodies and cytokines. Because the CIK cells share the same target cell group of malignant cells as CAR-T therapies, it could be an ideal model population for investigating methods that can be successfully used to preserve the cells between production and application to the patient.

CIK cells recognise tumour cells through the ligation of the Major Histocompatibility Complex (MHC)-unrestricted-, and T Cell Receptor (TCR)-independent-, NKG2D receptor by the target cells [55]. Once the latter receptor is ligated, the CIK cells release granules that take part in target cell death [56]. In agreement with that mechanism of action, it was shown that enriched CIK populations co-cultured with K562 cells displayed an increase in expression of NKG2C and NKG2E along with perforin genes in the CIK cells [57].

However, another mechanism, independent of granule formation, was also shown to exist when granule formation was inhibited by removing extracellular calcium, but cells showed cytotoxic activity [58]. This other mechanism is related to membrane bound tumour necrosis factor on the T cells [59], and has been shown to produce a much slower rate of death in the target tumour cells [60]. Although there are two known mechanisms of action of CIK cells, the different rates these take place at would indicate that if no difference in cytotoxicity is reported over a fixed duration of a cytotoxicity assay, then there is no difference in the method of action of the cells. The activated CIK cell must be capable of achieving a sequence of biochemical events in order effect a cytotoxic action on the target cell, which must be retained after preservation if the stored cell population is to be therapeutically useful.

2.3 Storage and transport of Cell and gene therapy products.

The current method for T cell CGTs is based on adoptive transfer and, as such, is an autologous therapy. The Cell and Gene Therapy Catapult review confirms that the majority of current trials use autologous cells [61]. In adoptive transfer a patient's own cells must be collected, isolated, cultured, and manipulated in-vitro to produce the intended effect, then returned to the patient by infusion [32]. The return to patient in a small scale clinical trial is often simple. In one example trial, T cells are harvested from culture, re-suspended in a simple medium, and infused into the patient 60 minutes later [62]. The effects on cell quality over this short period are reversible [20]. However, the chemical formulation of this simple medium, named Normosol, means that it is unlikely to sustain living cells for much longer. Another CGT product related to peripheral blood mononuclear cells is Provenge, a product that has an 18 hour shelf life at 2-8^{o}C when preserved in a medium containing sodium chloride, sodium lactate, potassium chloride and calcium chloride [63]. These basic preservation protocols and short times between manufacture and infusion to the patient are assumed to be sufficient for the small scale clinical trials, but are likely to be inadequate when the therapies are applied on a large clinical scale. For the current scale of CAR-T

application, the autologous products are cryopreserved and stored at cryogenic temperatures [4]. This use of cryopreservation is, perhaps, because the technology is more mature.

In part of a study using a cost-of-goods (COG) prediction model, cryopreservation was compared to preservation at 37^{o}C, shipping the cells as a fresh product, which found that the fresh preparations of cell products, with stem cells as the example, could cost $400 more than the cryopreserved equivalent [17]. The model included terms for: materials (both dry and wet consumables), labour, quality control, indirect cost, and annual throughput of the processing chain. The conclusion regarding transportation from this study was that cryopreservation is currently the more viable method to preserve cells during transportation, but non-frozen transportation could be more useful with improved supply chain logistics and a reduction in loss of product [17]. Supply of a fresh CGT product would result in a continuation of unattended culture conditions during transportation, which may be one driver of the increased cost because specialist management products would be required to achieve this method. However, cryopreserved products would also require specialist equipment for handling the ultra-low temperature goods [64]. It is possible that a clinic would require specialist equipment to deal with a CGT product irrespective of how it is stored and transported.

A COG study on centralised, international CAR-T cell production showed that transportation was susceptible to becoming the largest contribution to overall cost during sensitivity analysis as consumable costs were reduced [5]. Existing studies show that reagents can be reduced, particularly the use of lentiviral vector for transduction through the use of microfluidic arrangements [65]. That example suggests that a reduction in reagent costs can be readily achieved and, therefore, methods to reduce transportation costs should be investigated.

One possible factor that favours the development of methods to ship biological materials at warmer, ideally ambient, conditions relates to the energy impact of transportation. A

forward-looking study suggested that energy use, waste, and environmental impact could be important factors for deciding between centralised and decentralised manufacture [6]. Cryogenic refrigeration systems have been known to have low Carnot efficiencies [66], which is defined by:

$$\eta_{carnot} = \frac{W_{in}}{Q_{in}} = \frac{(T_h - T_c)}{T_h}$$

Equation 2-1

Liquid nitrogen has a high energy density [67], which, by the first law of thermodynamics, means that energy has to be placed into the system to produce the liquid nitrogen. Its use in cryopreservation may mean that this energy is not recovered. A 2-8ºC refrigeration unit could have higher efficiency. Efficient use of resources could be an argument for seeking alternative methods to cryopreservation for transportation and storage of biological products.

2.3.1 Viability of cells for use in cell and gene therapy products.

Viability is the measure of structurally intact cells that are present in a sample and is considered to be an important way of testing the success of a preservation method. The Food and Drug Administration (FDA) guidance requires that viability must be known before a cell based therapy product can be released, and that viability must be stable during storage [10] [68]. A review of a phase three trial considered the difference between the positive results from an animal model trial that used cells that were live and active straight after culture, compared to an unsuccessful clinical trial where thawed-cryopreserved cells were used with a cell viability release criterion of 70% trypan blue dye exclusion by cells [69]. That comparison suggested that the inclusion of a preservation step could be one reason why the latter trial was unsuccessful [69]. This shows the importance of having a storage method that keeps the cells in a condition similar to those used in initial testing. Thus, viability is a critical consideration for successful storage.

2.3.2 Number of cells required for cell and gene therapy doses.

Another important factor that must be considered for storing and preserving CGTs is that different applications may require a different number of cells for a successful treatment, which could depend on several factors, such as the target condition and cell type used. If a higher cell number is required to be infused in a suitable set volume, then the resulting high cell density is the level that cells must be preserved at to avoid the need for manipulation at the clinic. The cell density can be constricted for CIK cells where 10^9-10^{10} cells are infused into the patient in 300ml of a medium known as Normosol [62]. It is not clear what effect cell density will have on a chosen storage method [8], but an increasing density of metabolising cells will increase the demand for nutrients. For some common cell lines the specific oxygen consumption rate was not found to change with density [70]. This could mean the increase in nutrient demand may be directly proportional. It is assumed that the cells should be stored at high densities so that enough cells for an effective dose can be administered to the patient in an infusible volume, which means metabolic demands must be considered during preservation.

2.3.3 Preservation and storage of blood and blood products.

A biological product that is successfully preserved is red blood cells, although the development of effective storage methods for this cell type has taken 90 years [71]. Hess identified that red blood cells are the most frequently transfused blood product, possibly because a shelf life in excess of 1 month allows flexibility to balance supply and demand in a clinical environment [71]. This type of biological product has also been suggested to be a good source of evidence for making decisions on how to process CGTs [64]. The preservation of this cell type provides some insight into the need for an effective preservation medium because it is commonly stored in a simple medium of sodium, adenine, glucose, and mannitol (SAGM) at 4^{o}C for up to 42 days [71]. These cells experience reductions in adenosine triphosphate (ATP), sodium and potassium concentrations, and also cell damage

due to free radicals during preservation in SAGM [72]. Adenine is present and influences the metabolism of the red blood cell [73]. Mannitol works both as a free radical scavenger and membrane stabiliser [74]. Glucose is present to support the cell metabolism during storage and the amount required is related to the density of cells that can be stored [75]. However, when assessing the composition of blood preservation medium by Design of Experiment methods, with discoid morphology of red blood cells as the response, it was found that glucose was not required to benefit preservation [76]. Therefore, glucose may not be a universally required component of the preservation medium when storing biological materials under hypothermia. That same study also found that caspase inhibitors and anti-apoptotic agents were required to support the preservation of the whole blood components at ambient temperatures [76]. Non-toxic caspase inhibitors alone were shown to exhibit pharmacological effects in reducing brain damage and infections following a stroke [77]. The addition of biologically active components like these inhibitors to a CGT product could affect the therapeutic action of the biological product and may instigate further processing before application. The preservation of blood products that retain therapeutic effects seems to be possible with hypothermia, and for long durations.

2.3.4 Organ preservation and storage.

Organ preservation science has existed for a number of decades, with successful transplants of kidneys preserved for 24 hours with perfused blood in refrigerated conditions reported in 1960 [78]. The introduction of specialised preservation medium, University of Wisconsin solution (UW), in 1987 enabled the successful preservation of canine pancreases for up to 72 hours in hypothermic conditions [79]. UW is used today in preference to other organ preservation media because the negative effects produced by its high viscosity and high potassium level, which can cause hyperkalaemia in-vivo, can be easily managed [80]. The replacement of the hydroxyethyl starch and potassium ions by other media produce little benefit and may not drastically alter the costs of production once regulatory requirements

are considered [81,82]. The main beneficial component of UW solution was suggested to be the impermeant compound lactobionate, which averts hypothermia induced cell swelling [81,82]. UW contains raffinose, which was suggested to be an insignificant component that could be removed [81,82]. But more recent studies have identified this component to increase the levels of autophagy in cell cultures, which would affect cell metabolic functions [83]. Preservation of cardiac tissue in a modified UW solution that contained 25mM of phosphate with additive buffers *N,N-bis*(2-hydroxyethyl)-2-aminoethanesulfonic acid (BES), bicine, and histidine, each at a 90mM concentration, found that BES increased lactate production and also reduced the loss of ATP, which suggested the additions were affecting cell metabolism [84]. Falling ATP concentrations in cells within organs is a similar result to that found during preservation of blood products. Inorganic phosphate has some hydrogen ion buffering capacity but, compared to other buffers, has a particularly low acid dissociation constant (pKa) that is below physiological values and makes it a less effective buffer [85]. The true effect of phosphate in the UW solution may currently be unknown. Therefore, the unknown action of a number of components in existing preservation media suggest that further study on the individual effects of preservation media are required.

The previous organ preservation studies discussed in this Section used static cold storage (SCS). Hypothermic machine perfusion of organs is suggested to offer an improvement in preservation because it has been shown that the flow of storage medium maintained an oxygen concentration that was beneficial for increasing adenine nucleotide levels [86]. Further interventions to oxygenate the perfusing media, hypothermic oxygenated machine perfusion (HOPE), have shown beneficial results in preserving fatty liver grafts in both rats and humans by reduced oxidative stress and reduced nuclear injury, both of which demonstrated that lower levels of reperfusion injury had occurred [87]. Organs, particularly the heart, show improved post-preservation results when the storage medium is oxygenated [88]. Oxygenation also has benefits in SCS. The two-layer method, which releases oxygen

to the tissue through a layer containing a perfluorocarbon (PFC) compound, showed improved islet isolation from the stored material after hypothermic preservation of pancreatic tissue [89]. Therefore, oxygenation is important during organ preservation.

Current studies on the temperature at which organs are preserved are deviating from refrigerated conditions. Normothermic machine perfusion (NMP) achieves successful transplantation from previously unviable donors [90], but uses complex equipment that may limit use of NMP to a short period of hours through high electrical power consumption [91]. Aqix has a similar formulation to a physiological medium and is consequently used at a mild hypothermic condition of 30^{o}C for successfully preserving porcine kidneys, and has also been shown to be affective in preserving stem cells at 20 to 37^{o}C between 12-120 hours [92,93]. Alternatively, organ preservation at super-cooled temperatures just below 0^{o}C, using polyethylene glycol and glucose derivatives as cryoprotectants, extended the preservation of SCS by 1 day as demonstrated by increased survival after transplantation in a rat liver model [19]. Changing temperatures greatly from refrigeration can introduce complexity to preservation.

Pre-conditioning organs with a short period of ischemia is a common strategy to reduce the effects of ischemia/reperfusion injury on recovery, especially when considering hepatocytes and liver transplantation [94]. The mechanism of physical preconditioning of organs could be related to the release of reactive oxygen species, mainly through allowing a short period of oxidative stress to occur, because N-acetyl cysteine (NAC), a known glutathione precursor and radical scavenger [95], was shown to reduce the benefits of physical conditioning [96]. Another study on rats showed that physical preconditioning by ischemia led to reduced reactive oxygen species (ROS) production on reperfusion by reducing the conversion of xanthine dehydrogenase to xanthine oxidase during 90 minutes of ischemia [97]. A similar role of oxidative stress in surgical hypothermia and rewarming was also

shown to exist [98]. These studies suggest that the ROS levels increased upon reperfusion, not during the ischemia.

Pre-conditioning of livers by hypothermia mitigated multiple pathways during liver ischemia: both P13K/AKT/FOXO3a and P13K/AKT/GSK3β have been shown to be associated with the pre-treatment at 32^{o}C for between 2 and 6 hours [99,100]. It is difficult to precondition organs without invasive measures, and this led to attempts to achieve the same effects with pharmacological methods of treating the organ. One method used compounds that react with adenosine receptors: A2A adenosine receptor agonists (CGS-21680 at 10μg/kg) and A1A adenosine receptor antagonists (ZM-241385 at 100μg/kg) produced a similar response to preconditioning rats with 10 minutes of ischemia and reperfusion before attempting a full surgical procedure [101]. These results are linked to adenosine levels and the A2A receptor has a known role related to suppressing inflammation [102]. An addition of 5'-adenosine monophosphate, a product of hydrolysis of ATP [103], can be shown to induce a torpor-like state in mice upon infusion, and is actually produced by mice forced into torpor by constant darkness [104]. This compound might also interact with adenosine receptors: adenosine receptor blocker, aminophylline, inhibited the cellular level response to adenosine monophosphate (AMP) [105]. Also shown in that study was the administration of adenosine, adenosine diphosphate (ADP), and ATP had a similar effect compared to AMP in reducing cell activity [105]. AMP activated protein kinase (AMPK) signalling could be linked to the above studies of preconditioning organs for transplantation [106]. It has been demonstrated in a rat model that the inclusion of AMPK activator, aminoimidazole carboxamide ribonucleotide (AICAR), reproduced the effects of physical preconditioning [107]. Pre-conditioning organs affects multiple biochemical pathways.

Also related to conditional shocks on mammalian cells is the response of protein synthesis. Heat-shock protein (HSP) with a 70kDa molecular weight, HSP70, production was shown to induce thermal stability in rat cells, which survived temperature changes better than cells

without, suggesting that the gene protects cells from thermal stress [108]. This protection was suggested to be unrelated to the protein's ability to bind to ATP [108]. There is also a correlation between HSP70 production and the surviving cell fraction from hyperthermic exposure to 45^{o}C for 75 minutes [109]. Other HSPs exist with protective effects conveyed during hyperthermia [110]. The protective effects of hyperthermic pre-treatment were also found to benefit normothermic ischemia in livers in a rat model, although this was only a short duration with 15-minute exposure to 42^{o}C, recovery for 48 hours, followed by 45 minutes of normothermic liver ischemia. The livers in the model had higher intracellular ATP and normal levels of enzyme activity compared to an untreated control [111]. Not only do HSPs protect against ischemia, but they also protect against subsequent hypothermic exposure [112]. A five hour heat shock in a human fibroblast model resulted in the smallest decrease in fibroblast cell membrane integrity after hypothermia, which linked to a maximum HSP70 concentration produced after heat shock [112]. Before commencing the second shock, a period of recovery at normothermic conditions is required, which in the case of human fibroblasts was 2.5 hours [112]. This suggests that the physical presence of the HSP could provide a protective mechanism against hypothermia related stresses, and that preconditioning cells to a stressing deviation from normal conditions may be required in order to achieve prolonged exposure to the same stress, or possibly a different stress. HSPs do, however, appear to be related to physical stresses.

Organ preservation appears to suffer from greater limitations compared to the preservation of blood products discussed earlier and, as such, a greater number of parameters have been investigated. These studies do not appear to extend the period of viability much beyond the 72 hours originally offered by UW, but often markedly improve the quality of the product, which relates to increased survival post-transplant. However, preconditioning appears to involve inflammation, protein production, and signalling pathways, which suggests that the response could be due to any number of cellular changes. The results all seem to agree that

preconditioning of organs prior to transplant greatly improves functionality after completion of the procedure, whether it is hypothermia or ischemia.

2.3.5 Preservation of tissue engineered products.

The prescribing information for the engineered skin tissue, Apligraf™, states that the package the cells are in contains an atmosphere of air with 5% carbon dioxide, and a reservoir of culture medium and supplements dissolved in an agar hydrogel, and that the tissue sits at the interface between the two phases. The whole package must be kept between 20-23ºC [113]. The location of the tissue between the two phases is assumed to allow both gas transfer and nutrient diffusion through the very thin tissue, an assumption confirmed by modelling studies provided the tissue is thin [114]. The example of Apligraf™ provides an insight into how a suitably long shelf-life is required for therapies during commercial roll-out post approval. This product was initially launched in 1999 with a short shelf life of just five days [115]. However, the prescribing information for Apligraf™ now specifies a shelf life of fifteen days [113]. This increase suggests that a more effective storage method was found in the product development after regulatory approval. Apligraf™ is an example of an allogeneic therapy, which has the advantage that it is off-the-shelf and any piece of tissue can be applied to any patient to be treated, however this advantage is only realisable if that piece of tissue was not manufactured more than 15 days previously. A paper on transport strategies agrees that when quality, safety, efficacy, transportation, and application to the patient are considered, a preservation window of at least 7-10 days may be required [33].

It has been reported that wastage resulting from unsuccessful, unfrozen, cultured epithelial autografts was markedly reduced by adding a simple carrier, a chemically defined surface called MySkin, to the epithelial cells during culture. This reduced the time required to produce the graft and greatly increased the flexibility in logistics in the study [116]. This study demonstrated that cell-containing therapies can be transported without incurring unusable product with an optimised procedure, whilst avoiding cryopreservation. Apligraf

and MySkin are examples of products that are usable after preservation when stored in a culture medium supported by a suitable surface, which is simple compared to the specialised media and equipment required to preserve blood products and organs.

2.3.6 Summary table for preservation of cell-based therapies.

The findings from the investigation of methods that blood products, organs, and tissue engineered products utilise for preservation are shown in Table 2-1. This table highlights that there is uncertainty about the requirement for some components, even within a type of biological product. To exemplify this point, blood products have been stored successfully with, and without, the presence of glucose in the preservation media. Similarly, a range of temperatures has been tested with the products detailed, with no clear difference established between the different studies conducted. Table 2-1 clarifies that a suitable preservation window for CGT products should be at least of the order of days, with the minimum reported duration for organs being 3 days.

Biological product	Metabolite requirements	Oxygenation requirements	Sensitivity to physical effects	Temperature used for preservation	Current preservation window
Red blood cells	Arguments for and against depending on parameter investigated.	None tested.	Aggregation and sedimentation.	Mainly 4ºC, ambient, at 22ºC, with apoptosis inhibitors included.	42 days, new interventions increase quality not duration.
Organs	Arguments for and against depending on biological model investigated.	Oxygenation, either statically or dynamically improves a range of organs.	Pressure of the perfusate.	Supercooled, 4ºC, ambient, and 37ºC tested.	Maximum of 4 days, significantly less if donor is compromised. New interventions can increase duration.
Engineered tissue	Commonly stored in culture medium, which contains additives.	Oxygen and carbon dioxide are transferred from gas headspace.	Possibly, storage on solid substrate improves preservation.	Room temperature.	Up to 15 days.

Table 2-1 A summary table of the key findings from review of methods and research on the preservation of blood products, organs, and tissue engineered products.

2.4 Biology of the T cell.

To understand how CGTs that use T cells can be preserved, a rigorous understanding of the biology of the cell may be useful to identify methods in which preservation can be achieved.

T cells in the immune system can be said to go through three separate growth conditions: a homeostatic phase, where the cells are inactive and maintaining normal conditions; an activation and expansion phase that requires suitable antigens to be present for viable expansion; followed by T cell death once the antigen is cleared.

2.4.1 T cell activation.

In the T cell CGTs discussed in Section 2.2, the T cells require in-vitro activation to allow for transduction and expansion. Before activation, T cells are in a state of homeostasis that results from the limited space of the immune system within the human body [117]. T cell activation takes place through the T cell receptor (TCR), which must be activated for T cell expansion to take place [118]. This activation can be achieved by; anti-CD3/4-1BB antigen-presenting cells; which were found to be more effective than anti-CD3/CD28 cells; which in turn were better than anti CD3/anti-CD28 beads to generate CD8+ T cells [119]; although, cells activated with CD3/CD28 Dynabeads with a CD4+ and CD8+ population of cells were effective in trials against ALL [29]. The difference in ratio of helper to cytotoxic cell quantity in a T cell population could be related to how the antibody is presented to the cell. The lower percentage of CD8+ T cells can be altered by the way in which the CD3 and CD28 are presented to T cells, with 70um length 4.5um diameter lipid coated rods with CD3 and CD28 antibodies showing larger fold expansion and higher CD8:CD4 ratio compared to Thermofisher Dynabeads [120]. There could be many reasons behind this phenomenon with a number of differences between the agents. Both CD3 and CD28 antibodies are capable of generating traction forces in T cells when presented on a grid structure [121], which could suggest that the manner in which the T cell binds to the antigen affects how the T cell performs after activation. Also for consideration is the final population that has the best clinical performance: CD8+ cells displayed more cytolytic capacity with quicker tumor killing, but relapses occurred with that subset, whereas CD4+ showed more persistence and

remission [41]. There are many methods to activate T cells and they can alter the phenotype of the population

2.4.2 T cell anergy.

Anergy in T cells is defined as a state in which the cell is functionally inactive after an initial antigen load, but remains alive in a hypo-responsive state [122]. T cell anergy is linked to activation of AMPK signalling, which was shown to inhibit IL-2 and IFN-Y production, but not cell death, suggesting that a lack of ATP reserves will inhibit immunological function of the T cell [123]. Activation of AMPK in T cells can be achieved chemically by AICAR [123]. T cells can be recovered from the state of anergy by adding exogenous IL-2 to the suspension, which will affect all the T cells in the population and will also increase lactate production [124]. This a sign that the T cell is fully stimulated because it is displaying glycolytic metabolism, discussed in more detail in Section 2.4.4. T cells recovered from a chronically infected murine model expressed markers of exhaustion, programmed cell death protein one (PD-1) expression, and retained this exhausted expression after re-expansion, but produced secondary effector cells when challenged with an acute infection [125]. T cells can change their response without cell death, which a preservation method must avoid.

2.4.3 T cell death.

T cells can experience cell death on presentation with an antigen, known as activation induced cell death (AICD), which has been reported to occur when T cells were activated with phorbol 12-myristate 13-acetate (PMA), ionomycin, or a combination of CD3 and CD28 antibodies. This cell death was shown to occur in 12 to 20 hours depending on the method of activation [126]. Mature post-thymic T cells resist cell death related to antigens but become particularly sensitive to cell death when they start to pass through the cell cycle to begin a proliferative response. This sensitivity to apoptosis was shown to occur with cells blocked in the S phase of the cell cycle, whereas cells blocked in the G1 phase were less susceptible to cell death [127]. Autophagy has a role in both cell death and survival because

it is able to control nutrient recycling within the cells, directly cause cell death, and trigger apoptosis [128]. In-vivo, T cells were found to dynamically control autophagy during activation by viral infection: T cells displayed maximum accumulation of autophagy associated proteins between 5 and 8 days after activation without associated increases in related mRNA [129]. Conversely, it has been suggested that in-vitro T cell activation led to an increase in autophagy, which was shown to, at least, have a role in controlling the energy demand of the proliferating T cells [130,131]. T cells activated with CD3/CD2/CD28, sustained in culture medium initially with IL-2, then suffered from cell death that increased with frequency over 72 hours following withdrawal of the IL-2: this cell death was suggested to be controlled by autophagy [132]. Apoptosis was also reported to take place after withdrawing IL-2 from stimulated, cultured T cells [133]. Similar apoptosis and cell death was found after IL-2 is removed from proliferating T cells with the apoptotic response blocked by addition of the CD18 antibody [134]. This mechanism of cell death after withdrawal of cytokines is known as cytokine withdrawal induced cell death (CWID). With some mechanisms causing T cell death after antigen withdrawal, and others creating an apparent ability of T cells to enter an unresponsive state, methods to avoid activating these mechanisms may need to be developed in order to successfully preserve activated T cells.

2.4.4 The metabolism of activated T cells.

T cells experience a form of low metabolic rate that is primarily reliant on oxidative phosphorylation when in a quiescent, inactive state [135]. When T cells were activated with CD3 and CD28 there was an overall increase in metabolism, which, under high glucose concentrations, became biased towards glycolysis yet, under low glucose conditions, returned to being dominated by oxidative phosphorylation [136]. There was also an increase in mitochondrial activity when T cells were co-stimulated by CD28 antibody, as shown by an increase in maximum oxidative phosphorylation along with elongation of the mitochondria itself. CD3 stimulation without co-stimulation by CD28 resulted in the

population of memory T cells with low metabolic demand that failed to respond to a second load of antigens [137]. Activated T cells are metabolically active and highly proliferative, which may require interventions to achieve successful preservation of the cell type. The flow chart in Figure 2-2 depicts a summary of Sections 2.4.1 to 2.4.4.

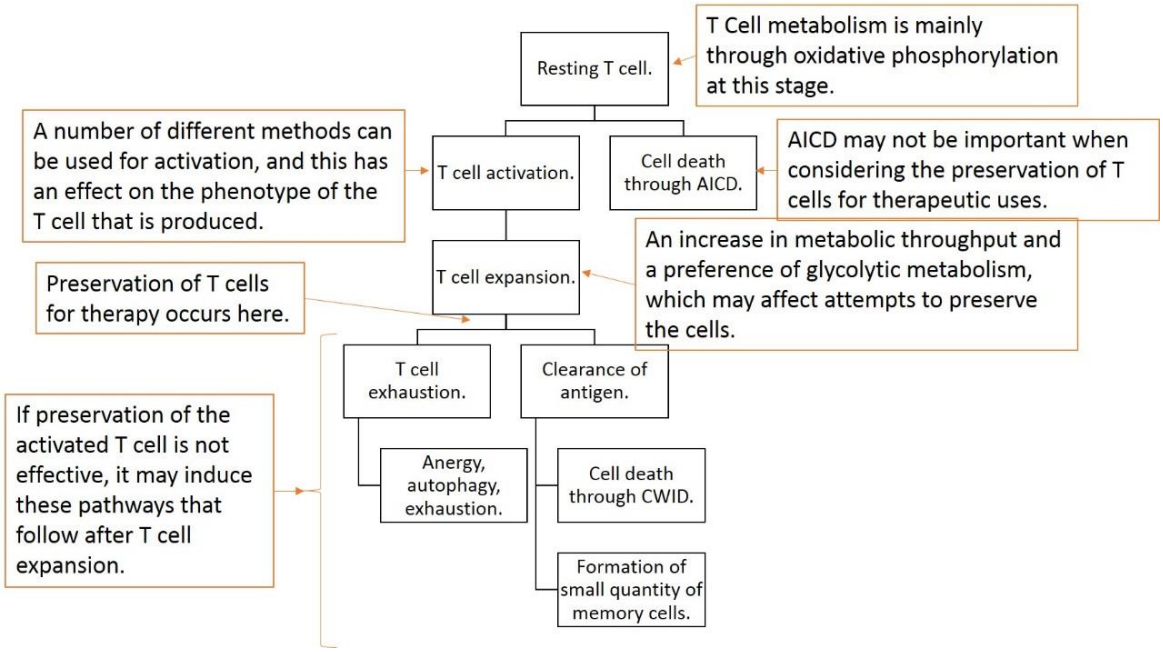

Figure 2-2 Progression of T cell activities in response to presence of an antigen. Memory cells adopt a similar resting metabolism compared to resting T cells until re-stimulation. Note how the first three processes are similar to some shown in Figure 2-1. It is likely that the steps following T cell expansion will occur if the therapy is not preserved correctly.

2.4.5 The adenosine triphosphate content of T cells.

The stimulation of T cells with CD3/CD28 Dynabeads caused a rapid increase in both extracellular and intracellular ATP, ADP, AMP, and adenosine: there was also a large increase in intracellular ATP after 30 seconds that stabilised at a peak of 4.5mM and then dropped towards basal levels, all in a cycle lasting approximately 5 minutes. The stimulated T cells produce more ATP and release a large quantity of this into the extracellular space. This release of ATP can be related to the formation of the immunological synapse (IS), as evidenced by the relocation of mitochondria towards the IS that forms during T cell activation [138]. When cultured with an excess of irradiated allogeneic PBMCs; a CD3 antibody; and IL-2, in a rapid expansion protocol for 14 days, the intracellular ATP concentration remained similar compared to that of freshly isolated cells, the baseline

detected in this study was 400-800 ng (ATP) /ml (lysate) [22]. During in-vitro culture, intracellular ATP remains constant in the long-term, with changes due to the activation process diminishing quickly.

Extracellular ATP has multiple effects on T cells depending on whether they are regulatory or activated sub-sets of the cells. Regulatory or activated CD4+ cells are not affected by physiological concentrations of extracellular ATP, but cytokine release was stimulated by 250nM of ATP in activated T cells and, when 1mM is reached, this causes inhibition of cytokine release and apoptosis, whereas regulatory cells are stimulated by 1mM of extracellular ATP [139]. Adenosine is a precursor to ATP, and extracellular concentrations of this compound can regulate naïve T cell activity. This was shown by Ohta et al. when differences in T cell homeostasis occurred in mice models with, or without, the A2A receptor gene that relates to one adenosine receptor: removing the effect of the gene resulted in a decline in number of naïve but not memory T cells [140]. Stimulation of A2A receptor resulted in a population of activated effector T cells that showed lower cytokine release, which suggests that, should extracellular adenosine be present, then immune function of activated T cells will be impaired [141]. ATP energy stores are sensed by AMPK [142]. As discussed in Section 2.4.2, activation of AMPK signalling stopped cytokine production by T cells, which suggests that maintenance of intracellular ATP in T cells is important. Due to the purinergic signalling that T cells take part in, exogenous nucleosides may not be ideal for preservation.

2.4.6 Reactive oxygen species in T cell biology.

Glutathione is one of the main compounds to have antioxidant effects within cells, and can have a role in controlling the concentration of ROS present within cells. Glutathione is converted to a reduced form and water by reacting with hydrogen peroxide, a reaction catalysed by peroxidases [143,144]. The inhibition of glutathione production by buthionine sulfoximine (BSO) was identified to permit T cell activation, but showed that these cells did

not move to a cell cycle distribution that represented proliferating cell distribution [145]. It has further been shown that, in mice that were genetically unable to produce glutathione or in cells that had glutathione production chemically blocked, the glutathione depleted T cells were initially activated by CD3/CD28 antibodies, but unlike normal cells, those without glutathione were unable to complete the rapid expansion that followed. The T cells without glutathione also suffered from higher levels of ROS production compared to the control samples [146]. In that study it was shown that the deficiency stopped mammalian target of rapamycin-1 (mTOR) activation and compromised expression of the nuclear factor of activated T-cells (NFAT), which reduced energy utilization and stopped the metabolic switch to favouring glycolysis [146]. Glutathione depletion was also shown to affect the CD4:CD8 ratio of murine T cells [147]. Those studies show that the effect of the removal of a natural antioxidant compound that occurs in cells relates to increased ROS formation and results in inhibited cell functions. Also for consideration is that some quantities of ROS are required for signalling purposes. Murine T cells, which lacked mitochondrial ROS, had lower proliferation capacity and produced IL-2 at reduced rates [148]. In conclusion, there appears to be a concentration of ROS that is required for activated T cells to function properly, although the studies that investigated the role of ROS have focussed on each extreme so that this range is not quantifiable by existing literature.

2.5 Cryopreservation.

Cryopreservation is a commonly used method to preserve cells for extended periods of time and uses ultra-low temperatures to completely stop cell activity. The cryopreservation process has been shown to cause manageable viability loss in many types of cell [7], but for some cell types such as stem cells, the cryopreservation process is detrimental [149]. The overall effect of cryopreservation on T cells has recently been established, and generally shows that cytokine release by cryopreserved, thawed, and then cultured T cells is reduced. A cryopreservation protocol using 10% (vol.) dimethyl sulfoxide (ME_2SO) in heat

inactivated foetal bovine serum (FBS) with an undisclosed cooling rate was shown to reduce the production of inflammatory cytokines of a CAR-T cell model, however, the cells were still shown to effect an insignificantly different amount of tumoricidal activity on RPMI826 and U266 target cell lines [12]. After activating a T cell culture and maintaining them under culture conditions, increasing the time point at which cryopreservation of these cultures occurs has been shown to increase the overall proliferative capacity of the cultured cells after completing a total in-vitro culture of 14 days [23]. The T cells that were cryopreserved before activation were shown to suffer significant changes to the cell phenotype after thawing and completing subsequent culture. The study used 10% (vol.) ME_2SO in FBS and the cooling rate was disclosed as -1^oC per minute and showed that IFN-y secretion remained stable with cryopreservation, but that IL-10 secretion was significantly reduced by cryopreservation [23]. The use of a similar cryopreservation recovery as above, except ME_2SO was at 15% (vol.), showed that cryopreservation of PBMC populations produced T cells that became sensitive to the activation compound when thawing and culturing, as demonstrated by significant changes to cytokines released by the cultures [150]. Furthermore, it has been shown that cryopreservation of T cells after an antigen-stimulated rapid-expansion protocol produced T cells that had a low concentration of ATP after cryopreserving and thawing, which required a period of antigen- and cytokine- free cell culture to recover intracellular concentrations [22]. That recovery period also allowed the T cells to produce inflammatory cytokines at a greater rate compared to that immediately after cryopreservation [22]. Once at a clinic, this period of recovery may be impractical to achieve. Cryopreservation of T cells is technically possible, but there are strict requirements for effective preservation.

It has been suggested that a better understanding of the effects that freezing and thawing have on cell function is required [151], which may be possible by further investigation into molecular based strategies to understand how the cell death occurs in a period of hours to days following recovery from preservation [15]. This is a longer-term effect often missed in

the cryopreservation studies performed on T cells specifically. This requirement for further work suggests that cryopreservation is an established technology, but may not be completely ready for large scale use.

The sub-zero temperature of cryopreservation means the cells would need thawing at the clinic, which is another process that has some adverse effects on the post-thaw quality of the cells. Ramachandran et al. reported that the slow addition of medium at 37^{o}C resulted in the increased recovery of cryopreserved peripheral blood mononuclear cells (PBMCs) [152]. In some cases, the cryo-protective agent, which is the component included to protect the cells from ice formation, may need removing due to some reported side effects of cryoprotection agents [31,153]. The need for removal introduces further undesirable processing at the clinic. The equipment required to maintain cryogenic temperatures in order to implement the method also requires consideration: cryopreserved cells could be transported with dry ice at -78^{o}C as coolant or in specially qualified vapour phase liquid nitrogen containers at -160^{o}C [154]. Although cryogenic temperatures are ultra-low, it is essential to maintain them at a constant level to avoid fluctuations that can cause a reduction in post-preservation viability [155]. This means using cryopreservation to transport biological therapies is a complex process. Also, there is a great dependence on the container that holds the cryopreserved cell suspension: it was identified that bags used for cryopreservation purposes suffered periods of inconsistent batch quality that led to container breaches, and consequently, high rates of bacterial contamination despite further interventions [156]. Cryopreservation allows for long term banking of samples; however, the trade-offs for achieving this may make it an unsuitable technology for delivery of CGTs to the clinic and subsequent storage there until application to the patient.

2.6 Hypothermic preservation.

Hypothermic preservation is the use of temperature below the normal culture condition of 37^{o}C to reduce the metabolic rate of cells and other biological materials [20]. Hypothermia

as a medical condition is defined as a drop in body temperature below 35^{o}C, which is a serious condition for a human being, whereas cells isolated from the human body usually have more tolerance for surviving cold conditions [157].

2.6.1 Temperature response of biochemical reactions.

All biochemical reactions that occur in a cell are known to respond to temperatures with an Arrhenius relationship: the oxygen uptake rate in isolated mitochondria from kidney cortex followed this Arrhenius relationship, where the reaction rate is exponentially related to temperature [158]. Jorjani and Ozturk agreed that oxygen consumption followed an Arrhenius relationship when testing other cell lines, but also concluded that cell types have different consumption rates [70]. These different rates suggest that different cell types may require a differing amount of oxygen during storage. A reduction in oxygen uptake rate is one biochemical reaction that can be taken to represent the general drop in metabolic rates with temperature due to the lack of intracellular oxygen store [20]. The demand for other nutrients will also be reduced at lower temperatures because other biochemical reaction rates will also slow with temperature reduction [159], suggesting that an optimum storage medium will contain components different to that of normal culture media. The reduction, but not cessation, of biochemical reaction rates at temperatures above 0^{o}C makes hypothermia a useful storage method, although the remaining activity must be managed successfully for an effective storage method. The preservation of robust cell lines at different temperatures of hypothermia in culture medium showed that low temperatures of 1^{o}C and 5^{o}C resulted in high rates of apoptosis and necrosis, with slightly lower levels of these parameters at 16^{o}C and 22^{o}C in an M2 cell line [160]. The more temperature is reduced, the less metabolite supplementation that may be required, but the trade-off could be more cell death occurring in the cell product.

2.6.2 Membrane ion pumps.

One reaction that is affected greatly by temperature is the activity of the membrane ion pump for sodium and potassium ions. Sodium-potassium adenosine triphosphatase (Na^+-K^+ ATPase) is reported to be at only 1% of its normal activity in some cell types when measured at 5^{o}C [161,162]. This lack of activity allows some ions to diffuse across the membrane when concentration gradients exist, meaning that ionic imbalances lead to osmotic cell swelling and eventually death: this is an early step in the pathway that leads to cell death during hypothermia [163]. A medium that has concentrations of ions matching intracellular levels used during hypothermia might avoid that mechanism of cell death. If the concentrations of sodium and potassium ions is correct there should be no driving concentration for diffusion between intra- and extra- cellular environment, thereby stopping the flux of ions once Na^+-K^+ ATPase activity is reduced.

2.6.3 Colloidal swelling.

The swelling of cells resulting from ionic imbalances was discussed in Section 2.6.2, but another mechanism that leads to cell-swelling in hypothermic conditions is oncotic pressure. The lack of function of the ion pump means that water fluxes can occur due to the colloids present inside a cell [20]. It has been demonstrated that the inclusion of colloids in the storage medium has a beneficial effect on the cornea during storage [157]. Experiments both with and without including the large molecular weight colloid, hydroxyethyl starch (HES), in a University of Wisconsin (UW) medium resulted in the successful preservation of organs, although the inclusion of HES produced slightly better results at longer storage times [164]. The component that provides the oncotic pressure relief needs to be determined: HES is used in several current generation mediums, but other research suggested components like polyethylene glycol can provide storage benefits [165]. Another study presents the comparison of red blood cell aggregation in mediums containing either polyethylene glycol (PEG) or HES, with HES causing more aggregation [166]. There are conflicting results to

the use of colloids during preservation: the impact of colloidal components on the preservation of cells during low temperature exposure must be determined.

2.6.4 Deoxyribonucleic acid damage and hypothermia.

Deoxyribonucleic acid (DNA) is the store of the information for the synthesis of proteins [167]. DNA fragmentation was shown to increase during hypothermic preservation of porcine hepatocytes, and this was significant when the cells were exposed to hypothermia at 4^{o}C in preservation mediums histidine-tryptophan-ketoglutarate (HTK) and Celsior, but not in PBS or UW [168]. A HMP study found that perfusion reduced the DNA damage when higher amounts of DNA repair enzymes and lower levels of oxidative stress were present, compared to a static control during preservation of canine hearts [169]. That study suggests that DNA damage during hypothermia is related to ROS. Furthermore, when catecholamines were used as additive compounds to preserved human umbilical vein endothelial cells with 4^{o}C hypothermia, it was found that those compounds with antioxidant activity reduced the amount of DNA and mitochondrial damage, as confirmed by terminal deoxynucleotidyl transferase dUTP nick end labelling (TUNEL) and Mitosensor staining respectively [170]. A sequence of mild cold shock of 25^{o}C and rewarming to normal temperatures also led to DNA damage, which was mitigated by the inclusion of the antioxidant NAC, a further indication that the damage was related to excess ROS production [171]. Conversely, hyperthermia has also been shown to increase DNA damage, which was suggested to be related to increased activity of ROS [172]. Temperature deviations from normal conditions appear to damage DNA through the effect of ROS activity.

2.6.5 Ribonucleic acid stability during hypothermia.

Ribonucleic acid (RNA) is the bridge between stored information and protein production [167]. A recent study investigating the stabilisation of RNA during transportation to allow easier sequencing showed that hypothermia at 4^{o}C produced stable complimentary DNA (cDNA) from the RNA in the preserved samples of murine kidneys over 3 days of

hypothermia [173]. There was, however, a large decrease in cDNA obtained on the fourth day without a decrease in the membrane integrity of cells in the preserved sample [173]. The mechanism for this instability on day 4 was given in a study of similar hypothermic conditions, which showed apoptosis caused instability in RNA before the complete death of the cells [174]. It has been shown that RNA was stable in Chinese hamster ovary K1 cells after exposure to 27^{o}C hypothermia in a standard cell culture medium, whereas, at 37^{o}C specific RNAs showed degradation, both of which were evidenced by examining the quantities of RNA detected during the application of the temperature treatment [175]. It has been shown that the cold inducible RNA-binding protein (CIRP) mRNA was produced in the greatest amounts at 32^{o}C, with a large quantity remaining at 25^{o}C but much lower levels detected at 15^{o}C, which suggested that some changes in RNA can occur at warmer hypothermic temperatures [176]. Isolated RNA can be stored for even longer periods with specialised buffers that allow the sample to be dried. Drying protects the RNA from hydrolysis and nuclease degradation [177], but, given that lyophilisation does not yet result in acceptable recovery of cells compared to cryopreservation [178], stabilisation of the RNA by dehydration would be an unsuitable method for preserving biological products that contain whole cells. Therefore, in the aqueous state, some RNAs are altered in response to temperature changes, but only above 15^{o}C. Preservation strategies at moderate temperatures may suffer from altered RNA transcriptomes during the preservation, whereas the RNA should remain stable at low temperatures.

2.6.6 Protein production is dependent on temperature.

Proteins are responsible for carrying out the majority of cellular activity [167]. The amount of protein production during hypothermia is dependent on the temperature that cells are exposed to: decreasing temperature of exposure from 37^{o}C to 20^{o}C with 30 hour incubation showed that protein production at the lower temperature was much lower than production at normal temperature [175]. This result was shown by the radio labelling of the newly

produced protein followed by scintillation counting. Protein production stops at temperatures below 15^{o}C, although existing proteins remain stable [175]. That study also showed that production was restored from all hypothermic temperatures on rewarming, although a qualitative difference was evident in the size range of 50-75kDa. Proteins related to temperature shock response have also been shown to exist in isolated cell models that have molecular weights within the previous range [112]. Another study in a W126 cell line found the ratio of LC3 I and II proteins was altered towards one that indicates autophagy during exposure to 25^{o}C, and went further towards autophagy with short term recovery to normal temperatures, but this subsided after 24 hours [171]. Intracellular proteins are stable at low temperatures; however, preservation at moderate temperatures may cause instability.

2.6.7 Metabolite requirements during hypothermia.

The presence of glucose in preservation solution at 4^{o}C, with or without insulin, greatly reduced the recovery of beating, immature cardiomyocytes and increased the release of lactate dehydrogenase [179]. However, glucose did increase ATP and ADP concentrations in adult rat cardiac myocytes and improved survivability of the cells after short-term 4^{o}C hypothermia: fructose-1,6-bisphosphate further increased those parameters [180]. However, after 48 hours of hypothermic preservation, the inclusion of both metabolic compounds produced only a small effect on ATP levels; only 6% higher than a control without the compound [180]. Fructose-1,6-bisphosphate is produced from glucose by cell metabolism requiring ATP for the progression of the reaction. The addition of fructose-1,6-bisphosphate to permeabilised glucose-starved cells inhibited the upregulation of AMPK signalling [181]. Therefore, the addition of metabolites to hypothermic preservation medium can stimulate metabolism, but this may be short-lived.

Another saccharide, raffinose, induced autophagy in human keratinocytes at a 100mM concentration, as measured through production of microtubule-associated protein 1 light chain 3 (LC3) [83]. The same result was seen with sucrose and trehalose [83]. Raffinose is

included in the formulation of UW medium [81,82], and sucrose is included in another specialist preservation medium, Hypothermosol (HTS) [21]. In both those studies, the saccharide is suggested to act as a cell impermeant and reduce hypothermia induced swelling. However, the links to autophagy suggests that the compounds may also affect cell metabolism. Saccharides can both support and reduce cell metabolism during hypothermia. The metabolism of T cells, both glycolytic and mitochondrial, has been suggested to regulate their immunological function [148]. Hypothermia can also affect cell metabolism through both temperature effects and additive compounds [180]. Therefore, the most important quality to consider when evaluating the successful preservation of T cells may be to assay the cytotoxic activity that cells produce after preservation.

2.6.8 Adenosine triphosphate in low temperature exposure.

When temperature is reduced, not all cellular processes continue their use of ATP in the same manner as during physiological conditions [162,182]. An early event that leads to cell death at reduced temperatures is an imbalance of ATP supply and demand [163]. It has been suggested that this imbalance be resolved by the inclusion of ATP precursors adenosine [183] and phosphate ions [184], both of which have benefits to tissue during hypothermic exposure for preservation. However, it was discussed in Section 2.4.5 that T cells may react negatively to extracellular nucleosides, therefore, their use in T cell preservation methods may not be straightforward. This suggests that investigations need to be performed into the inclusion of ATP reactants to improve the storage of cell based therapies, and to determine whether it will have any effect during storage or the recovery of the cells, or induce negative response relating to T cell biology.

2.6.9 Generation of reactive oxygen species during hypothermia.

DNA is particularly sensitive to ROS that is generated during hypothermia and on recovery, as discussed in Section 2.6.4. This section investigates the mechanisms of ROS that affect a cell during exposure to cold conditions through the generation of free radicals that oxidise

the cell [185]. This exposure to cold temperatures causes an imbalance in reaction rates between the production of intracellular ROS and the rate of bio-chemical reactions that remove them, causing the ROS to damage the cell [20]. This damage may occur during the cold storage period itself or after return to normal temperatures [186], therefore, the damage may continue after the cells are recovered from preservation. Many studies have investigated the inclusion of different antioxidant compounds during hypothermic storage to mitigate this damage, including; 6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid (trolox) [187], glutathione [188], ascorbate, and deferoxamine [189]. Lipid peroxidation has been suggested to exist during the application of hypothermia. This was shown by the increase of malonyl dialdehyde, a product of lipid peroxidation, observed when isolated hepatocytes were stored in hypothermia for 24 hours [190]. However, lipid peroxidation was reduced during hypothermic preservation of dog kidneys compared to a 37^{o}C incubation of the ischemic organ [191]. This was shown by significant increases in conjugated dienes, lipid peroxides, and Schiff bases. The lipid peroxidation displayed in the kidneys was further reduced by the addition of antioxidant compound, trolox, which improved their function after recovery from hypothermia. Hence, it was suggested that reperfusion may increase the oxidative stress [191]. In that study, one marker related to oxidative stress did not produce a response to the onset of hypothermia or the addition of the antioxidant compound [191]. Furthermore, recent studies detected oxidative stress but minimal release of damage markers, which indicated that damage related to ROS in recovery of human kidney and heart models may also be linked to other mechanisms as well [192]. Cell damage related to ROS can occur both at hypothermic temperatures and during subsequent recovery to normal temperature conditions, but this oxidative stress may not result in immediate necrotic cell death after preservation.

Glutathione is normally synthesised within cells in all mammalian tissues [193]. Glutathione has several roles within the cells, where it; takes part in antioxidation through GSH

peroxidase-catalysed reactions [194], which is particularly important in the mitochondria where most of the reactive oxygen species are produced through oxidative phosphorylation [195]; modulates redox signalling through the oxidation of protein cysteine residues [143]; and promotes the expansion of T cells post activation [146,196]. Glutathione has been tested in many biological models where reduced temperature is desirable for preservation, particularly whole liver, heart, and kidneys [82,197]. It was shown that glutathione increased the intracellular ATP concentration and reduced the lactate dehydrogenase (LDH) release of the preserved cells in these models. The export of glutathione from the cell under physiological conditions is tightly controlled by cell membrane proteins [198], although, during hypothermia, glutathione was depleted from rat hepatocytes [199]. These studies suggested that the cell membrane became permeable to this compound when exposed to hypothermia. Further studies have been conducted on the "Gold standard" [200] components of the UW composition, one of which found that glutathione could be replaced by NAC in a mouse liver model [201]. That study did not establish how the change to NAC affected ROS production and tested only one specific biological model. The concentration range of glutathione present within the cells is known to be between 1 to 10mM [143]. The complex reactions that glutathione is involved with; the cold-induced permeability; and different intracellular concentrations, all suggest that there is a cell-specific optimum concentration of glutathione supplementation in preservation medium. This potential specificity could explain the occurrence of both positive and neutral results above.

Glutathione is the most predominant component with antioxidant abilities within cells, but other compounds can achieve similar effects. Novel compounds with chemistry similar to vitamin E can also have beneficial effects on cell preservation. Hajmousa et al. have identified a novel compound with a structure modified from vitamin E to be water soluble [202]. They found this compound; had an antioxidant effect; increased ATP production by increasing the activity of certain mitochondria complexes, but this occurred only in initial

stages of hypothermic preservation; and, overall, was found to significantly improve the hypothermic preservation of adipose derived stem cells with reduced necrosis and apoptosis [202]. This study suggests that, in addition to DNA damage and lipid peroxidation, compounds with antioxidant function can also affect mitochondria activity. Because mitochondria are the main source of ROS and also the production of ATP [203], it may be important to investigate how the inclusion of antioxidants affects the cell metabolism during hypothermic preservation and whether potential effects are compound specific.

Other derivatives of water-soluble vitamin E have been shown to benefit hypothermic preservation; ETS-GS in a rat liver model; and trolox in the preservation of adherent cell lines [21,204]. The natural form of vitamin E, α-Tocopherol (ATOC), has also been shown to have anti-apoptotic roles in avoiding ischemic reperfusion injury in hepatic cells [205]. Vitamin E is lipid soluble and, thus, must be solubilised into micelles within the intestinal tract before it can be absorbed into endothelial cells [206], and is then transported around the body by plasma lipoproteins [207,208]. Therefore, extracting the benefits of ATOC may be dependent on a suitable vehicle to increase the bioavailability of the compound.

2.6.10 Preservation by encapsulation.

As discussed in section 2.6.3, a high viscosity of the preservation medium negatively affects the hypothermic preservation of some organ types. The requirement of viscosity properties for preservation of isolated cell types needs to be identified. Stem cells are sensitive to vibrations when suspended in a normal culture medium and subjected to mechanical load [209]. Reports state that the encapsulation of stem cells in an alginate hydrogel is capable of providing effective preservation of the cells at room temperature [210]. That report does not explain the mechanism behind the effectiveness of this method and does not identify if the encapsulation can mitigate the effects from shear stresses. After hypothermic preservation at 15^{o}C of adipose derived stem cells (ASC) encapsulated in an alginate gel system, the total recovery, which was a calculation of viable cell number, metabolic activity, and cell

attachment, was significantly greater than liquid controls [211]. Cells were released from the alginate by dissolving the mixture in 100mM trisodium citrate for 5 minutes, then diluting the citrate, followed by centrifugation to recover the cells [211]. That study also indicated that encapsulation using a hydrogel-cell culture medium combination, or a liquid culture medium, outperformed the specialist hypothermic preservation medium, HTS. It is also worth noting that the ASCs from the 15^{o}C culture medium control were able to return to proliferation with increasing cell numbers after preservation, which suggests that this cell type is particularly able to survive hypothermia at a suitable temperature without a great deal of intervention. Complete encapsulation of hepatocytes in gelatin improved their response to hypothermic preservation by the prevention of haloing, a condition where cytoplasm exists outside of the cell membrane [212]. This enabled the cells to maintain high membrane integrity for 7 days. Recovery was completed by liquefying the gelatin at 37^{o}C [212]. Complete encapsulation may not be required for adherent cells to survive hypothermia. MCF-10 and GLC-82 immortalised cell lines were shown to survive hypothermia with greater viability and maintained proliferative capacity when preserved in a compacted sediment by centrifugation with a quantity of micro-particles, whereas liquid media controls did not maintain such a high viability or proliferative capacity [213]. In that study, UW preservation medium outperformed the culture medium control. The cells were then recovered from the compacted state by re-suspending the sediment and removing the micro-particles from suspension by a magnet. The hypothesis from that study was that the assembling ECM was blocking the communication by cell-to-cell contact that could occur in the liquid controls [213]. This mechanism may be the same for complete encapsulation because all the encapsulation methods produced the same results assessed by the same parameters. Another study suggests that the mechanism of preservation by encapsulation could be related to a suppression of metabolism [214]. None of the encapsulation studies examined the therapeutic action of the cells on recovery. Furthermore, it is not yet

determined if there would be an effect due to the materials used in each study because the mechanism of action for this preservation method has not been established.

2.7 Improving preservation protocols by quality by design methodologies.

From the review of hypothermia and preservation so far in this Chapter, it can be suggested that there are a number of different mechanisms that can affect cells during hypothermia, and a number of methods that could influence these effects. However, these methods were tested on a range of different biological models, which could mean that the results are not directly transferable between cell models. A more robust approach for performing the investigations in to preservation is required. Quality by design (QbD) approaches, including Design of Experiments (DoE) and Taguchi methods, are widely used in process industries and have shown beneficial results in their use within bioprocessing [76,215–217]. Taguchi methods are a particular application of orthogonal array design tables which minimise test runs and allow relative comparison between multiple effects [76]: these methods were used recently to optimise the preservation of the oldest biological product, blood, at ambient conditions, although, one-factor-at-time (OFAT) trials with apoptosis inhibitors were required to successfully maintain all quality parameters tested [218]. DoE response surface methodology and Taguchi methods have been directly compared on a single process and both produced similar results, which suggested that any QbD method is suitable and can produce the benefits of identification of interactions, reduced trial runs, and relative comparison of effects [219]. The reason to use these methodologies relates to the theory of DoE. OFAT investigations will not allow interaction terms to be established between the factors, which can be better understood when completing DoE [220]. Also, the method in which the effect of a factor in DoE methodology is analysed is more robust than OFAT because the response of one individual factor is measured over a range of conditions, therefore, the result produced by the DoE analysis is valid over a wider range of conditions than a result produced by OFAT [220]. The majority of hypothermic preservation methods

previously presented in this Chapter were the result of OFAT investigations to develop an understanding of preservation sciences. The wealth of knowledge established may now best be compared using QbD methodologies to understand the most important effects described and produce optimum preservation strategies.

2.8 Summary.

This review has established the importance of CGTs for treating conditions that do not respond to existing medical interventions, however, the processing chain of these therapies is complex and requires improvement in order to reduce the cost and increase the availability of such therapies. The process of preservation to achieve storage and transportation of the live CGT product is less understood and methods more efficient than cryopreservation must be found. The concept that if the cells are not frozen then they are continued in culture leads to wastage from unused product, which is the main hindrance of preservation at $0^{o}C$ and above. The study of the preservation of blood and organ products suggests methods that may facilitate the preservation of CGTs more suitably for transportation, but the complexity of many parameters and equally as many biological models makes comparison of results difficult. DoE methodology may therefore provide a suitable means of investigating these parameters on CGT models to understand the main requirements for non-frozen preservation. The use of these methods may also help to improve the understanding of the mechanisms of established preservation strategies because new techniques in isolation are suggesting that components involved may have multiple effects on the biology of cells.

The overall conclusion from the study of current understanding of low temperature biology was that, as temperature is reduced below that of physiological conditions, processes that damage the cell occur. There is a lack of studies that quantify if there is a relationship between temperature and damage, whereas a relationship of reducing cell activity with reducing temperatures is well established. Therefore, a balance between cell damage and

provision for remaining cell activity must be created in a cell-preservation strategy, which is visualised in Figure 2-3.

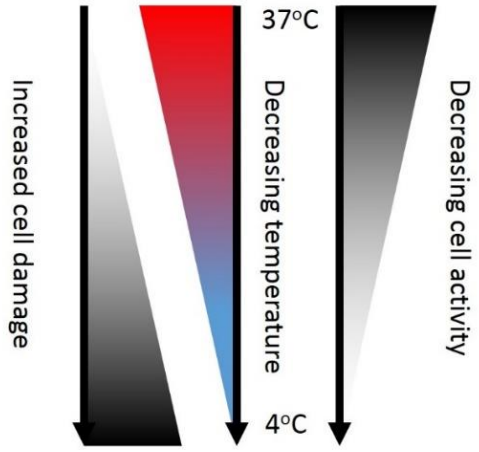

Figure 2-3 Graphical depiction of how cell activity and cell damage may change as a result of the application of hypothermia, as evidenced by existing literature. The suggestion is that, as temperature is decreased below 37°C but remains above conditions of freezing water, overall biochemical activity of cells will be reduced, but the trade-off can be suggested to be an increase in cell damage and death produced by several mechanisms.

The behaviour of T cells in response to antigen presentation, rapid proliferation, and T cell death resulting from antigen withdrawal, and the mechanisms these processes involve, is shared with some of the pre-conditioning strategies that are important in protecting organs during preservation. These similarities may hinder the transfer of existing methods of preconditioning and pre-treating biological products to improve post-preservation quality, because the mechanisms behind T cell anergy and pre-conditioning organs appear to share molecular pathways. Therefore, further methods may need to be developed to facilitate improved preservation of activated T cell CGT products.

The literature reviewed in Chapter 2 poses the research questions: can the hypothermic methods used to preserve existing cell-containing therapy-products be directly translated to achieve a short-term preservation strategy for activated T cell CGT products? Alternatively, will novel preservation strategies be required to overcome the challenges presented by the unique T cell biology, which relate to the drastic changes that occur in the cells upon presentation with antigens?

3 Development of T cell Preservation Medium using Design of Experiments.

3.1 Introduction.

University of Wisconsin (UW) solution is reviewed by Stewart as being the "gold standard" for organ preservation [200][81,165,221,222]. This medium has been tested on isolated cell types as a model replacement for whole organs and tissues when developing the composition and investigating the modification of the preservation medium [81,82]. These existing studies confound different treatments and biological models in an attempt to justify the composition. Other preservation media exist, such as HTS, the basic composition of which shares some similar components to UW [21]. The HTS base medium is often supplemented with caspase inhibitors to reduce apoptosis [223]. This supplementation increases the number of components in HTS required to achieve a truly effective preservation medium. The UW medium consists of 10 components and HTS of 15 components. The high number of components may make it difficult to identify those components that are beneficial to the preservation of individual biological models when one-factor-at-a-time (OFAT) experiments are performed. OFAT experiments were typically used in the studies that attempted to justify components within preservation media. The multicomponent complexity of the existing preservation media, when examined with OFAT tests used originally to develop these media, suggests that more rigorous tests may be required to justify the components for a truly effective composition.

The preservation medium sodium-adenine-glucose-mannitol (SAGM), used for red blood cells (RBC), and the medium citrate-phosphate-dextrose-adenine (CPDA), used for whole blood, each contain some similar components to the above cell and organ preservation media [224]. The names of both blood preservation media indicate that each contains four components only, a simplification compared to UW and HTS. These blood preservation media are typically combined with refrigerated temperatures to preserve the blood and its components. In a recent study by Sandlin et al., the 4 components of SAGM were

supplemented with an antioxidant compound and HEPES buffer, and the resulting 6 component system was examined using Taguchi methods to find an optimum composition for preservation of RBCs within whole blood [76]. Taguchi methods is one example of a quality-by-design (QbD) method, which is an alternative to OFAT tests. The viability of the Leukocyte population within the blood was only improved by the addition of an anti-apoptotic agent, which was identified by OFAT experiments. This demonstrates that improvements can be made to these long-established preservation methods, both by OFAT and QbD methods.

Taguchi methods of process optimisation can be applied to any process in order to investigate its beneficial components and provide robust statistical optimisation [225]. It has been demonstrated by Baust et al. that individual cell types require individual preservation medium [21]. The aim of the study for Chapter 3 was to investigate the hypothermic preservation of an activated T cell line, specifically CIK cells, for which no studies exist in literature. Combining this objective with the evidence that old formulations require improvement suggests that all the components of a preservation medium should be investigated for suitability in the previously untested hypothermic preservation of the CIK cell type.

The previous discussions in this introduction drove the investigation of using engineering methods and QbD approaches to optimise the basal composition of the hypothermic preservation medium. The Design of Experiment (DoE) method of fractional factorial design (FFD) was tested as a method to complete the multi-component analysis with a reduced number of trials of the widely tested UW medium composition. The FFD method should identify whether components have a positive or negative effect and establish the relative importance of each factor. It was sufficient to use concentrations of components from the original UW formulation for the purpose of this FFD to identify components required for T cell preservation. The knowledge generated by this study might indicate that all the original

medium components are required for the previously untested T cell model, but this will be achieved in a rigorous way providing a more robust argument to justify using the existing formulation. The exemplar process to produce each DoE model is described in the Section 3.2 along with the methods used to define model responses. The predictions produced by the DoE model were tested with independent trials. Further to this, more advanced study was completed to identify mechanisms that explain the results produced in the DoE model, particularly if the concentration of the most important component from the FFD had an effect on the response parameters.

3.2 Materials and methods.

3.2.1 Cell culture, cryopreservation and hypothermic preservation.

PBMCs were purchased from ATCC (PCS-800-011). Supplied cryo-vials were thawed according to manufacturer instructions. The PBMCs were cultured according to a protocol to develop CIK cells, as detailed by Bonano et al. [16]. Thawed cells were re-suspended to $1x10^6$ live cells/ml in RPMI 1640 media (RPMI) (Thermofisher) with 10% (volume) of foetal bovine serum (FBS) (Thermofisher). T cells were activated with a combination of soluble CD3 antibody (Miltenyi Biotec) to 50ng/ml, CD28 antibody (Miltenyi Biotec) to 50ng/ml, and IFN-γ (Peprotech) to 1000 IU/ml. Cultures were maintained by bolus addition of 300 IU/ml of IL-2 (Peprotech) and dilution to $1x10^6$ live cells/ml every 3 days. After 14 days, cultures were counted for total cell quantity, washed, re-suspended to $10x10^6$ live cells per ml, and then cryopreserved. Cryopreservation medium was 90% (vol.) Foetal Bovine Serum (FBS) with 10% (vol.) dimethyl sulfoxide (ME_2SO). Cell suspensions were cooled using a Biocision Coolcell to obtain a 1^oC per minute cooling rate. Cryo-vials were transferred to vapour phase liquid nitrogen tanks the following day. To recover cryopreserved cells, vials were rapidly thawed in a 37^oC water bath, washed by centrifuging at 300g for 3 minutes, re-suspended in RPMI 1640 cell culture medium supplemented with 10% (vol.) FBS to a density of $5x10^6$ cells per millilitre, rested for 24 hours without

exogenous antigen, diluted to $1x10^6$ cells/ml, and then supplemented with the previous concentration of IL-2. Hypothermic preservation was achieved by re-suspending cells to a density of $10x10^6$ total cells/ml in medium formulations, described later. Cell suspensions were transferred to multi-well plates so that the volume did not exceed maximum working volume. All wells were filled with PBS to reduce moisture loss from samples, and then the plates were sealed against water vapour loss with Parafilm® tape. The plates were then directly transferred to a controlled, refrigerated temperature of 4°C, or to a bench-top incubator controlled at a room temperature of 22°C. Cells were recovered from preservation by gently pipetting to re-suspend and then assayed by techniques as described below. Both the duration and temperature of preservation are indicated on the Figures when required.

3.2.2 High-throughput cell counting and membrane integrity assay.

Membrane integrity of T cells was measured by exclusion of Draq7 (Abcam), a membrane impermeable compound that labels nuclei of membrane-compromised cells. Labelling was performed according to manufacturer instructions. Draq 7 labelling identified dead cells. Total cells were counted by labelling with acridine orange (AO) (Sigma Aldrich UK) at a final concentration of 2µM. The far-red fluorescence channel did not show staining of single stranded DNA or RNA by AO in the subsequent image analysis. 20µL of each stained cell sample was mounted on to glass microscope slides, covered with a cover slip and imaged on a Molecular Devices Spectramax system. Softmax software, coupled to the system, was used for image analysis to count both the total number of cells and the number of dead cells by setting thresholds on fluorescence intensity to identify individual cells over the background of the image. Total cells and viabilities were calculated at each data point so that final cell viability after preservation was calculated as:

$$Viable\ cell\ number\ on\ day(x) = \frac{Total\ live\ cells\ on\ day(x)}{Total\ live\ cells\ on\ day(0)} \quad \text{Equation 3-1}$$

and;

$$Total\ live\ cells\ on\ day(x)\ = 1 - \left(\frac{Draq\ 7\ positive\ cells\ on\ day(x)}{Total\ cells\ on\ day(x)}\right)$$

Equation 3-2

Live cell count produced by this high-throughput method correlated strongly with that of standard haemocytometer and trypan blue counts with a Pearson correlation of 0.9547, which had a statistically significant probability of the trend calculated to be 0.0031.

3.2.3 High-throughput metabolic activity assay.

Alamar blue was purchased from Thermofisher. $1x10^5$ total cells in 10ul of suspending medium were transferred to a 96 well plate. This suspension was diluted to 100ul with a working assay mixture. For positive controls, the working mixture was 10µl of Alamar blue stock with 80µl of complete RPMI medium for each sample. For negative controls, the working mixture was 10µl of Alamar blue stock, 70µl of complete RPMI medium and 10µl of 10% (vol.) Triton-X100 in complete RPMI medium for each sample. These mixtures where then incubated for 4 hours at 37^{o}C. Reduction of Alamar blue was measured by fluorescence according to manufacturer protocol. Negative background controls were subtracted from the reading of each sample to differentiate cell activity from background reduction. The incubation at 37^{o}C was taken to indicate metabolic activity on recovery of cells from tested low temperature conditions.

Inhibiting the activity of the electron transport chain (ETC) by controlling aspartate synthesis was linked to inhibition of cell proliferation, which suggested a relationship between this part of cell metabolism and DNA synthesis [226]. Activity of the ETC and other reactions in cell metabolism can be measured by the reduction of Alamar blue, because the oxidation reduction potential of the compound allows reaction with compounds involved in the four mitochondrial complexes as well as cytochromes [227]. Therefore, reduction of Alamar blue was interpreted as the metabolic activity of the T cells in this study.

3.2.4 Exemplar process of design of experiments optimisation.

Design Expert 7 software was used to complete the design and analysis of the DoE fractional factorial design. The 10-component composition of UW solution would require 2^{10} individual runs to complete a full factorial experiment. The main aim of this study was to investigate the main effects of the individual components of the UW preservation medium, therefore a resolution IV experiment was adequate because the main effects are not confounded. The 10 factors investigated with a resolution IV experiment would test $2^{10-5} = 32$ different combinations, as shown in Table 3-1.

The results were collected in specified standard order using the selected measure of the response. In this study the responses were viable cell number, see Section 3.2.2, and metabolic activity characterised by the reduction of Alamar blue, see Section 3.2.3. Metabolic activity after preservation was compared to that obtained from samples in each standard on day 0 of the experiment. Response values were then analysed to identify which effects were significant in the DoE software. The initial selection of significant terms was performed by graphing the half-normal effects against a standard effect, shown in Figure 3-1. The half-normal meant that only the magnitude of the effect was displayed. The selection of terms using this plot followed the rationale that effects from a sample population against the true population will form an approximate straight line thus, if the samples are from a normal distribution, they should form a straight line when plotted against a theoretical normal distribution. Components that do not lie on this straight line are likely to have had a significant effect in changing the normal distribution of the population overall population.

After the important effects that should cause a significant difference were chosen from the half-normal plot, the resulting model was then assessed for probabilities of significant differences caused by those effects through analysis of variance tests (ANOVA). Should the model identified be insignificant then the effects that were least significant were removed in an iterative procedure alternating between the half-normal plot and ANOVA. Once the

ANOVA result was satisfactory (overall *P*-value less than 0.05) then a model of the chosen factors was produced with regression fit coefficients for unit changes in the response of the factor. These coefficients were obtained while holding the other factors in the model constant. An example of the ANOVA table is shown in Table 3-2 and predictive models are discussed in the following results and discussion section.

Std	A:Lactobionic acid	B:Hydroxyethyl starch	C:Potassium phosphate	D:Magnesium sulphate	E:Raffinose pentahydrate	F:Adenosine	G:Allopurinol	H:Glutathione	J:Dexamethasone	K:Insulin
	g/l	g/l	g/l	g/l	g/l	g/l	g/l	g/l	g/l	mIU/L
1	0	0	0	0	0	1.34	0.14	0.92	0.02	25
2	36	0	0	0	0	0	0	0	0	25
3	0	50	0	0	0	0	0	0	0.02	0
4	36	50	0	0	0	1.34	0.14	0.92	0	0
5	0	0	3.4	0	0	0	0	0.92	0	0
6	36	0	3.4	0	0	1.34	0.14	0	0.02	0
7	0	50	3.4	0	0	1.34	0.14	0	0	25
8	36	50	3.4	0	0	0	0	0.92	0.02	25
9	0	0	0	1.23	0	0	0.14	0	0	0
10	36	0	0	1.23	0	1.34	0	0.92	0.02	0
11	0	50	0	1.23	0	1.34	0	0.92	0	25
12	36	50	0	1.23	0	0	0.14	0	0.02	25
13	0	0	3.4	1.23	0	1.34	0	0	0.02	25
14	36	0	3.4	1.23	0	0	0.14	0.92	0	25
15	0	50	3.4	1.23	0	0	0.14	0.92	0.02	0
16	36	50	3.4	1.23	0	1.34	0	0	0	0
17	0	0	0	0	17.83	1.34	0	0	0	0
18	36	0	0	0	17.83	0	0.14	0.92	0.02	0
19	0	50	0	0	17.83	0	0.14	0.92	0	25
20	36	50	0	0	17.83	1.34	0	0	0.02	25
21	0	0	3.4	0	17.83	0	0.14	0	0.02	25
22	36	0	3.4	0	17.83	1.34	0	0.92	0	25
23	0	50	3.4	0	17.83	1.34	0	0.92	0.02	0
24	36	50	3.4	0	17.83	0	0.14	0	0	0
25	0	0	0	1.23	17.83	0	0	0.92	0.02	25
26	36	0	0	1.23	17.83	1.34	0.14	0	0	25
27	0	50	0	1.23	17.83	1.34	0.14	0	0.02	0
28	36	50	0	1.23	17.83	0	0	0.92	0	0
29	0	0	3.4	1.23	17.83	1.34	0.14	0.92	0	0
30	36	0	3.4	1.23	17.83	0	0	0	0.02	0
31	0	50	3.4	1.23	17.83	0	0	0	0	25
32	36	50	3.4	1.23	17.83	1.34	0.14	0.92	0.02	25

Table 3-1 DoE design table showing combinations of factors that were used to complete the resolution IV experiment. The table shows the concentration in grams/litre (g/l) that each component was included in the combination to make up the mixture for each standard run.

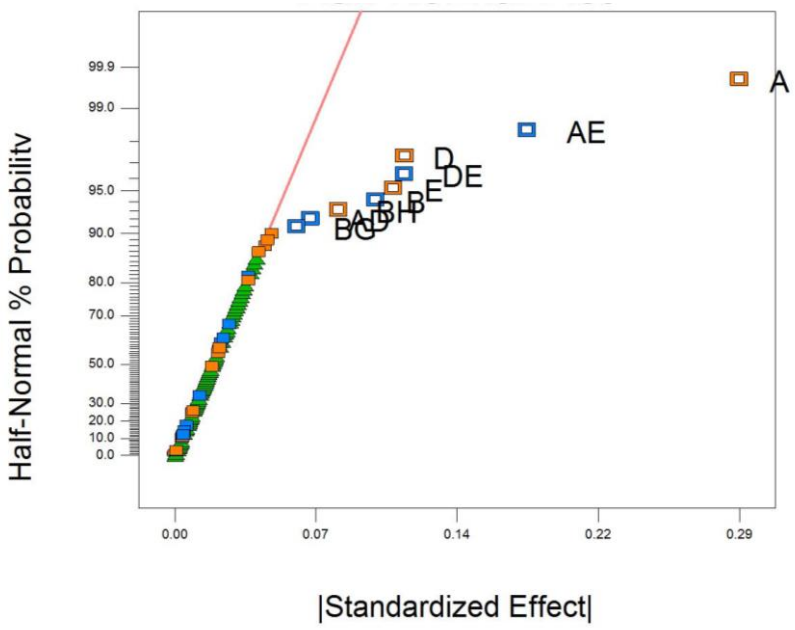

Figure 3-1 Example half-normal plot of effects produced by the factors shown in Table 3-1. Significant factors are likely to lie off to the right-hand side of the plot, with remaining insignificant factors on a straight line formed by the assumption that the population is characterised by a normal distribution.

ANOVA for selected factorial model						
Analysis of variance table [Partial sum of squares - Type III]						
	Sum of		Mean	F	p-value	
Source	Squares	df	Square	Value	Prob > F	
Model	4.421633	11	0.401967	21.78305	< 0.0001	significant
A-Lactobionic acid	1.992783	1	1.992783	107.9913	< 0.0001	
B-Hydroxyethyl starch	0.252248	1	0.252248	13.66961	0.0004	
D-Magnesium sulphate heptahydrate	0.330457	1	0.330457	17.90788	< 0.0001	
E-Raffinose pentahydrate	0.299018	1	0.299018	16.20414	0.0001	
G-Allopurinol	0.008723	1	0.008723	0.472685	0.4936	
H-Glutathione	0.058732	1	0.058732	3.182753	0.0780	
AD	0.115237	1	0.115237	6.244809	0.0144	
AE	0.775344	1	0.775344	42.01679	< 0.0001	
BG	0.093126	1	0.093126	5.046629	0.0273	
BH	0.167272	1	0.167272	9.064685	0.0034	
DE	0.328693	1	0.328693	17.81225	< 0.0001	
Residual	1.550068	84	0.018453			
Lack of Fit	0.311734	20	0.015587	0.805558	0.6977	not significant
Pure Error	1.238334	64	0.019349			
Cor Total	5.971701	95				

Table 3-2 Example of ANOVA table generated during the DoE analysis of the metabolic activity and membrane integrity response of T cells to the compositions shown in the design table, Table 3-1

The model was also subject to a variety of diagnostic procedures interpreted through the following plots;

(1) normal probability plots, to check that the data fits a normal distribution;

(2) a plot of residual versus predicted values, to confirm constant variance;

(3) a plot of residuals versus run number, to confirm that the experimental order has not affected results;

(4) a plot of actual values versus predicted values, to confirm that the model fits the data range well;

(5) interaction plots are examined for non-parallel lines, to confirm that interaction between components exists.

3.2.5 Formulation of the design table compositions.

Individual components as detailed in the DoE design table, see Table 3-1, were dissolved in deionised water to produce concentrated stock solutions. Lactobionate, potassium phosphate, magnesium sulphate, and glutathione were dissolved to 10-fold increased concentrations; the remaining components were at 5-fold increased concentrations. These stocks were adjusted to a pH of 7.4 by addition of 1M hydrochloric acid or 1M sodium hydroxide. The HES stock was autoclaved at 121^{o}C and 1 atmosphere pressure to sterilise it, and all other stock components were 0.22µm filter sterilised. A diluting deionised-water stock was prepared by adjusting its pH to 7.4 and then filter sterilising. Post-sterilisation, the stocks were combined together to form the design table compositions, which were maintained under aseptic conditions and stored at 4^{o}C until use. Recovered T cells were then re-suspended to fixed densities in each composition before commencing hypothermia.

3.2.6 Measuring osmolality by freezing point depression.

A Gonotec osmometer was used to assess medium osmolality by freezing point depression. The machine was calibrated using two points; first, deionised water to calibrate 0 mOsm/kg, and second, a reference standard of 300mOsm/kg. Once calibrated, 50µL of medium was transferred to a sample container and tested in the osmometer to assess the sample osmolality.

3.2.7 Investigating metabolic inhibition by phosphate.

T cells were cultured as described in Section 3.2.2. The cells were then re-suspended in 3 separate fresh media to investigate the effect of phosphate addition. The first was complete medium alone to act as a control. The second medium had sodium phosphate added at a fixed concentration. The third medium included sodium chloride at a concentration to achieve the same total osmolality compared to the second medium. Re-suspended cells were cultured for a further 3 days at 37ºC, measuring viable cell number, as above, and collecting supernatant for metabolite analysis, as below.

3.2.8 Enzymatic measurement of glucose and lactate compounds.

300µL of cell culture medium containing cells was collected from cultures. Cells were then pelleted by centrifuge at 300g for 3 minutes. 200µL of supernatant was transferred to a sample container. Supernatant from the cell culture medium was de-proteinised by centrifugation in a 10kDa molecular weight cut-off spin column at 10000g for 10 minutes. Samples were stored frozen until the time of assay. To measure glucose, a commercial kit from Sigma Aldrich (GAGO-20) was used. The method was adapted for a 96 well-plate by linear correction of volumes from the manufacturer's instructions. Samples were diluted 1:50 with deionised water and 50µL of the resulting mixture was transferred to a 96 well- plate. 100µL of the assay reaction mixture was added to this and then the plate was incubated for 30 minutes at 37ºC for 30 minutes. After incubation, 100µL of 12N sulphuric acid was added to stop the chemical reaction. Absorbance was measured at 540nm on a Molecular Devices Spectramax plate reader and converted back to a concentration of glucose by a standard curve. Lactate was measured using a kit purchased from Trinity Biotech Ltd. Volumes were modified for a 96 well-plate format by linear reduction of reaction volumes. 2µL of sample was mixed with 100µL of assay reagent and incubated for 10 minutes at room temperature. Absorbance was read at 540nm on the same plate reader and the concentration was obtained from comparison to a standard curve. The chemical reaction of these assays involves the

generation of hydrogen peroxide, therefore, if the supernatant to be tested contained compounds with peroxide scavenging ability, such as glutathione or alpha-tocopherol (ATOC), then the effect of these compounds was removed by including them when generating standard curves for the assay.

3.2.9 Measurement of pH.

Preservation medium pH was measured at room temperature using a 3-point standard calibrated Mettler Toledo micro pH probe (Manufacturer number 51343160). The design of the probe meant that samples did not require agitation.

3.2.10 Statistical analysis.

If the data produced significant differences in variance as shown by Levene's tests, then the raw data was transformed with a $\log_{10}$ function to test for avoiding difference in variance. For data that did not differ in variance, was normally distributed, and where two factors were varied in the dataset, two-way ANOVA was used with Tukey post-hoc follow up tests to examine significant differences. After confirming two-way significant difference, individual differences were identified by one-way ANOVA with Tukey post-hoc testing to examine significant differences between the two conditions. Data that retained unequal variances after transformation was assessed by pair-wise comparisons using t-tests, assuming unequal variance to establish significant differences. For non-normal data that was found by Shapiro-Wilks testing, non-parametric Kruskal-Wallis testing followed by pairwise Mann-Whitney tests were used to establish significant differences. Statistical testing was implemented in Microsoft Excel with the open source Real Statistics Resource Pack available online. The number of independent replicates are detailed in the caption of each of the Figures that follow. The graphs of these figures depict original data for clarity with either; (1) box-plots with minimum, first quartile, median, third quartile, and maximum values for non-normally distributed data; or (2) bar charts for normally distributed data that show the average values ± the standard deviation of the replicates. A *P*-value of less than 0.05 was considered to be

significant, which was the same for DoE testing. Comparisons between samples are marked on the graphs of all figures included in this thesis by:

- Square bridges between datasets with asterisks, indicating a significant difference between the two individual conditions.
- Flat bars joined by square bridges with asterisks, showing a significant difference between group comparisons.
- Flat bars with asterisks, indicating the result from omnibus testing between all samples of group.
- Asterisks above a data set with no additional marker, signifying the comparison to a day 0 control, which was from before commencing treatment.

The number of asterisks included with a label represents the value of the significant probability of difference between datasets, with: * = $P < 0.05$; ** = $P < 0.005$; *** = $P < 0.0005$; **** = $P < 0.00005$. No asterisk or a not significant label indicates indicate a calculation where $P > 0.05$.

3.3 Results and discussion.

3.3.1 Model equations from design of experiments analysis show that useful components of preservation medium are consistent with duration and temperature.

Equation 3-3 and Equation 3-4 show the model equations developed from significant model effects for the activated T cell responses of metabolic activity and membrane integrity after 24 hours of 4^{o}C hypothermia. These models, and the ones that follow, are obtained by following the methodology as described in the materials and methods section 3.2.4 above. The models use coded factors so that each effect varies between 0 and 1 to allow comparison amongst the difference components. The square root transformation was a result of needing to transform the data for the DoE software to make ANOVA assumptions valid. The square root transformation is often applied to counting data, such as the membrane integrity

response and amount of metabolic activity recovered. These equations from the DoE analysis show the importance of each effect, as given by the magnitude of the coefficient, and whether it was positive or negative, and the findings are discussed below.

Potassium lactobionate (A) was one of the most significant positive terms to benefit T cells during this short period of exposure to hypothermic conditions, for both viability and metabolic activity on recovery. A had the most significant positive effect on metabolic response. Raffinose (E) was slightly more important for maintaining viability because of the 0.01 higher coefficient of this component compared to A but, given how much higher both A and E were compared to the rest of the significant terms, both were considered important. E was also important for maintaining metabolic activity on recovery, with the term holding significant probability of difference in ANOVA. The magnitude of the A coefficient in Equation 3-4 was less than Equation 3-3, suggesting a stronger importance for the component to maintain metabolic activity compared to membrane integrity. The large positive coefficients of both A and E suggest that these components were the most important over short term hypothermic preservation. Potassium phosphate (C) also had a relatively strong positive effect on membrane integrity with a coefficient of 0.029, which suggested that the term is very likely to be significant. The effect of C on metabolic activity was not significant and thus does not appear in Equation 3-3. Magnesium sulphate (D) was an important factor to maintain both membrane integrity and increase metabolic activity after short term 4^{o}C hypothermia. Hydroxyethyl starch (B) had a negative effect on both metabolic activity and membrane integrity after one day of exposure in combination with 4^{o}C hypothermia. Other terms not discussed above were insignificant, which suggested that these had small effects on either cell membrane integrity or metabolic activity after short term preservation. Equation 3-3 and Equation 3-4 show that some interaction terms had a significant effect on the responses of T cells after the short-term preservation. These effects mainly related to the compounds that would increase the osmolality of the preservation

medium and, because many of these interactions had negative effects, this could suggest that a high osmolality had a negative effect on T cell preservation.

$$\sqrt{(Metabolic\ response_{D1,4^oC})} = 0.72 + 0.14A - 0.051B + 0.059D + 0.056E - 0.09A \times E - 0.059D \times E$$

Equation 3-3

$$\sqrt{(Membrane\ integrity\ response_{D1,4^oC})} = 0.52 + 0.05A - 0.021B + 0.029C + 0.024D + 0.051E - 0.041A \times B - 0.041A \times D - 0.045A \times E - 0.031D \times E$$

Equation 3-4

$$\sqrt{(Metabolic\ response_{D1,22^oC})} = 0.56 + 0.19A - 0.058B + 0.038C + 0.050D + 0.02E - 0.058A \times E - 0.044D \times E$$

Equation 3-5

$$\sqrt{(Membrane\ integrity\ response_{D1,22^oC})} = 0.53 + 0.019A - 0.022B + 0.039C + 0.033D + 0.048E - 0.048A \times B - 0.021A \times E$$

Equation 3-6

Compounds that were effective at 4°C were also similarly effective at 22°C. Equation 3-5 shared the majority of significant terms with Equation 3-3 for metabolic activity recovered and a similar statement can be made for the membrane integrity response. The difference was that C was significant and positive at preservation of 22°C. There were more differences between the temperatures related to membrane integrity at 22°C. Equation 3-6 does not have significant interacting terms of potassium lactobionate with magnesium, or magnesium with raffinose, whereas Equation 3-4 showed that these were significant.

The constant terms suggest that some difference occurred between the temperatures that could not be corrected by components in this model, because many of the terms related to additive components having similar coefficients. There was little difference reported between membrane integrity responses of the two temperatures, with constant terms of Equation 3-4 and Equation 3-6 only having a 0.01 difference. Conversely, there was a large

difference between metabolic activities recovered between the two temperatures: the constant term of Equation 3-5 was 0.56, which was much lower than that of Equation 3-3. Over short preservation durations, it is suggested that similar components of the preservation medium benefit both 4^{o}C and 22^{o}C hypothermic preservation of T cells.

Equation 3-7 showed that overall metabolic activity was reduced after increasing hypothermic preservation at 4^{o}C to a duration of 3 days, shown by the lower constant term of 0.44 compared to 0.72 of Equation 3-3. This suggested that none of the components tested in the model avoided a decline in the metabolic activity of cells after preservation. A remained an important positive effect after extended preservation to 3 days, the magnitude of the coefficient only decreasing by 0.01. The negative effect of B on metabolic activity was reduced after adding a further 48 hours to the preservation time: the coefficient of this term roughly halved to 0.026 at 3 days. C became an important positive effect on cell metabolic activity after the three day preservation duration and remained a positive effect on cell membrane integrity. D continued to display an important effect on recovery of metabolism with increased preservation duration, whereas the strength of the effect of E decreased between 1 and 3 days of preservation. Another term that became important with increased time of 4^{o}C preservation was glutathione (H). The significant term H is shown in Equation 3-7, which shows the response of metabolic activity. The combination of A and E remained significantly negative at 3 days of 4^{o}C preservation. Membrane integrity remained stable during increased preservation duration with constant coefficient at 0.56, similar to the value after only 1 day of preservation. Terms related to lactobionate, phosphate, magnesium, and raffinose remained positive, and had a reasonably similar magnitude to day 1 when comparisons were made between Equation 3-8 and Equation 3-4. The differences resulting from extended preservation were that; there was an increasing magnitude of the negative effect of the hydroxyethyl starch component, B; and the effect related to adenosine, F, became significantly negative: for both of these effects, see Equation 3-8. This suggested

that the components that benefit short term preservation are mainly the same as those that benefit long term hypothermic preservation. The exceptions to this are that the negative components have an increased magnitude of effect with time, and that some initially insignificant components become important negative effects with increased duration. This suggested that B had a negative effect that increased with increased exposure time.

$$\sqrt{(Metabolic\ response_{D3,4^oC})} = 0.44 + 0.13A - 0.026B + 0.041C + 0.059D + 0.02E + 0.03H - 0.048A \times E$$

Equation 3-7

$$\sqrt{(Membrane\ integrity\ response_{D3,4^oC})} = 0.56 + 0.052A - 0.041B + 0.021C + 0.033D + 0.033E - 0.028F - 0.05A \times B - 0.036A \times D - 0.046A \times E + 0.024C \times D$$

Equation 3-8

$$\sqrt{(Metabolic\ response_{D3,22^oC})} = 0.26 + 0.12A + 0.038C + 0.032D + 0.036H - 0.032A \times C$$

Equation 3-9

$$\sqrt{(Membrane\ integrity\ response_{D3,22^oC})} = 0.52 + 0.024A - 0.054B + 0.032C + 0.05D + 0.043E - 0.077A \times B - 0.038B \times E + 0.027C \times E$$

Equation 3-10

Increasing the duration of 22ºC hypothermic preservation to 3 days decreased the overall metabolic activity further, compared to 4ºC, to the lowest level of overall normalised cell activity of 0.26 that of the initial metabolic activity on day 0. A, C and D retained important positive effects on metabolic activity with extended 3 day 22ºC preservation, see Equation 3-9. However, E lost importance with extended preservation at 22ºC. The negative effect of hydroxyethyl starch, B, also lost its significance at this longer time point. H also became a significant positive effect that was not present on day 1, which was the same as that at 4ºC hypothermia. Membrane integrity remained stable at 22ºC, with a similar constant term between Equation 3-10 and Equation 3-6 from 1 day of preservation. A, C, D and E all

remained significantly beneficial to maintaining membrane integrity of the T cells after hypothermic preservation, see Equation 3-10. The addition of B was negative after 3 days of preservation at 22^{o}C. Unlike 4^{o}C, the presence of adenosine did not produce a significant negative term for the model so could be less important at this warmer temperature.

These results from Equation 3-3 to Equation 3-10 all suggest that only a subset of components from the original UW medium formulation are beneficial to the hypothermic preservation of T cells, and that two components, hydroxyethyl starch and adenosine, had a negative effect on cell membrane integrity and metabolic activity on recovery after preservation. Those that are negative or insignificant have been identified and can be removed from the preservation medium to optimise the formulation. This showed that not every component of a preservation medium will be beneficial for every cell type. The components with positive effects were common to 4^{o}C and 22^{o}C preservation with only a few changes that occurred with time. Therefore, the selection of components to include in a preservation medium was straightforward because there was no difference between the conditions. The final formulation resulting from this DoE study is shown in Table 3-3, which was based on components that appear consistently in the previous equations. This result shows that, by application of engineering design processes to existing preservation medium, the formulation has been greatly simplified, and only components that improve quality parameters of T cells during hypothermic preservation can be included to improve the hypothermic preservation outcomes. The result also suggests that this type of quality-by-design principle should be applied to individual cell types as they are required to be preserved. This principle provides a robust method to formulate the individual medium compositions and supplements for different biological models, whereas the existing literature reviews of Southard et al. and Cobert et al. [81,82,228] are less convincing about medium formulations they recommended by comparing multiple, individual OFAT studies. The use of DoE provides a method that facilitated the investigation of a complex 10

component medium within 32 individual trials: a significant saving over completing a OFAT time study. This meant that all components could easily be investigated on one biological model and one set of response criteria; in this case membrane integrity and metabolic activity on recovery.

Component	Concentration (g/L)
Potassium lactobionate	36
Hydroxyethyl starch	0
Potassium phosphate	3.4
Magnesium sulphate	1.23
Raffinose pentahydrate	17.83
Adenosine	0
Allopurinol	0
Glutathione	0.92
Dexamethasone	0
Insulin	0

Table 3-3 Composition of modified preservation medium predicted to be optimal for 3 days of hypothermic preservation based on DoE analysis Equation 3-3 to Equation 3-10.

A drawback of previous studies was the confounding of the results by different quality parameters and different biological models for which the response was assessed. This led to Southard et al. concluding that raffinose is an insignificant component of the composition of a hypothermic preservation medium [81]. Raffinose was included in their formulation to reduce hypothermia induced swelling, which led to necrotic death of cells caused by its impermeant nature. This would suggest that the only effect raffinose should have would be on membrane integrity. However, its possible insignificance, and its membrane-integrity-only response, were shown to be incorrect by the results in this Section for the preservation of CD3/CD28 activated T cells. Here, raffinose was shown to be one of the most important components in the medium for preserving activated T cells. This Section showed that raffinose not only improved cell membrane integrity, but also had a positive effect on the metabolic activity of T cells on day 0 and subsequently after the application of hypothermia. The membrane integrity result was to be expected because Marsh et al. identified that the compound functions to stop hypothermia induced swelling [229]. The increase of cell

activity on day 0 shown in this Section was unexpected given the cell membrane impermeant nature of raffinose. Musatto et al. established that the component is a non-digestible saccharide that benefits humans as a dietary fibre and prebiotic [230], which would suggest that it cannot be metabolised by isolated human cells. It has, however, been shown by Chen et al. to induce autophagy when included in isolated cell cultures. This was in a manner independent of mTOR pathways, which was demonstrated in human keratinocyte cell lines. This biochemical effect was not linked to a breakdown of the trisaccharide into its constituent saccharides under the in-vitro conditions [83]. Also, Seglen at al. found that radiolabelled raffinose must be loaded into cells by electro-permeabilization [231], which suggested that the compound is not cell membrane permeable. In the autophagy study, no attempt was made to permeabilise the cell to the saccharides, so it may be possible that the effect of the raffinose was extra-cellular. It is not yet understood whether the oligosaccharides, saccharides with 3-10 sugar moieties, are present in a cell after exogenous loading. Overall, the studies above do suggest that raffinose affects the activity of cells. The results of this Section indicate that raffinose appeared to have a positive effect on both membrane integrity and metabolic activity of T cells before the application of hypothermia and when recovering the cells after hypothermic preservation. Despite having links to autophagy and not being able to enter the cell, raffinose upregulated the metabolic activity of T cells when examining the composition of medium for hypothermic preservation. Raffinose not only improves T cell membrane stability during hypothermia, but also improves the cell activity after preservation.

Potassium lactobionate was previously identified to be one of the most important components of UW medium by the original investigators, Southard et al., which the results of this Section agreed with. Southard et al. showed that lactobionate was cell impermeant and prevented hypothermia induced cell swelling [82]. Impermeant components were also one of the most important parts of the preservation medium used in the trials to preserve

activated T cells in this Section. However, so far in this Section, it has not been established if the effect of lactobionate is related to concentration.

The use of DoE in the results above has also identified that there was some significant interaction between some of the components, which may not have been identified if one factor at a time was investigated. The interaction terms between the components of the UW solution found in Equation 3-3 to Equation 3-10 mainly related to the compounds that would increase osmolality of the preservation medium. Results of Marsh et al. suggest that lactobionate should reduce the amount of swelling under hypothermia due to osmotic pressure related to intracellular impermeants [229]. However, the interactions of multiple components were negative in the results of this Section. This suggested that increased osmolality negatively affected the preservation of T cells. This will be investigated further in Section 3.3.3.

The DoE experiment also showed that potassium phosphate from the original UW formulation benefitted the preservation of T cells, with positive effects on both cell membrane integrity and metabolic activity on recovery after hypothermic preservation. Phosphate is known to act as a poor buffer under hypothermic conditions [85]. A high extracellular concentration of phosphate was shown by both Mozar et al. and Marco et al. to inhibit receptor activator of nuclear factor k B signalling in osteoclast like cells, and downregulated annexin II and impaired angiogenesis in endothelial cell-based assays [232,233]. Both those studies suggest that high phosphate could have an inhibitory effect on a range of cells. However, this DoE study showed that inclusion of phosphate increased metabolic activity on recovery after three days of hypothermic preservation, and also improved membrane integrity. Given that the inhibitory raffinose and phosphate compounds increased cell metabolic activity after hypothermic preservation, the conclusion could be that inhibiting T cells, in addition to reduced reaction rates through low temperatures, improves

the outcomes of preserving the cells. The role of phosphate is discussed further in Section 3.3.5.

The final component with positive effect for discussion was that of glutathione. After 1 day of hypothermic preservation, glutathione had no significant effect on either cell membrane integrity or metabolic activity on recovery. However, by day 3, it was shown to significantly impact the metabolic recovery of T cells after completing hypothermic preservation. Vreugdenhil et al. showed that extracellular glutathione maintains intracellular glutathione concentrations in rat hepatocytes, suggesting that exogenous concentrations maintain endogenous concentrations under 2-8ºC hypothermia [222]. No further cell parameters were investigated in that study. Mak et al. showed that glutathione has an important role in managing reactive oxygen species that are produced during the activation and expansion of T cells [146]. It could be suggested that glutathione has a similar antioxidant role in recovery of T cells after hypothermic preservation. Results of this Section showed that glutathione can benefit metabolic recovery after return to 37ºC, but this only becomes apparent after completing 3 days of hypothermia. The role of antioxidants is discussed in greater detail in Chapter 4.

Although many of the components of the UW solution were positive, some had insignificant effects, suggesting that they could be removed from the composition with no noticeable impact. This Section found two components to have a negative effect. The first was hydroxyethyl starch (HES), which had a strong negative effect throughout hypothermic preservation. HES can induce the aggregation of red blood cells [166]. Ozturk et al. showed that HES reduced the expression of inflammatory cytokines, and maintained concentrations of CD4+ and CD8+ positive T cells when HES was used for haemodilution after coronary bypass graft surgery [234]. This suggested an anti-inflammatory response of HES. CD3/CD28 activated T cells typically produce inflammatory cytokines such as IFN-γ and IL-2. Therefore, it is suggested that the anti-inflammatory effect of HES has a negative effect

on the inflammatory activated T cells during hypothermic preservation. Furthermore, solutions containing HES are being withdrawn from clinical use because of concerns about patient harm caused by them [235]. Therefore, the negative effect found by DoE in this Section, and this evidence of the published studies, suggest that it should be removed from a preservation medium of cells that will be applied as a therapy.

The second negative consideration was that of adenosine, which began to have a negative effect after prolonged hypothermic exposure. Purinergic signalling is important in development of naïve T cells. Both Trabanelli et al. and Cekic et al. showed that a lack of adenosine receptors on genetically modified regulatory T cells (Tregs) had increased proliferation, coupled with increased apoptosis [139,140]. Tregs make up a small percentage of the whole CD3+ T cell population. Ohta et al. and Huang et al. showed that extracellular adenosine inhibited the expansion of a more general T lymphocyte population when attempts were made to trigger the TCR [141,236]. The results presented in this Section suggest that prolonged exposure to adenosine under hypothermia at 4^{o}C appeared to trigger a negative response of adenosine related to a general population of CD3+ T cells. It is suggested that, when preserving T cells, the addition of nucleoside signalling compounds should be avoided. Southard et al. suggest that reducing components with the aim of simplification may not ultimately reduce the cost of a T cell preservation medium because the medium must still be manufactured and regulated [81]. Results of this Section showed that two of the components displayed significant negative effects on both membrane integrity and metabolic activity of the preservation of T cells. Therefore, they should be removed to improve post-preservation outcome for this cell type. In conjunction with this major change, other, insignificant compounds can justifiably be removed. There were few differences in the effect of the compounds tested between parameters of both the duration of preservation and the temperature of preservation. This suggests that, from the complete formulation of UW medium tested here, the components reported to be positive by Equation 3-3 to Equation

3-10 can be selected for use in the modified preservation medium. This final formulation is shown in Table 3-3.

3.3.2 Confirmation that the modified UW medium improves the preservation of activated T cells.

The optimised medium, shown in Table 3-3, resulting from the DoE screening study was tested with independent trials to confirm the improvement predicted from the resulting models shown in the previous equations. Results shown in Figure 3-2 confirmed that, after preservation at 4^{o}C in the modified University of Wisconsin medium (mUW), there was a significant increase in membrane integrity of cells compared to the complete formulation of UW medium. This was true after both 1 and 3 days of preservation. Both mUW and complete composition conditions resulted in a significant decrease in membrane integrity compared to day 0 values after completing 3 days of preservation at both temperatures, whereas, the mUW composition displayed no significant loss of integrity after 1 day of hypothermia at either temperature. Both compositions of preservation medium had a similar pattern of results when T cells were preserved at 22^{o}C compared to 4^{o}C; a small significant improvement from the simplified medium after 24 hours and a significant improvement after 72 hours. There was no significant difference between the two temperatures of 4^{o}C and 22^{o}C when comparing the mUW with respect to membrane integrity. Results in Figure 3-3 confirmed that the mUW composition also increased the metabolic activity on recovery after 72 hours of hypothermic preservation at both temperatures of 4^{o}C and 22^{o}C. However, results in Figure 3-3 also showed that there was a large decrease in the metabolic activity recovered after preservation compared to day 0 from day 1 onward. This decrease was irrespective of temperature.

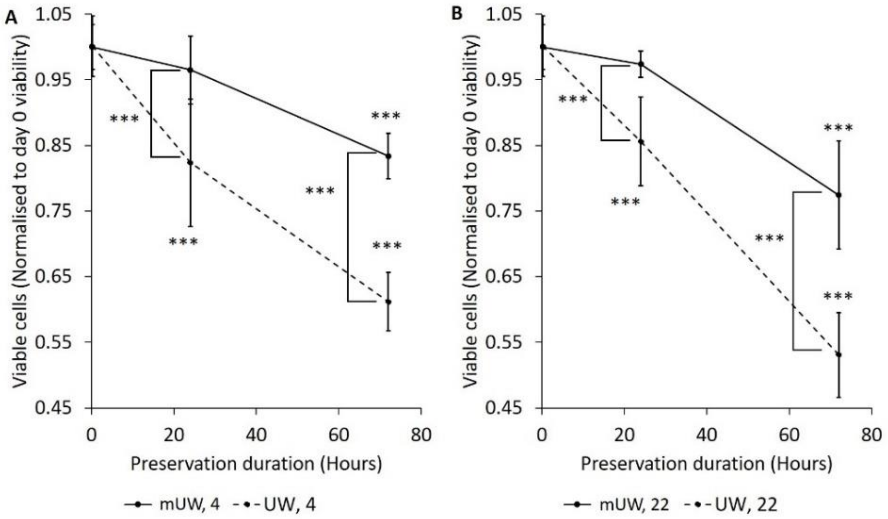

Figure 3-2 Viable cell count of activated T cells after different durations of hypothermic preservation in modified preservation medium. Viable cell count was compared between modified composition proposed by DoE (mUW) and the complete composition of UW. Viable cell count includes cell exclusion of Draq 7 and change in total cell count. Results are normalised to a day 0 control of activated T cells suspended in PBS. Statistical analysis was performed by suitably valid ANOVA and Tukey post-hoc testing. A) Modified UW medium (mUW) and complete composition in combination with preservation at 4ºC. B) mUW and complete composition in combination with preservation at 22ºC. See Section 3.2.10 for the explanation of statistical analysis and consequent labelling. N = 6.

The mUW preservation medium improved membrane integrity and metabolic activity after hypothermic preservation, which confirmed that DoE is a useful tool for modifying multiple component systems that occur during hypothermic preservation. This DoE study used UW as a base model for preserving T cells for three reasons. Firstly, because of its consideration by Stewart as a "gold standard" for organ preservation [200]. Secondly, it has been tested by Southard et al. with isolated cell lines during its development [81]. Thirdly, it continues to be investigated by Juriasingani et al. in conjunction with new interventions such as hydrogen sulphide treatment [237]. The hydrogen sulphide is suggested by Bouma et al. to induce torpor in non-hibernating animals [103]. The optimised formulation produced by the DoE study was confirmed by results of this Section to maintain membrane integrity and slightly increase the metabolic activity on recovery of CD3/28 activated T cells. The results of this Section also confirmed an observation that was inferred from the DoE study that, although membrane integrity was being preserved, there was a significant decrease in metabolic activity that could not be recovered on re-warming of the T cells. There is a decoupling of

metabolic activity from viable cell number that was shown by a quicker reduction of metabolic activity compared to membrane integrity of the T cells. This indicates that further investigations must be conducted with the aim of increasing the metabolic activity of T cells after recovery from hypothermic preservation, which is one motivation for the studies of Chapter 4 and Chapter 5.

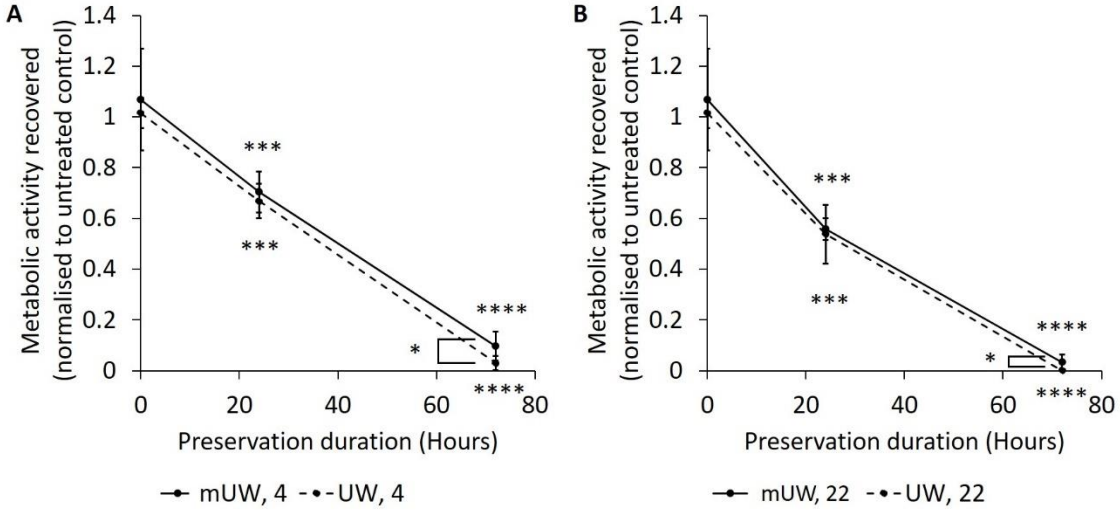

Figure 3-3 Alamar blue reduction of hypothermic preserved T cells with modified UW (mUW) medium compared to original formulation. Alamar blue was interpreted as metabolic activity of activated T cells. Activity was assessed after different durations of hypothermic preservation. Results have background reduction subtracted and are normalised to a day 0 control of activated T cells. A) Modified UW medium (mUW) and complete composition in combination with preservation at 4°C. B) Modified UW medium (mUW) and complete composition in combination with preservation at 22°C. Statistical analysis was performed with $\log_{10}$ transformed data to validate ANOVA and Tukey post-hoc testing assumptions. See Section 3.2.10 for the explanation of statistical analysis and consequent labelling. N = 6.

3.3.3 Osmolality of electrolyte composition and preservation viability.

The concentration of lactobionate in the modified composition was altered to investigate the effect of osmolality on membrane integrity during preservation. Results in Figure 3-4 show that, over the range of 211 to 275 mOsm/kg, the slightly hypo-osmolar to physiological total concentrations do not significantly impact membrane integrity. However, hyperosmolar concentrations of 372 mOsm/kg demonstrated a significant decline in membrane integrity. These results confirm that, under hypothermic temperatures, T cell membrane integrity responds negatively to high osmolality, which was generated by changing the concentration of the most beneficial components found in Section 3.3.1.

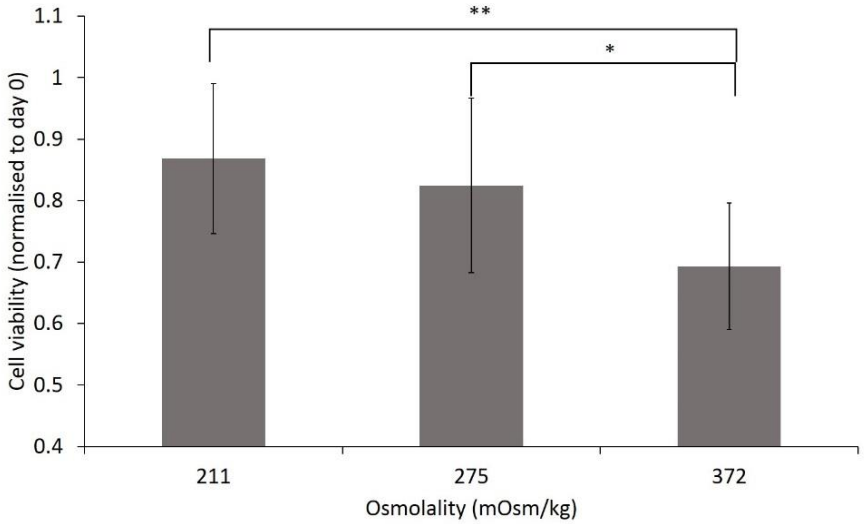

Figure 3-4 The response of cell viability to changing osmolality and hypothermia at 4°C. Cell viability was measured by total cell count, membrane integrity by Draq 7 exclusion and quantity of cells in control samples. Osmolality was changed by varying the concentration of potassium lactobionate. Osmolality was measured by freezing point depression, and viability by the cell count and exclusion of Draq 7. See Section 3.2.10 for the explanation of statistical analysis and consequent labelling. N = 6.

The complete formulation of UW is hyperosmotic to prevent cell swelling that occurs during hypothermia and has an osmolality of 320mOsm/kg [79]. HTS has a higher osmolality of 360mOsm/kg, which is achieved through components with similar properties, cell impermeants [238]. The results of Figure 3-4 showed that T cells respond negatively to hyperosmolality either through the complete UW formulation or by increasing the concentration of the beneficial impermeant component of lactobionate. Veale et al. and Sparrow et al. reported a similar requirement for low osmolality preservation medium when red blood cells (RBC) were preserved at 4°C in hypotonic medium, where RBCs benefitted from increased membrane and osmotic resilience, which was a measure of how the cells survive lysis in hypotonic buffer. These cells were dehydrated after the storage, the effect of which was hypothesised to be related to different behaviour of ion channels under reduced temperatures [239,240]. UW medium was developed for complete organs and, as such, has been tested on whole organs, tissue sections, and cells adhered to surfaces [79,82]. The RBCs used in the published studies, and the T lymphocytes examined in this thesis, were derived from blood and are examined in liquid medium without adherence to any surface. Wallas et

al. and States et al. showed that adherent cells in kidney tissue and RBCs have no Na+K+ATPase activity during exposure to hypothermia, so the transport of ions is uncontrolled during hypothermia for both these categories of cells [182,241]. The other factor that could play a role in response to different osmolalities in the apparently inactive membrane transporter state of hypothermia could be related to protein concentration of the cell. Human T lymphocytes have a lower protein of 14.1% ± 1.79% [242], compared with RBCs around 20%, and muscle and liver cells ranging from 20% toward 30% [243]. This difference might be explained by the extracellular fluids these cells are exposed to. Serum has a high protein content, whereas interstitial fluid has a low protein content mainly because of the low permeability of capillaries to proteins [20]. The whole basis of hypothermia induced cell swelling relates to the presence of macroscopic impermeable proteins inside the cell that cannot move across the cell membrane, producing the Gibbs-Dunan effect, which is the increased concentration of ionic components in the cell. This allows for water uptake in to the cell, in turn drawing in more ions, which increases osmolality and eventually leads to water uptake that ruptures the cell [20]. A lower protein content would reduce this effect. The other difference in T lymphocytes compared to adherent cell types is the small diameter of T cells, which may be another reason for the low osmolality requirement of preservation medium that T cells and RBCs share. This may make them susceptible to removal of intracellular water. The results of Figure 3-4 and discussion here suggest that, in cells which have lower protein concentrations, a high concentration of cell impermeant compounds is not required to benefit the preservation of these cells. The requirement for a lower osmolality compared to existing preservation medium showed that the base components of a preservation medium do need to be examined for individual cell lines.

3.3.4 Phosphate and its impact on cell preservation.

In Section 3.3.1, phosphate was repeatedly shown to be an important component of the preservation medium of T cells. Historically, phosphate was considered to act as a buffer in

preservation mediums, although it only has weak buffering capacity [82,244], and some studies advocate the avoidance of this anion in preservation strategies [93]. Results in Figure 3-5 showed the final pH recorded after 3 days of hypothermic preservation with similar compositions that either contain phosphate or do not. There was a decrease in extra-cellular acidification of the samples containing phosphate at both 4 and 22ºC, but the difference in pH after 3 days of preservation was insignificant. Another result displayed by Figure 3-5 was the larger acidification of the samples preserved at room temperature, presenting one mechanism to investigate further when deciding on how to improve the success of 22ºC preservation: biochemical reaction rates are not being suppressed as effectively, allowing processes that led to acidification to happen to a greater extent.

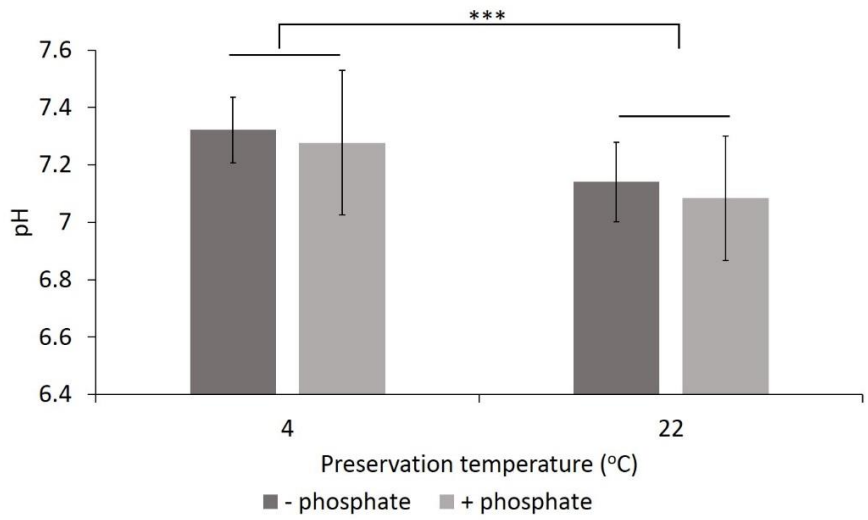

Figure 3-5 Post-preservation pH of medium compositions that contain phosphate, and those that exclude the component. All mediums were adjusted to pH 7.4 before use for preservation. Measurement of pH was taken using a potassium chloride probe designed for testing low volumes of sample, hence small volume samples did not require agitation. Graph shows pH after both refrigerated preservation and controlled room temperature preservation. See Section 3.2.10 for the explanation of statistical analysis and consequent labelling. N = 8

Inorganic phosphate will competitively and reversibly inhibit a number of enzymes, and it will also promote the glycogenolysis of cells in preservation of cardiac tissue[84]. Furthermore, extracellular phosphate inhibits angiogenic capacity of endothelial cells [233]. Phosphate appears to be inhibitory on some cell biochemical reactions. This may provide an explanation as to how the phosphate is having a beneficial effect on preserving T cells if the biochemical interaction between the chemical compound and T cell is similar. Therefore,

the effect of phosphate on T cell metabolism was investigated, and results are shown in Figure 3-6. The addition of both phosphate and sodium chloride components increased the osmolality by 29 mOsms/kg above the cell culture medium osmolality, and both significantly reduced the glucose consumption of the T cells compared to a normal culture control. Phosphate addition to the culture reduced the glucose consumption 0.31-fold compared to that of the same osmolality control of sodium chloride additive; a further significant decrease of the phosphate containing test. For glucose related results, see Figure 3-6A. The addition of both sodium phosphate and sodium chloride reduced the rate of lactate production as well, but only the addition of sodium chloride reduced the consumption significantly compared to normal culture conditions. Phosphate addition resulted in lactate production that was 0.90-fold lower than that of a standard culture, yet was 1.39-fold higher than that of a culture with the same osmolality provided by the addition of sodium chloride. Data related to lactate production is shown in Figure 3-6B. The effect of increased osmolality has suppressed metabolism, but the addition of a phosphate compound to activated T cell suspensions produced a substantial shift in the cell metabolism away from glucose consumption to a form of metabolism that increased the ratio of lactate production.

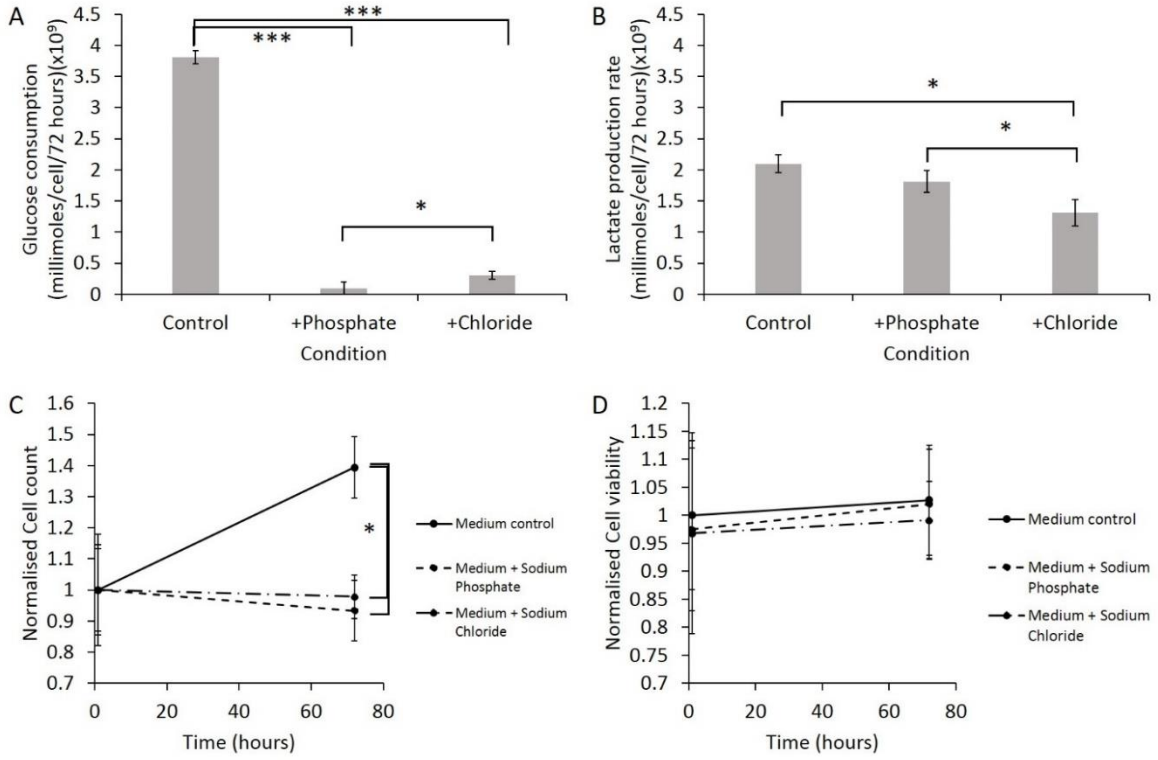

Figure 3-6 Results of including extra inorganic phosphate or sodium chloride on T cell metabolism during 37ºC, antigen stimulated culture. T cells were re-suspended into fresh medium with IL-2, and with sodium -chloride or –phosphate, or without further additives, and incubated for 3 days. A) Glucose consumption rate of T cells suspended in three different conditions at 37ºC. 1) An unchanged medium control. 2) Culture medium with 10mM sodium phosphate added. 3) Culture medium with 34mM of sodium chloride added. B) The same conditions with data showing lactate production rate. C) Normalised cell count, to Day 0, of each of the cell suspensions in media the same as A). D) Fraction of live cells counted in each test normalised to Day 0 number of live cells in the medium control, cells suspended in the same medium compositions as A). See Section 3.2.10 for the explanation of statistical analysis and consequent labelling. N = 5.

All three of these conditions retained a stable, or increased number of live cells, Figure 3-6C, with the medium control resulting in a significant 1.39-fold increase compared to the modified conditions of phosphate and sodium chloride, which showed a small fold decrease, 0.97 and 0.93, respectively. These small differences show that the hyper-osmolality is having a negative effect on cell expansion during IL-2 supplemented cell T culture. All three conditions tested resulted in stable cell membrane integrity of the T cells in culture, see Figure 3-6D. Therefore, cell viability should not have affected the change in metabolite concentrations. These changes were considered sufficiently small not to have affected the results of lactate and glucose reaction rates that are shown by concentration changes in Figure 3-6A and Figure 3-6B.

Figure 3-6 demonstrated that addition of phosphate has inhibited glucose uptake of activated T cells under in-vitro culture conditions without a significant decrease in lactate production. Phosphate also had a large positive effect on post-preservation viability and metabolic activity on recovery after either temperature of 4^{o}C or 22^{o}C hypothermia; this result is shown in Section 3.3.1. Some other sources consider phosphate to act as a pH buffer [93,244,245]. However, the results of Figure 3-5 showed that the inclusion of phosphate only had a small effect on the extracellular pH of the cell suspension after 5 days of preservation. Instead, it was hypothesised that phosphate was benefitting preservation by affecting the T cell metabolism, suggested by its ability to reversibly and competitively inhibit enzymes. Pulis et al. have shown that the addition of extra buffer components to a normal UW medium also has a similar effect on cell metabolism by promoting glycogenolysis for cardiac tissue: a further point is that glucose is also avoided in bio-preservation due to intracellular acidification [20,84,179]. One additional pathway presented by the results of this Section is that phosphate altered the T cell metabolism in a manner beneficial for hypothermic preservation. This alteration was a reduction of glucose consumption, while avoiding decreased cell viability. It is possible that this inhibitory effect of phosphate on glucose uptake and cell proliferation without negatively affecting viability could lead to improved cell viability and activity after recovery from hypothermic preservation because of the non-destructive pausing of cell activity. Addition of phosphate components increased the relative amount of lactate production, suggesting the metabolism is not oxidative, so methods to increase the ability of cells to make more use of oxidative metabolic pathways would be desirable to supply more energy.

3.4 Summary.

A range of hypothermic preservation media exist that were developed with a one-factor-at-a-time approach. These media contain a range of components that may be capable of mitigating cellular stresses that occur during hypothermia. There was existing data, which

suggested that response to hypothermia may be cell specific. This supported the need to perform engineering quality-by-design studies to evaluate the hypothermic preservation of activated T cells. No evidence was found of existing studies preserving the activated T cells, commonly used in adoptive transfer therapies, by the reduced temperatures of hypothermia beyond 18 hours. Therefore, an approach that investigated the effect of individual components was vital to identify which components were required. DoE was shown to be capable of testing all the components of the UW preservation medium, with the reduced number of data points required by a fractional factorial approach reducing the demand on costly laboratory resources. The proposed optimum composition was validated with independent studies to confirm the DoE prediction. Mechanisms behind the action of some components have been proposed and updated, and other components have been identified for further in-depth analysis. Compounds that were suggested to be inhibitory on cell activity were found to increase the metabolic activity of T cells when reverted to normal temperatures after exposure to hypothermia. This study demonstrates the need to include quality-by-design principles in order to establish individualised mediums to support the preservation of different cell types. The main point for further investigation was shown to be identification of methods to improve T cell metabolic activity on recovery. Having identified beneficial components in the original formulation of the UW medium, these can be investigated in greater detail and combined with further additives so that a complete preservation medium and process can meet all requirements to maintain therapeutically active T cells.

4 Effect of Antioxidants on T cell Preservation.

4.1 Introduction.

The aim of this study was to investigate if compounds with antioxidant activity have a beneficial effect on hypothermic preservation of T cells.

One part of the study investigated glutathione that was already present in the formulation of the modified University of Wisconsin (mUW) medium. In Chapter 3, glutathione was reported to improve metabolic activity of T cells on recovery to 37^{o}C only after completing 3 days of hypothermia at a temperature of either 4^{o}C or 22^{o}C. Glutathione is an endogenous compound that has antioxidant function [143]. The literature review, Chapter 2, established that the role of reactive oxygen species is important when considering the initial expansion of T cells after mitogen activation, particularly that the presence of glutathione is required in T cells to achieve their rapid expansion [146,147]. It was also established that exogenous glutathione affects cells during hypothermia. The presence of exogenous glutathione reduced the depletion of intracellular concentrations, and reduced hepatocyte necrosis under low temperatures [246,247]. However, the concentration of glutathione within each cell type is different, with hepatocytes containing larger concentrations of glutathione compared to other cells [143,248]. Therefore, part of Chapter 4 investigated the role of glutathione in preserving T cells in hypothermic temperatures, focussing on how the concentration of exogenous glutathione affects the recovery of T cells. The concentration range tested was set to a maximum by the high concentration found in hepatocytes and to a minimum by the low concentrations more comparable to those found within T cells specifically.

It is known from literature that including water soluble analogues of vitamin E in a medium for the preservation of adherent cells lines obtained from different tissues can improve the results from hypothermic preservation. 6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid (trolox) was shown to increase metabolic activity after recovery to in-vitro conditions from hypothermia [21,191,221]. Trolox is one example of a water-soluble vitamin E

analogue. (6-hydroxyl-2,5,7,8-tetramethylchroman-2-yl)(4-(2-hydroxyethyl)piperazin-1-yl)methanone (SUL-109) is a second example of a water-soluble vitamin E analogue. SUL-109 was shown in a study by Hajmousa et al. to improve recovery of adipose derived stem cells by maintenance of membrane integrity and mitochondrial membrane potential [202]. In that study, the mechanism was thought to be related to stimulation of mitochondrial complexes providing ATP during hypothermia, but this was only demonstrated after very short periods of 1 hour. Their application of SUL-109 was achieved in culture medium, which means that compounds required for complete in-vitro culture were present in the tests. The application with culture medium suggests simplicity, but the complete product of cells and medium may not be readily infusible into the patient.

The compounds trolox and SUL-109 appear to stimulate cell metabolism at different stages of the application of hypothermia and can reduce apoptosis in a range of stressing conditions. A further possible candidate is alpha-Tocopherol (ATOC), which has a similar role to the other compounds identified above [205,249].

The studies previously discussed in this introduction identified that antioxidant compounds can affect cell metabolism. However, the base preservation medium developed in Chapter 3 did not contain a compound that is known to be metabolised by T cells. It could be possible that the addition of antioxidant compounds alone to the mUW, without a usable metabolite, may not produce a complete benefit of the compounds. Therefore, part of the study in Chapter 4 investigated the combination of glucose with ATOC and trolox, and utilised the ability of Design of Experiments (DoE) to analyse if any interactions occurred between these three compounds during hypothermic preservation. This would help establish if complete T cell metabolism can persist when incubated in temperatures below 37^{o}C. Further, this would identify if the preservation medium must be supplemented to support cell metabolism. The range of concentrations tested for the antioxidant compounds was set to match those previously tested by Hajmousa et al. [202]. Use of these pre-existing concentrations should

identify if the same metabolic effects can be reproduced in T cells. Glucose was tested as the metabolite in this study, set to a maximum concentration as found in standard culture media. The exact data points were set by the DoE methodology to produce the most reliable results. The main purpose of this study was to understand if the metabolism of activated T cells could be stimulated to continue during hypothermia, and subsequently, whether this stimulation maintained the pre-preservation energy state of the cells.

A further part of this study also investigated the role of apoptosis during preservation in hypothermic conditions in the specific case of T cells. Once activated, T cells undergo cell death after antigens are removed, which is known as cytokine withdrawal induced cell death (CWID) [133,134]. These in-vitro observations reflect how the human immune system works [117]. This part of the study would establish if the cell death mechanisms, related to immune responses of the T cells, could cause cell death when attempting to preserve activated T cells under hypothermia. It is known that hypothermic temperatures generally allow biochemical reactions to continue [20]. But it is not yet known if it may be possible for the cells to respond to removal of antigens during reduced temperatures. This part of the study aimed to understand if this response is possible during hypothermic temperatures.

4.2 Materials and methods.

4.2.1 Exemplar design of experiments response surface modelling.

This study utilised the response surface methodology (RSM) of Design of Experiments (DoE), which is applicable once key parameters have been identified, and it is desired to find optimum levels of components that achieve the required effect on the response parameter. The method chosen for the study of ATOC, trolox and glucose was a central composite design (CCD). The design table used to investigate the preservation duration and temperature affects is shown in Table 4-1. The analysis of a CCD follows the same methods as described for partial factorial designs described in Chapter 3 to establish significant model terms. The main difference between CCD and factorial methods of Chapter 3 is the design table and

number of levels included for each factor to be investigated: the inclusion of plus or minus alpha points allows the calculation of quadratic terms to fit the response values and then further calculation to examine if they hold any significance. The particular design chosen for this analysis was a circumscribed CCD with the design points at ±1 levels and alpha calculated to maintain rotatability of the design so that there was no bias on certain regions of the response surface. The addition of design points at ± alpha levels allows potential curvature of the response to be estimated. The responses analysed for the design were; viable cell number, see 3.2.2; metabolic activity, see 3.2.3; activity of ROS, see 4.2.3; intracellular ATP, see 4.2.4; and amount of apoptotic cells, see 4.2.5.

4.2.2 Formulation of the supplemented preservation medium.

Glutathione, glucose, trolox, α-Tocopherol (ATOC) and ME_2SO were purchased from Sigma Aldrich UK. For the DoE study, concentrated stock solutions of the basal medium composition were prepared in a similar method as described in Section 3.2.5. Glucose was dissolved to a concentration of 200mM. This stock solution was filter sterilised by 0.22µm filter. Trolox and ATOC were dissolved in dimethyl sulfoxide in a range of concentrations so that ME_2SO concentration across all final supplemented solutions was 1% (vol.). These stocks were then combined together to form the compositions shown in Table 4-1. These compositions were then tested in combination with the activated T cells and hypothermic conditions in the same way as Section 3.2.1. The chemical structures of the antioxidants glutathione, ATOC and trolox are shown in Figure 4-1.

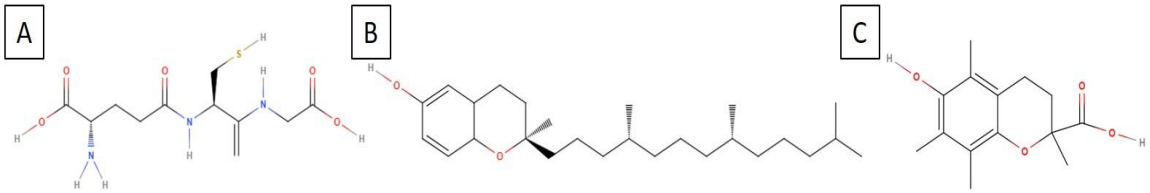

Figure 4-1 Images showing the chemical structures of the antioxidants investigated in this Chapter. A) Glutathione. B) α-Tocopherol. C) Trolox. Images created using MolView software.

For the OFAT trials with glutathione, stock solutions of this compound were prepared by dissolving in formulated basal preservation without glutathione to a concentration of

200mM, filter sterilised and then added to further base preservation media in different volumes to obtain the concentrations as discussed in the results below. This supplementation replaced the concentration of glutathione shown in Table 3-3.

	Factor 1	Factor 2	Factor 3
Std	A:Glucose (mM)	B:Trolox (mM)	C:ATOC (mM)
1	2.106712	2.106712	2.106712
2	7.993288	2.106712	2.106712
3	2.106712	7.993288	2.106712
4	7.993288	7.993288	2.106712
5	2.106712	2.106712	7.993288
6	7.993288	2.106712	7.993288
7	2.106712	7.993288	7.993288
8	7.993288	7.993288	7.993288
9	0.1	5.05	5.05
10	10	5.05	5.05
11	5.05	0.1	5.05
12	5.05	10	5.05
13	5.05	5.05	0.1
14	5.05	5.05	10
15	5.05	5.05	5.05
16	5.05	5.05	5.05
17	5.05	5.05	5.05
18	5.05	5.05	5.05
19	5.05	5.05	5.05
20	5.05	5.05	5.05

Table 4-1 Design table for CCD study of antioxidant and glucose compounds showing concentration of each component.

4.2.3 Reactive oxygen species detection.

Activity of ROS species was measured using 2′, 7′-Dichlorofluorescein diacetate (DCF), which becomes fluorescent upon oxidation by ROS. For capacity assays, DCF was added to 50µL of PBS to be tested to a final concentration of 10µM to form the assay buffer. Positive controls also included hydrogen peroxide to a concentration of 40µM. 50µL of $10x10^6$ cells/ml were transferred to a 96 well-plate and the assay buffer added to this volume. The samples were incubated at 37^{o}C in the dark for 2 hours, then examined for fluorescence on a Molecular Devices Spectramax plate reader with excitation and emission at 485nm and 535nm, respectively.

4.2.4 Measurement of intracellular adenosine triphosphate.

ATP was measured by ATP determination kit purchased from Thermofisher and used according to manufacturer instructions. ATP was extracted by lysing cells in boiling, deionised water. $5x10^5$ total cells were lysed in 500µl of water. Samples were centrifuged at 10000g for 15 mins and supernatant was used with the Thermofisher kit. The extraction method was adapted from Yang et al. [250]. This method was adapted to measure lower quantities by linearly reducing the lysing medium to match quantity of cells present.

4.2.5 Apoptosis measurement.

Apoptosis was prepared for measurement by two methods. Firstly, by using commercial Abcam kit, ab176749, in accordance with manufacturer instructions. $0.5x10^6$ cells were collected per sample. Suspensions were spun at 500g for 5 minutes and supernatant discarded. Cells were re-suspended in 200µL of sample buffer prepared with 2µL of Apopxin Green indicator and 1µL of 7-Aminoactinomycin D. Cell samples were incubated for 30 minutes at room temperature and the samples then topped up to 500µL using PBS. The second method used intracellular propidium iodide (PI) staining. This PI staining followed the same method as described in Section 5.2.3. The samples for each method were then measured on a BD FACScalibur system and their analysis was completed in Flowing Software 2 open source flow cytometry software.

4.2.6 Mitochondrial membrane potential identification.

To assess mitochondrial membrane potential (Δψ), the Abcam kit JC-1 - Mitochondrial Membrane Potential Assay Kit (ab113850) was used following manufacturer instructions. Briefly: $2.5x10^5$ cells were used per sample; washed and stained with 10µM of JC-1 buffer; incubated for 1 hour; then read for monomer and aggregate forms with excitation at 475nm and emissions at 530nm and 590nm, respectively. Results after preservation were compared to an in-vitro control.

4.2.7 Previously used methods.

Detailed methods for the measurements of; cell membrane integrity, metabolic activity, ATP concentration, enzymatic measurement of metabolites, and statistical analysis, can be found in Section 3.2. Cell culture and hypothermic preservation methods are also detailed in Section 3.2.

4.3 Results and discussion.

4.3.1 Viable cell number shows no differences amongst the design of experiments design table.

Viable cell number was investigated to understand if the effect of the additives alleviated the small amount of cell death that was shown to occur in Chapter 3. Supplementation of the modified University of Wisconsin solution (mUW) had no significant effect on membrane integrity determined by ANOVA within the DoE software, as can be visually observed by the small differences in cell membrane integrity shown in Table 4-2.

Processing these results in the CCD showed no significant effect from any of the 3 components at either temperature of 4^{o}C or 22^{o}C after preservation for 1 or 3 days. Table 4-2 showed that most of the conditions tested fell within the 6 replicates of standards 15 to 20. Analysis between each dataset for the DoE design table showed that there was no difference between temperatures of 4^{o}C and 22^{o}C on day 1. Day 3 results, combined together, produced lower viability at 4^{o}C compared to day 1, with a p value of 0.037 when comparing all 20 conditions. This may relate to the slightly reduced membrane integrity of some standards, which was deemed not be significant in the DoE analysis of the 4 individual data sets. This significant difference with time was not seen at 22^{o}C.

Std	Run	A: Glucose (mM)	B: Trolox (mM)	C: ATOC (mM)	D1, 4°C	D1, 22°C	D3, 4°C	D3, 22°C
1	17	2.1	2.1	2.1	81.53%	81.87%	79.11%	80.93%
2	14	8.0	2.1	2.1	84.22%	85.93%	75.07%	82.18%
3	11	2.1	8.0	2.1	81.25%	88.43%	73.11%	82.05%
4	12	8.0	8.0	2.1	80.72%	83.99%	76.82%	79.52%
5	3	2.1	2.1	8.0	83.12%	83.60%	76.80%	75.28%
6	5	8.0	2.1	8.0	83.80%	84.47%	74.78%	72.15%
7	9	2.1	8.0	8.0	83.64%	84.08%	73.44%	74.91%
8	15	8.0	8.0	8.0	83.63%	83.01%	82.57%	80.97%
9	18	0.1	5.1	5.1	82.26%	84.12%	63.78%	70.21%
10	6	10.0	5.1	5.1	83.37%	83.54%	76.77%	74.41%
11	10	5.1	0.1	5.1	83.29%	83.07%	75.30%	74.09%
12	2	5.1	10.0	5.1	83.66%	82.16%	69.34%	77.54%
13	8	5.1	5.1	0.1	81.44%	85.06%	67.71%	78.37%
14	13	5.1	5.1	10.0	87.32%	81.99%	61.27%	72.58%
15	19	5.1	5.1	5.1	83.88%	94.46%	73.72%	75.06%
16	16	5.1	5.1	5.1	84.36%	84.50%	78.12%	73.15%
17	4	5.1	5.1	5.1	80.47%	79.63%	67.56%	77.80%
18	1	5.1	5.1	5.1	79.41%	82.46%	73.65%	78.22%
19	20	5.1	5.1	5.1	78.95%	86.55%	80.27%	79.74%
20	7	5.1	5.1	5.1	81.80%	81.34%	77.56%	83.54%

Table 4-2 Viable cell number (4 right hand columns) of T cells preserved with varying concentrations of glucose, trolox and ATOC. Cell count and membrane integrity was performed as described in 3.2.2 The viable cell counts are presented as the average of 3 biological replicates.

4.3.2 Reactive oxygen species activity was dominated by increased levels from addition of trolox.

ATOC and trolox can have an antioxidant capability, which was investigated in this section by their response on concentration of ROS produced. Glucose and ATOC supplementation as individual components produced no significant effect on ROS activity over the concentration range of 2.11 to 7.99mM tested, whereas, the interaction term of these two compounds produced a significant effect on the ROS activity response, the heat map of which is shown in Figure 4-2A. This interaction showed that, under initial incubation on day 0, matched concentration levels of both glucose and ATOC were essential to produce the lowest levels of ROS activity.

It was shown that ATOC and glucose produced no significant effect on ROS activity with the application of hypothermia at either temperature or any duration, but that trolox levels significantly increased ROS activity, shown in Figure 4-2B. The response to trolox was

initially quadratic over the concentration range tested, which suggested that increased concentrations led to ever increasing ROS activity. Trolox supplementation continued to increase ROS activity over the subsequent application of all hypothermic conditions, Figure 4-2C as a representative example, but the order was reduced to a linear relationship. This was the same for the other 3 datasets from the application of hypothermia. The complex interactions that occur on day 0 appear to give way to a much simpler, linear response of only one component with the onset of hypothermia, which suggested a simplification in cell response once cells have been exposed to temperatures below 37^{o}C. This is particularly observable in Equation 4-1 through to Equation 4-5, which start off with a complex amount of terms on day 0 but only contain a linear term related to trolox (B) thereafter. The lower constant terms of these equations for results at 22^{o}C suggested that cells preserved over a short duration at this temperature retain more of an un-supplemented antioxidant capacity, although, they are closer in magnitude to 4^{o}C hypothermia after 3 days of preservation. This could suggest that the addition of antioxidant compounds had less of an effect at preservation temperatures of 22^{o}C.

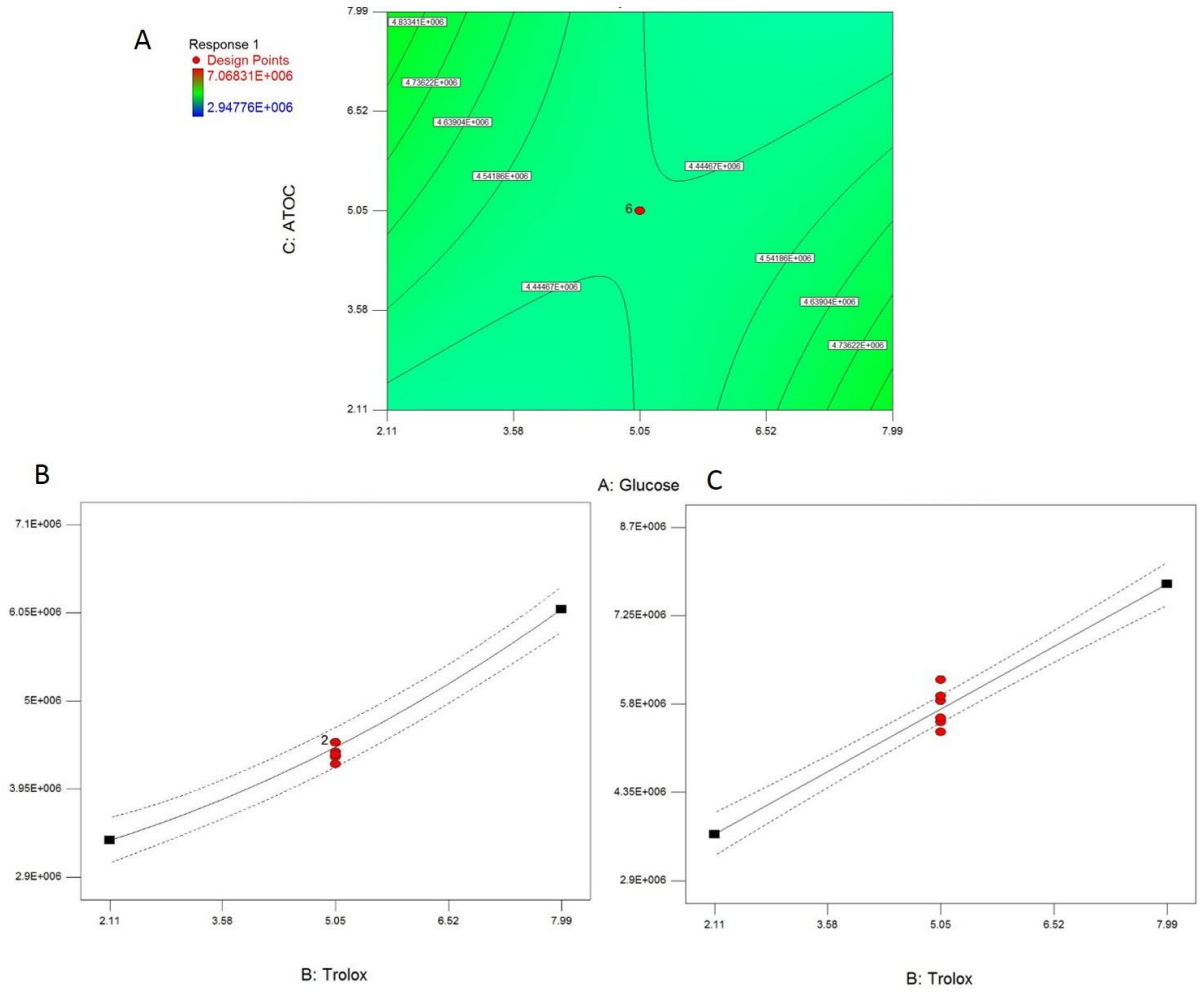

Figure 4-2 Reactive oxygen species produced by T cells supplemented with glucose, alpha-tocopherol and trolox. Time points include before commencing preservation and after 1 day of hypothermic preservation shown as a representative example of subsequent days and grades of hypothermia. Figures show response surfaces or individual response with relative fluorescent units as the scale of the response. ROS activity on A) day 0, the interacting term between glucose and ATOC. B) Day 0, the individually significant effect of trolox. C) Day 1, 4°C, trolox had the only significant effect, which was representative of all further time points and temperatures.

$$ROS\ response_{D0} = 4.45 \times 10^6 - 2.21 \times 10^4 A + 1.38 \times 10^6 B - 8.44 \times 10^2 C - 2.61 \times 10^5 A \times C + 1.99 \times 10^5 A^2 + 2.65 \times 10^5 B^2$$

Equation 4-1

$$ROS\ response_{D1,4^oC} = 7.00 \times 10^6 + 2.38 \times 10^6 B$$

Equation 4-2

$$ROS\ response_{D1,22^oC} = 5.72 \times 10^6 + 2.05 \times 10^6 B$$

Equation 4-3

$$ROS\ response_{D3,4^oC} = 6.51 \times 10^6 + 1.95 \times 10^6 B$$

Equation 4-4

$$ROS\ response_{D3,22^oC} = 6.54 \times 10^6 + 1.64 \times 10^6 B$$

Equation 4-5

4.3.3 T cell metabolic activity on recovery to 37ºC was significantly reduced by addition of trolox.

It was understood that the additive components could influence cell metabolism, therefore this was investigated through the examination of metabolic reduction of Alamar blue. Increasing the concentration of trolox decreased the Alamar blue reduction by T cells, which was significant in both linear and quadratic terms of the model. The quadratic model showed that, as the concentration of trolox was increased, the decline in metabolism was relatively greater. This decrease in activity can be seen from the negative coefficient of the trolox (B) term in Equation 4-6 resulting from the lowered values of trolox supplementation shown in Equation 4-2. Figure 4-3 shows a cubic representation of the ±1 levels of the three compounds of the study. The corners of these cubes display the processed values for the response of the three compounds on metabolic activity on recovery and provide a visualisation of the possible effect of each compound. The glucose term, A, in the DoE model had a small coefficient in Equation 4-6. This small coefficient showed that supplementation of the preserving medium with glucose had no independent significant effect on the day 0 metabolic activity of the cells. The interaction term between glucose and trolox, AxB, held a P-value of 0.0594 suggesting that it was important. Metabolic activity in the presence of increasing concentrations of both trolox and glucose was progressively inhibited on day 0, see the heat map shown in Figure 4-4. ATOC alone, term C of Equation 4-6, was an important positive term, with p=0.0590, because the coefficient of C was only slightly less in magnitude compared to B, which was significant. Therefore, an increased concentration of ATOC led to a slightly higher metabolic activity.

The reduced metabolic activity from trolox addition remained the same with application of hypothermia at both temperatures tested and showed no change with time. This reduced activity remained significant throughout the DoE models generated from the datasets shown in Figure 4-3.

Glucose switched from slightly increasing metabolic activity on day 0 to an insignificant decrease with completion of 1 day of hypothermia at either of the tested temperatures, see the negative A coefficient of Equation 4-7 and Equation 4-9. This insignificant decrease was observed for day 3 of hypothermia at 22^{o}C, see Equation 4-10. Because the effect of glucose was small, the glucose term was removed from the model at 4^{o}C after 3 days of hypothermia to allow prediction of significant differences, see Equation 4-8.

Supplementation with ATOC had an effect that changed with both time and temperature. After 1 day of hypothermia at 4^{o}C, ATOC retained an ability to slightly increase metabolic activity, see the positive coefficient for C in Equation 4-7, but after 3 days of hypothermia ATOC supplementation insignificantly decreased in metabolic activity at increased concentration, see C coefficient in Equation 4-8. At 22^{o}C, ATOC behaved similarly to glucose with the increased activity on day 0 switching to a decrease from day 1 onwards, see Equation 4-9 and Equation 4-10. Some effects on metabolic activity were detected, but the desired increase that ATOC provided was small and short-lived and therefore may not be required for improving cell metabolism after 3 days of exposure to hypothermia.

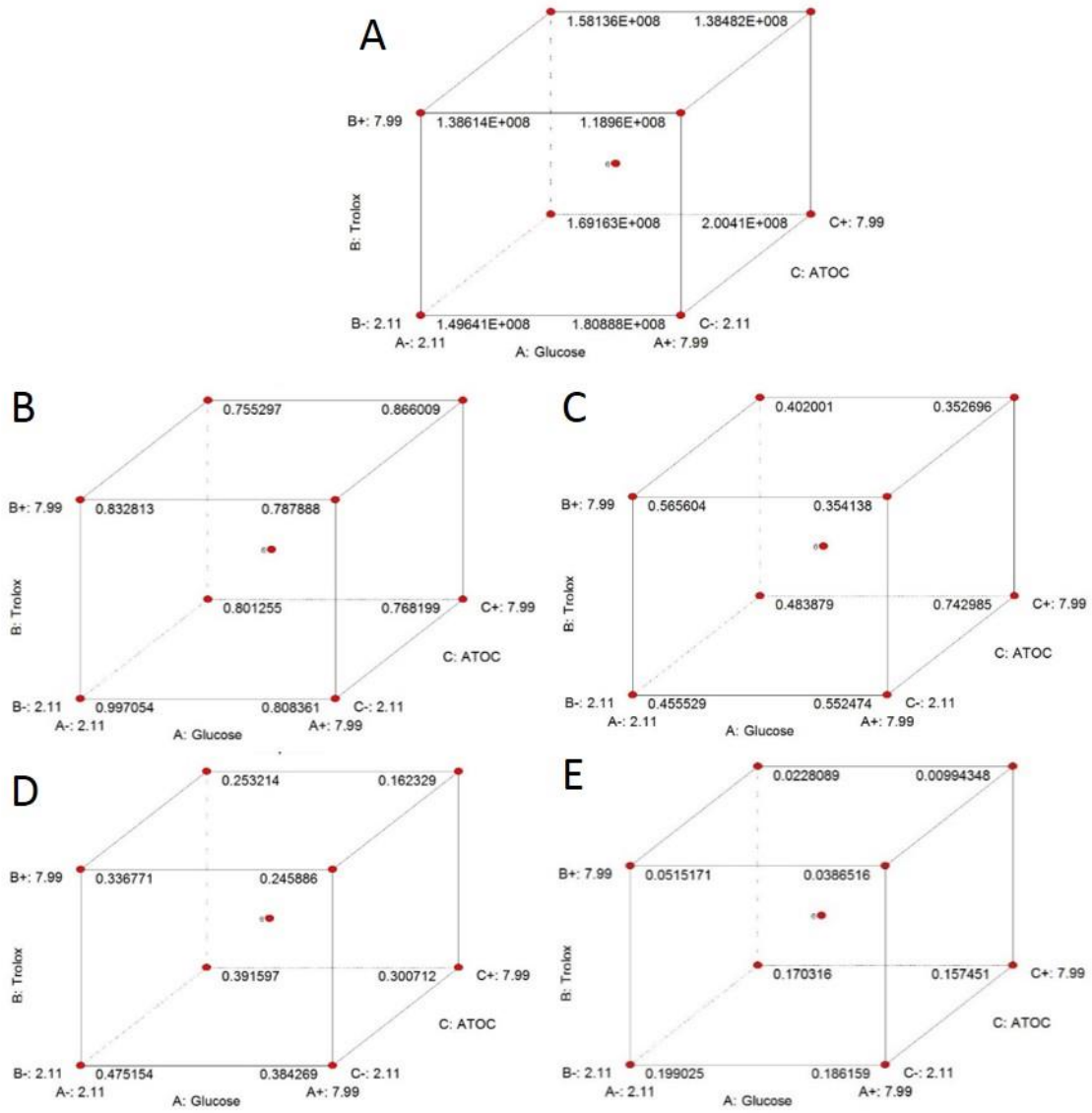

Figure 4-3 Metabolic activity of T cells recovered at 37°C before, during, and after 3 days of hypothermic preservation supplemented with glucose, alpha-tocopherol, and trolox. Alamar blue was used as measure of metabolic activity due to its potential to be reduced by several biochemical reactions. Results were corrected to remove background reduction from compounds added during the test. Figures show design data points with results at the ±1 levels at the corners of the cube, ±α levels used to detect linearity are not shown. Metabolic activity on A) day 0, B) day 1, 4°C, C) day 1, 22°C, D) day 3, 4°C, E) day 3, 22°C

$$Alamar\ blue\ response_{D0} = 1.68 \times 10^8 + 2.90 \times 10^6 A - 1.82 \times 10^7 B + 9.76 \times 10^6 C - 1.27 \times 10^7 A \times B - 1.14 \times 10^7 B^2$$

Equation 4-6

$$Alamar\ blue\ response_{D1,4^oC} = 1.29 \times 10^8 - 1.85 \times 10^6 A - 1.92 \times 10^7 B + 2.80 \times 10^6 C$$

Equation 4-7

$$Alamar\ blue\ response_{D3,4^oC} = 6.56 \times 10^7 - 1.47 \times 10^7 B^2$$

Equation 4-8

$$Alamar\ blue\ response_{D1,22^oC} = 7.37 \times 10^7 - 2.50 \times 10^6 A - 3.25 \times 10^7 B - 5.93 \times 10^6 C$$

Equation 4-9

$$Alamar\ blue\ response_{D3,22^oC} = 1.77 \times 10^7 - 2.54 \times 10^6 A - 1.31 \times 10^7 B - 1.6 \times 10^6 C$$

Equation 4-10

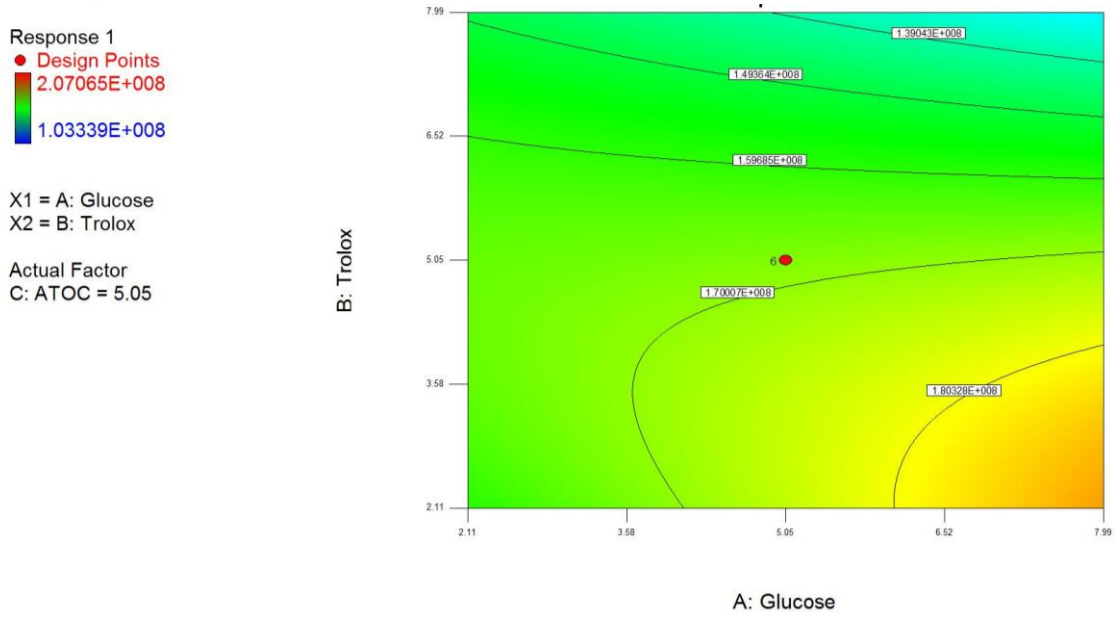

Figure 4-4 Heat map showing the day 0 interaction between A, glucose, and B, trolox, over the ±1 concentration of 2.11mM to 7.99mM. Component C, ATOC was held at a fixed concentration. The graph shows that the combination of both components results in lower Alamar blue reduction, which was the response parameter tested.

4.3.4 Intracellular adenosine triphosphate concentration of T cells was depleted by addition of trolox.

If the components added were capable of influencing cell metabolism during the application of hypothermia, then a response should be detected in the intracellular ATP of the T cells. Glucose produced no significant effect on concentration of intracellular ATP on day 1 at 4^{o}C, the P-value in DoE ANOVA was 0.7877, see Figure 4-5 for raw values. The effect of glucose on ATP was negative and small because the coefficient of term A was the smallest by nearly an order of magnitude compared to the coefficients for the two other variables, see Equation 4-11. Glucose retained a very small effect with increased duration to 3 days of 4^{o}C hypothermia, see Equation 4-12. The effect of glucose on ATP concentration at 22^{o}C was

also negative and insignificant, shown by the small A coefficients of Equation 4-13 and Equation 4-14.

Trolox was found to have a significant negative effect on ATP concentration throughout the models at different temperatures and time points. The response of ATP to concentration of trolox supplementation was linear at 4^{o}C for day 1, see Equation 4-11, and became a squared term with increased duration of hypothermia, see Equation 4-12 for term B. The concentrations of trolox tested here reduced the ATP concentrations and inhibited metabolism, which became a stronger negative effect with increased duration of preservation. Therefore, trolox is unsuitable for the preservation of T cells.

Short-term hypothermia of 1 day at 22^{o}C produced significant interactions between all three of the components. The combination of glucose and trolox multiplied the individually negative effect on ATP. There was a positive interaction between glucose and ATOC, which suggested that these two components together were able to maintain slightly higher ATP concentrations after completing the short-term preservation, see Equation 4-13.

This interaction effect decreased after 3 days of preservation at 22^{o}C, and the effect of trolox was wholly negative on ATP and had a quadratic profile, suggesting the greater the concentration of trolox the much greater the decrease in ATP concentration. ATOC had a small effect on the ATP response, which was best fitted with a quadratic profile, but the coefficients were such that a maximum concentration of ATP was detected around 5.25mM of ATOC, and the increase was not detected to be significant.

The response of ATP was measured as a ratio to the concentration on day 0, indicating the relative amount of ATP that had been lost. The constant term of Equation 4-11 had already dropped to 0.33 after 24 hours of 4^{o}C, a result of the lower ATP concentrations observed throughout the data points shown in Figure 4-6. This decrease suggested that an effect unrelated to the factors of glucose, trolox, or ATOC caused a large drop in ATP concentration over the first day of hypothermic exposure. The constant of Equation 4-12

reduced to 0.21 over the next two days of preservation at 4°C. Therefore, the largest drop not controlled by this model happened over the first 24 hours. The constant term of Equation 4-13 showed that the largest drop in ATP at 22°C occurred over the first 24 hours, and was a greater decrease compared to 4°C. Unlike 4°C hypothermia, the decrease was quite large when the duration of hypothermia was extended to 3 days, when ATP levels decreased to only 0.09 of day 0 concentrations, see Equation 4-14. Some effects on cell metabolism were detected, but these were short lived and do not benefit longer-term T cell preservation.

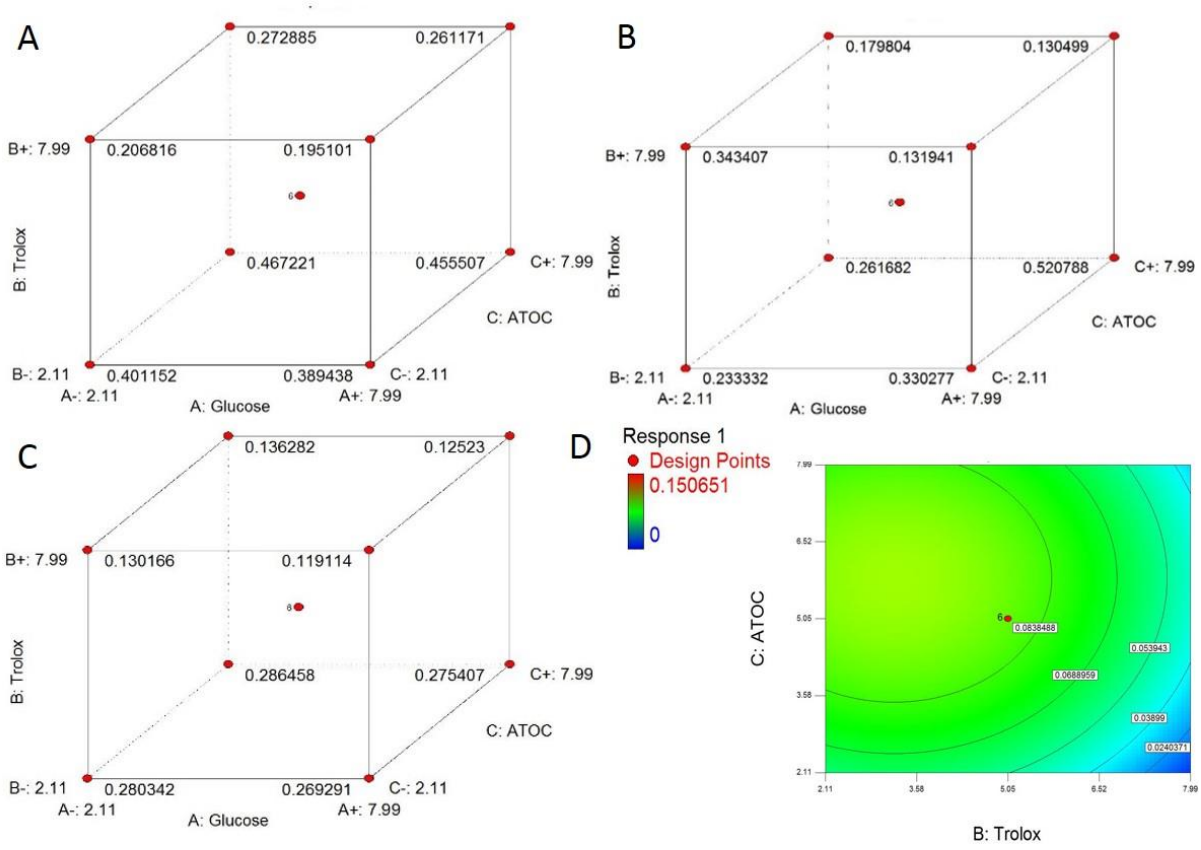

Figure 4-5 Intracellular ATP content of T cells during preservation with glucose, alpha-tocopherol, and trolox at different concentrations. Results were normalised to quantities of ATP present in T cells on day 0 before commencing hypothermia, therefore they show how much ATP was depleted. Figures show design data points with results at the ±1 levels indicated at the corners of the cubes, ±α levels used to detect linearity are not shown. ATP concentration on A) day 1, 4°C, B) day 1, 22°C, C) day 3, 4°C, D) day 3, 22°C. D shows the heat map of the remaining two components required to establish a significant model on day 3 after preservation at 22°C.

$$ATP\ response_{d1,4^oC} = 0.331161 - 0.00586A - 0.09717B + 0.033035C$$

Equation 4-11

$$ATP\ response_{d3,4^oC} = 0.202786 - 0.00553A - 0.07509B + 0.003058C$$

Equation 4-12

$$ATP\ response_{d1,22^oC} = 0.266466 - 0.01191A - 0.07005B + 0.006727C - 0.0771A \times B + 0.04954A \times C - 0.04799B \times C$$

Equation 4-13

$$ATP\ response_{d3,22^oC} = 0.08939 - 0.02505B + 0.012147C - 0.02009B^2 - 0.02302C^2$$

Equation 4-14

4.3.5 Alpha-tocopherol significantly reduced apoptosis of T cells after hypothermic preservation.

It was discussed in the literature review at Chapter 2 that antioxidants can effect apoptosis, hence the response of apoptosis was investigated. Glucose supplementation of the preservation medium was shown to increase the amount of apoptosis detected by phosphatidylserine exposure at both 4°C and 22°C. This increase in apoptosis was greater at 22°C, as shown by comparisons of the coefficients of the glucose (A) terms in Equation 4-15 and Equation 4-16. Supplementation with trolox also increased apoptosis during preservation at both temperatures. The effect of trolox was not as strong compared to glucose, with lower coefficient shown for the trolox (B) term in the same equations. However, the effect of both A and B was stronger when T cells were preserved at 22°C, although it should be noted that both of these effects produced by glucose and trolox were insignificant on the response of apoptosis.

The addition of ATOC to the preserving medium had a different effect on apoptosis between the two temperatures after 3 days of hypothermia. At 4°C the effect was a significant inhibition of apoptosis that linearly increased with concentration, see term C in Equation 4-15 and Figure 4-6 for numerical data points. The linearity of the ATOC term over the 2.11-7.99mM concentration range examined suggested that the frequency of apoptosis continually decreased through increasing concentration ATOC. The ±α term of 10mM of ATOC did not detect that the apoptotic response to supplementation of ATOC was reaching a maximum.

The coefficient for the ATOC term had the lowest value at 22°C, see Equation 4-16. At 22°C ATOC had a weak negative effect on apoptosis at this warmer temperature.

In general, preservation at 22°C showed higher amounts of apoptosis overall compared to 4°C. This can be seen from the constant terms of Equation 4-15 and Equation 4-16. The background response without any modifications at 4°C indicated that approximately 84.9% of cells were free of apoptosis. The 22°C preservation had a lower constant term of 46.0%, this shows that apoptosis can occur at a greater frequency at 22°C.

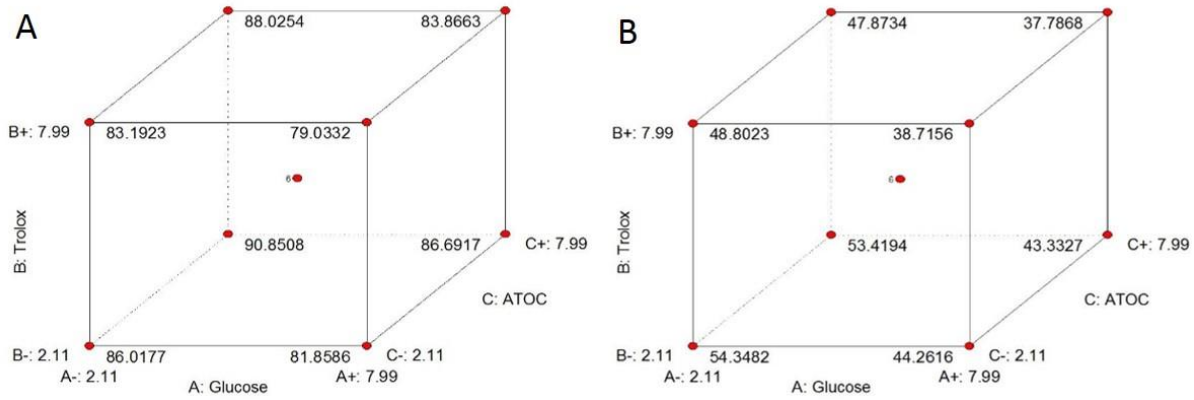

Figure 4-6 Apoptosis of T cells during preservation with glucose, alpha tocopherol, and trolox at different concentrations examined by exposure of phosphatidylserine on the outer side of the cell membrane. Values shown are the percentage of non-apoptotic intact cells. Figures show design data points with results at the ±1 levels at the corners of the cubes, ±α levels used to detect linearity are not shown. Apoptosis negative cells for A) day 3, 4°C, B) day 3, 22°C

$$Non-apoptotic\ cell\ response_{D3,4^oC} = 84.942 - 2.08A - 1.41B + 2.42C$$

Equation 4-15

$$Non-apoptotic\ cell\ response_{D3,22^oC} = 46.07 - 5.04A - 2.77B - 0.46C$$

Equation 4-16

4.3.6 Design of experiments model discussion: glucose does not improve the metabolism of T cells under hypothermia.

This DoE study of Sections 4.3.1 to 4.3.5 showed that supplementation of T cells with glucose during hypothermia had little effect on a range of cellular characteristics related to overall cell metabolism after preservation. The addition of glucose did increase the metabolic activity on day 0 and only slightly increased in the metabolic activity on recovery after 1 day

of 4°C hypothermia. Glucose had little or negative effect on metabolic activity at the other time points and temperatures tested. However, glucose was shown to increase the frequency of apoptosis at 22°C. The short-term increase in metabolic activity produced by glucose was not large enough to increase the intracellular ATP present after completion of 3 days of hypothermia. The inclusion of glucose did not have the intended effect of sustaining cell metabolism during hypothermia because ATP was depleted after completing either 1 day or 3 days of preservation. The preserving medium buffer did contain phosphate, which was shown in Section 3.3.5 to reduce glucose consumption by T cells at normal temperature, in-vitro conditions. This could be one reason that glucose supplementation had little effect on cell preservation, with the phosphate buffer in the preserving medium inhibiting glucose uptake. Kobayashi et al. have shown no difference in rat survival after transplanting liver tissue preserved in UW solution containing phosphate and either glucose, sucrose, or raffinose [251]. An effect that was observed in that study was that the lower molecular weight compound of glucose did allow more water to enter the tissue, but this appeared to produce no lasting result. There is some evidence that the glucose requirement could be cell type dependent. Wheeler et al. showed that glucose and fructose supplementation of a phosphate-containing preservation medium improved preservation of myocardial cells with regards to maintaining higher cell ATP content, albeit only for short durations [180]. In that study, metabolite supplemented media showed no differences compared to controls after 72 hours. This short-term benefit was comparable to the short duration effect that glucose was shown to produce in Section 4.3.3. No increase in ATP was found and only slightly higher metabolic activity was achieved after short-term exposure to the preservation medium supplemented with glucose. Furthermore, Sandlin et al. found that glucose was not required to achieve optimum characteristics of red blood cells after preservation of whole blood at ambient conditions [76]. This result was not discussed in the rest of that study for the preservation of the whole blood. The base medium in that study used a HEPES buffer rather

than phosphate. The use of a different buffer would avoid the effect of phosphate on glucose consumption discussed in Chapter 3. Results in Sections 4.2.1 to 4.2.4 suggested that glucose does not benefit preservation because it was unable to stimulate long-term T cell metabolism during the reduced temperature conditions. From the results and evidence discussed here, it is concluded that glucose supplementation is not a key aspect to preserving activated T cells.

4.3.7 Design of experiments model discussion: trolox supplementation inhibits metabolism and forces adenosine triphosphate consumption.

Trolox, despite being a water-soluble analogue of ATOC, produced opposite effects over the same concentration ranges. Including trolox decreased metabolic activity, severely reduced ATP, and increased ROS level towards that of antioxidant-free controls. Wattamwar et al. showed that trolox increased ROS within endothelial cells when concentration was increased above 0.499mM [252]. Results of Section 4.3.2 showed that the increase in ROS production continued to increase through the concentration range from 2.1mM to 8.0mM and produced no other desirable effects. Section 4.3.2 also tested a minus alpha level of 0.1mM that reported no improvement in the tested characteristics from inclusion of trolox. Inclusion of trolox reduced the effectiveness of hypothermic preservation of activated T cells. The negative effects of trolox on cell metabolism and ATP reduction did not translate to decreased membrane integrity or significantly increased apoptosis after completing the hypothermic preservation of the T cells.

4.3.8 Design of experiments model discussion: explaining the interaction between terms in the study.

One purpose of the DoE study was to investigate if there were any positive interactions between the supplementation with glucose, ATOC, and trolox. These components were all known to individually alter cell metabolism from evidence presented in pre-existing studies [21,180,205,251,253]. However, these pre-existing effects were with limited temperature and very short durations. It was not known if supplementation of the preservation with one

component may benefit from an interaction with another component. This DoE study showed that there were many interacting terms between the three components on day 0 in some of the parameters tested. There were no significant two component interactions detected after the onset of 4^{o}C hypothermia. However, there was a significant positive interaction between ATOC and glucose after preservation at 22^{o}C for day 1 that increased the amount of ATP present in the T cells. This positive effect lost significance as preservation duration increased. Reducing temperatures below 37^{o}C affects biochemical reactions rates to different extents. Southard et al. found breakpoints in Arrhenius plots of decreasing mitochondrial activity with reduced temperatures and suggested that changes in rate-limiting reactions of biochemical processes took place [158]. Sections 4.3.1 to 4.3.4 showed a decreasing amount of interactions detected with decreased temperature and increased preservation duration. Both of these points together show that hypothermia removed the ability for biochemical reaction sequences to interact. This could be due to the large number of intermediate steps that occur in cells being affected differently by temperature. Guy et al. found that the majority of metabolite constituents of a preservation medium for human kidneys were unaltered during 4 hours of preservation [254]. The minority that experienced changes in that study were increased glucose concentration and decreased glutathione concentration in the perfusate. Overall, this body of evidence shows preservation strategies that aim to sustain cells with metabolites may not be effective because the complete metabolic pathways cannot be used effectively. Therefore, a better strategy may be to use extra interventions to reduce T cell activity during the application of hypothermia.

The component ATOC was suggested to benefit long-term preservation by reducing the amount of apoptosis at 4^{o}C. This result requires further investigation, as presented in Section 4.3.9.

4.3.9 Design of experiments model confirmation: alpha-tocopherol supplementation reduced apoptosis at 4ºC and has a small effect on cell metabolism on recovery to 37ºC.

The main positive result to confirm from the DoE model prediction was that supplementation with ATOC reduced the frequency of apoptotic cells during 4ºC hypothermia. A secondary result to confirm was that 22ºC hypothermia resulted in a higher frequency of apoptosis compared to 4ºC and ATOC supplementation had no significant effect on this frequency. It was shown that ATOC supplementation significantly reduced apoptosis in independent trials compared to the frequency produced at 4ºC in control medium, see Figure 4-7. This effect was confirmed to be unrelated to the ME_2SO vehicle. Supplementation with ATOC limited the increase in apoptosis from an average of 11.1% ± 3.0% on day 0 to 13.7% ± 4.6% on day 3 with 4ºC hypothermia. Supplemented and un-supplemented conditions at 22ºC resulted in frequencies of apoptotic cells of an average 55.8 ± 9.2% and 52.1% ± 5.9%, respectively. Supplementation of the preservation medium with 8mM of ATOC in ME_2SO carrier limited apoptosis at 4ºC, but had no effect at warmer hypothermia, 22ºC. In conjunction with apoptosis reported by phosphatidylserine exposure, see Figure 4-7, the amount of sub-G1 peak cells from intracellular PI staining was examined, see Figure 4-8. This figure displays the amount of condensed nuclei, a morphological change known to be a result of apoptosis [255]. The increase of condensed nuclei confirmed the result of Figure 4-7 because there was a small but significant increase of condensed nuclei at 4ºC compared to the control series, whereas 22ºC suffered from a much higher population of sub G1 peak cells compared to the control.

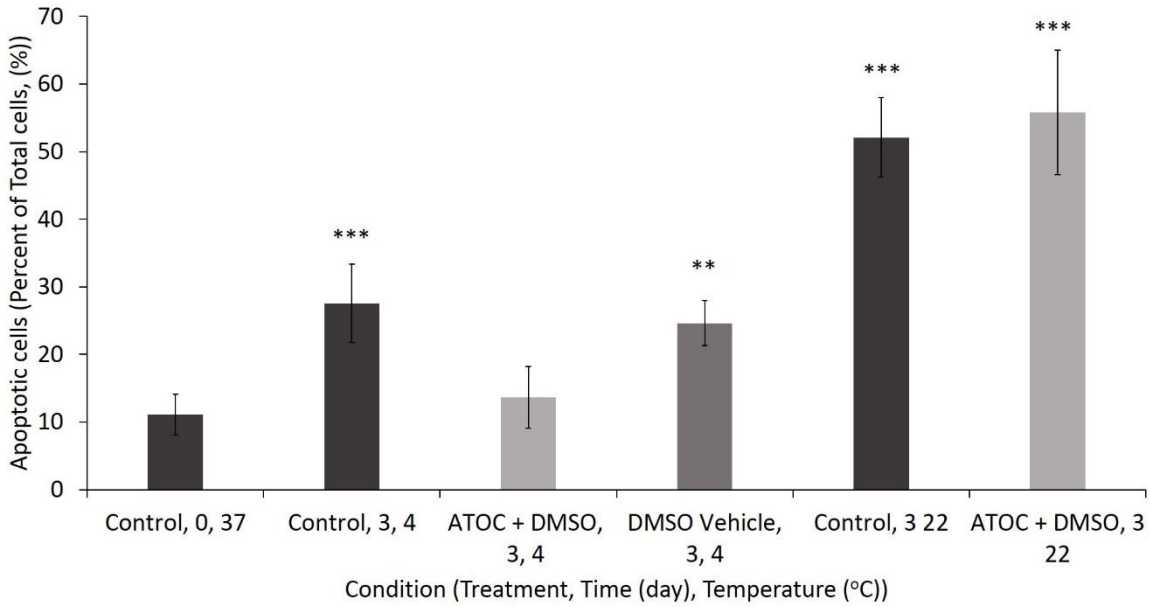

Figure 4-7 Percentage of apoptotic cells in response to the application of hypothermia with the supplementation of alpha-tocopherol in dimethyl sulfoxide vehicle at two grades of hypothermia. Apoptosis was measured by phosphatidylserine exposure on the extra-cellular side of cell membrane by binding of Apopxin green and fluorescence examined by flow cytometry. Control group did not include glutathione in the medium formulation. See Section 3.2.10 for the explanation of statistical analysis and consequent labelling. N = 6.

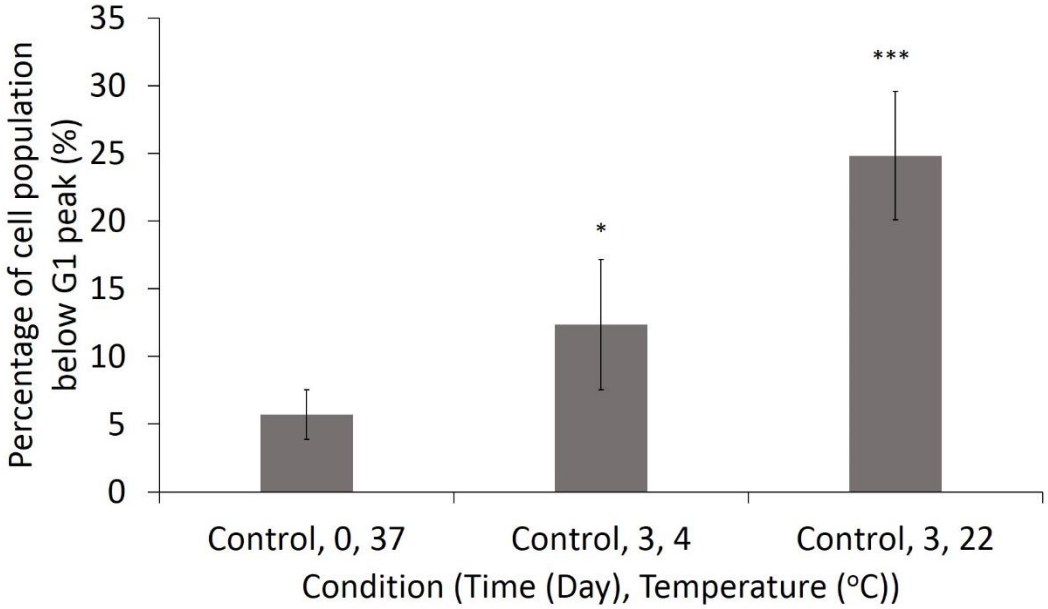

Figure 4-8 Percentage of apoptotic cells in response to the application of hypothermia at two different temperatures of preservation. Apoptosis was measured by the percentage of cell nuclei that appeared just below the G1 peak of cells, but above background events, which was examined by intracellular PI staining, and data collected by flow cytometry. See Section 3.2.10 for the explanation of statistical analysis and consequent labelling. N = 6.

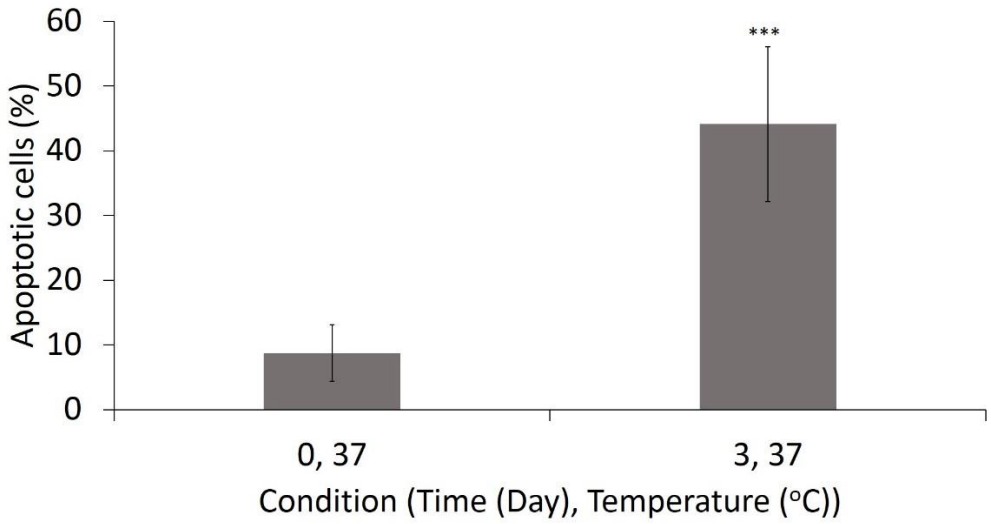

Figure 4-9 Percentage of apoptotic cells in response to cytokine and antigen removal and then continued culture under in-vitro conditions. Apoptosis measured by phosphatidylserine exposure on extra-cellular side of cell membrane. Statistical analysis used T tests assuming unequal variances. See Section 3.2.10 for the explanation of statistical analysis and consequent labelling. N = 6.

Figure 4-9 shows the significant increase in apoptosis that occurred when activated T cells were withdrawn from the antigens used to expand them in-vitro and retained at temperatures of 37ºC. The incidence of apoptosis was comparable to the amount observed after hypothermic preservation at 22ºC. The quantity of apoptotic cells measured in response to presence of stimulating antigens present within the preservation medium is shown in Figure 4-10. The antigens were the same as those used for in-vitro activation and expansion of the cells. These results showed that no combination of the antigens affected apoptosis during preservation within temperature groups because no significant difference was found amongst the combinations of antigens. In the absence of ATOC, 4ºC preservation displayed a slight increase of apoptosis over the day 0 control and 22ºC displayed drastically increased amounts of apoptosis. The lack of difference in apoptosis observed with antigens present in the preservation medium suggests that the high apoptosis observed after preservation at 22ºC was not related to removal of the antigens.

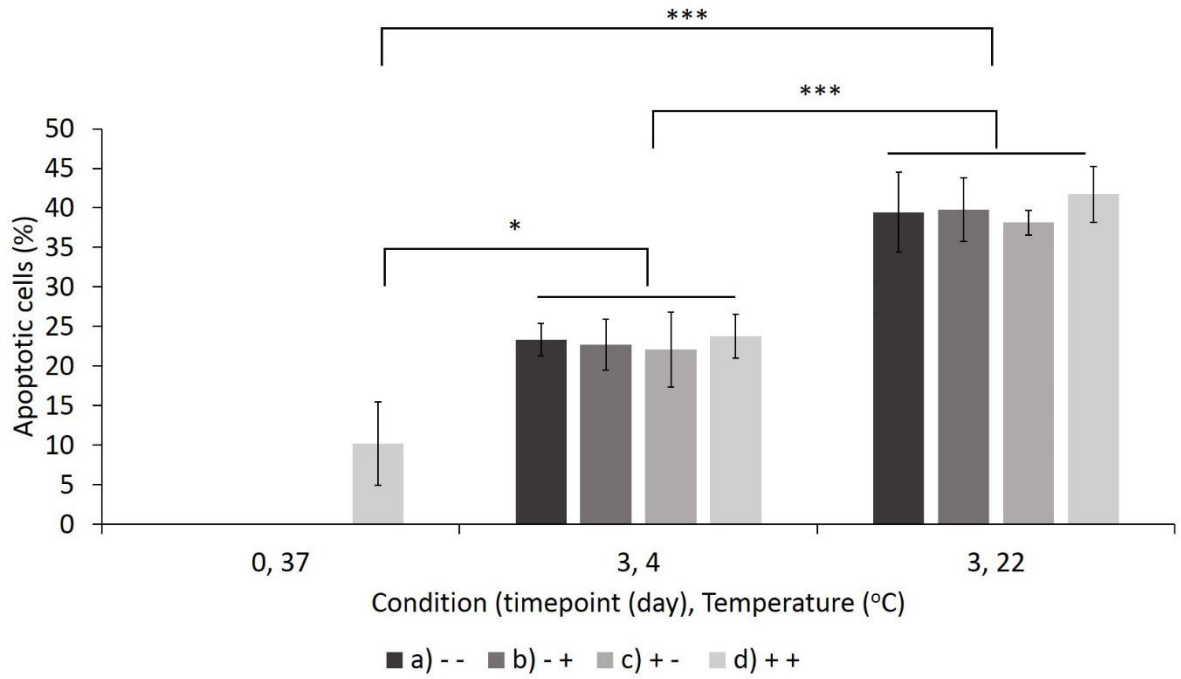

Figure 4-10 Apoptosis with antigens present in the preservation medium. A) - - indicates no antigen present, B) - + indicates that only IL-2 was present. C) + - indicates that only that only CD3/CD28 antibodies were present. D) ++ indicates that both antibodies and IL-2 were present in the preservation medium. Apoptosis was measured by exposure of phosphatidylserine by binding with Apopxin green and flow cytometry. See Section 3.2.10 for the explanation of statistical analysis and consequent labelling. N = 6.

Results in Figure 4-11 confirm those predicted by the DoE model concerning the inclusion of ATOC and lack of difference in ATP content of the T cell after long term hypothermia. After 3 days of preservation there was no significant difference in terms of the intracellular concentration of ATP between the control preservation mediums and the mediums supplemented with 8mM of ATOC in ME_2SO at both temperatures. Results in Figure 4-11 also provided a visualisation of cells exposed to hypothermia at 22°C that had significantly less ATP remaining after 3 days compared to cells exposed to 4°C. There was no difference in viabilities observed, which confirmed the data recorded for the DoE model shown in Table 4-2.

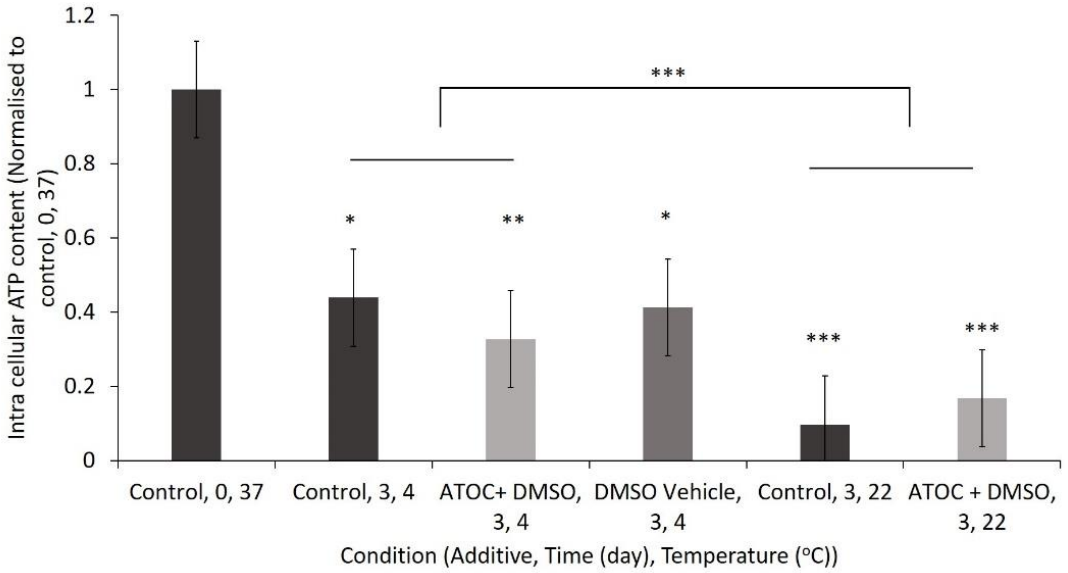

Figure 4-11 Intracellular ATP content of T cells treated with alpha-tocopherol and preserved with hypothermia. This was examined by extraction of ATP by boiling deionised water followed by analysis of the lysate for luminescence with a firefly luciferase assay. The data points show how the application of two temperatures of hypothermia reduced ATP after preservation and the lack of affect of including ATOC in ME_2SO vehicle. Control group did not include glutathione in the medium formulation. See Section 3.2.10 for the explanation of statistical analysis and consequent labelling. N = 6.

The DoE model predicted that ATOC supplementation would produce an increased response on metabolic activity of T cells during short-term exposure. This was confirmed by the data in Figure 4-12, which showed a small significant (P-value = 0.0194) increase in Alamar blue reduction by T cells in the preservation medium supplemented with 8mM ATOC in vehicle. The normalised median of metabolic activity significantly increased from 1.00 with the control to 1.34 when supplemented with ATOC. This increase was not a result of the ME_2SO vehicle, which was no different to the control. Another prediction from the DoE study was that the effect of ATOC supplementation on cell metabolism would decrease in magnitude after completing 3 days of hypothermic preservation. This was confirmed by the large decrease of metabolic activity recovered with hypothermia after both 4°C and 22°C. There was a slight increase in medians from 0.26 in the control 4°C cells to 0.27 with ATOC at the same temperature, although this increase was not significant. The ME_2SO vehicle alone significantly decreased activity. After preservation at 22°C, ATOC still had no significant effect on cell metabolism individually. ATOC was capable of increasing cell metabolism

while T cells were maintained at 37°C before hypothermia with an effect that reduces with the application of hypothermia. This compound was not able to produce a large enough effect after preservation to recover most of the starting metabolic activity.

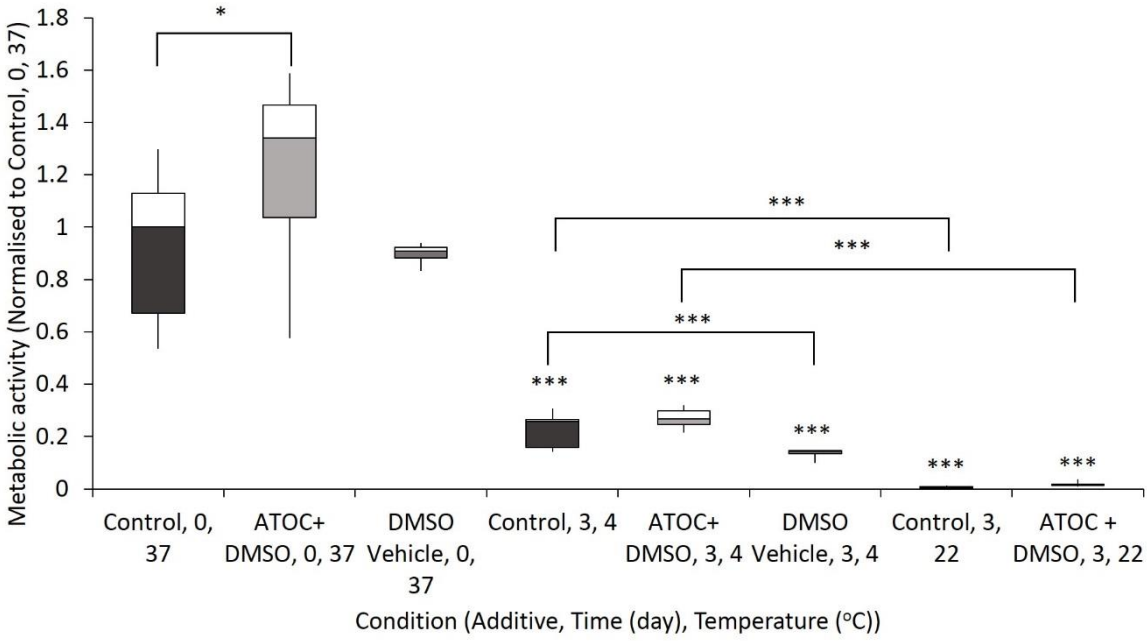

Figure 4-12 Normalised Alamar blue reduction of T cells treated with alpha-tocopherol and preserved with hypothermia. Alamar blue reduction was interpreted as metabolic activity produced by T cells. Results include incubation at 37°C with the application of ATOC in ME_2SO vehicle before and after the application of hypothermia, which was at two temperatures. Statistical analysis was performed by Kruskal-Wallis test followed by pairwise Mann-Whitney tests because of non-normal data. Control group did not include glutathione in the medium formulation. See Section 3.2.10 for the explanation of statistical analysis and consequent labelling. N = 6.

The DoE model predicted that either glucose or ATOC would reduce the ROS activity within T cells. This was confirmed to be due to ATOC supplementation, which reduced DCF fluorescence at 8mM to a normalised average of 0.72 ± 0.12 from an untreated control that showed 1.00 ± 0.23, a significant difference, see Figure 4-13. A step decrease between post-preservation samples ROS values compared to day 0 control was observed, regardless of the preservation temperature or treatment with the antioxidant compound. Except for an insignificant decrease with ATOC supplementation with preservation at 4°C, this step decrease was significant compared to the day 0 controls for all the preserved sample conditions with varying degrees of probability. There was no significant difference between the individual samples treated with ATOC or controls at either temperature of preservation.

This indicates that ATOC was having some effect on ROS activity but, after preservation, the effect was much less than before.

The decrease in ROS activity that was apparent with the application of hypothermia is suggested to be a result of the assay and not a decrease in ROS activity. Fitton et al. showed that ROS activity increased after hypothermia and induced cell damage on recovery [169]. The controls in the test presented in Figure 4-13 were produced from a medium free of glutathione and show that, despite the removal of this exogenous antioxidant, the ROS activity detected by the DCF compound was decreased. This was most likely a result of the requirement for the DCF to be deacetylated by cellular esterases before it can react with ROS to produce fluorescence [256]. Figure 4-12 shows that there was a decrease in cell activity after hypothermic preservation. This may make comparisons between different temperatures difficult.

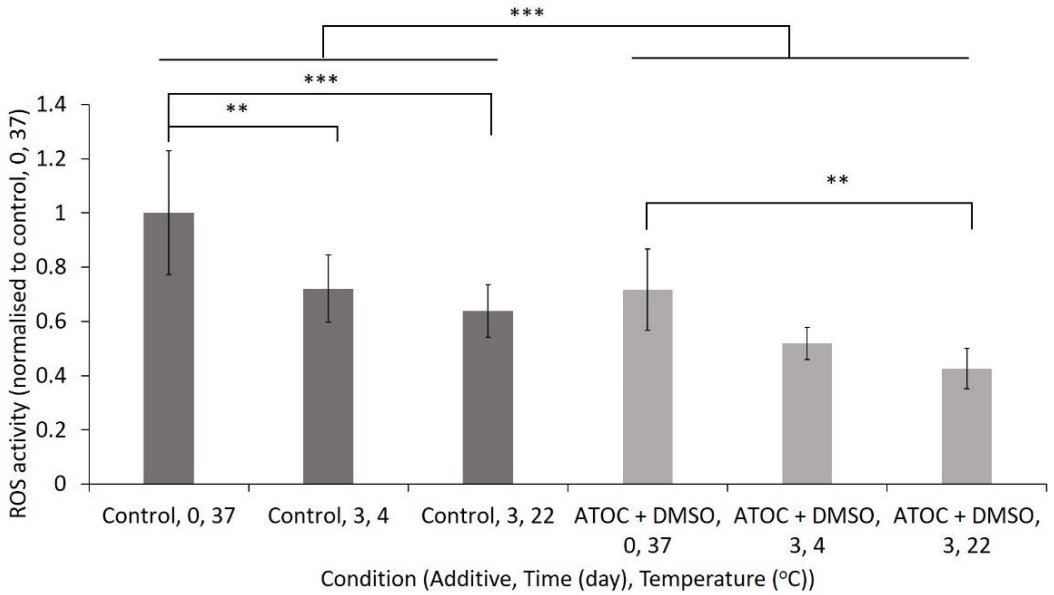

Figure 4-13 Reactive oxygen species activity produced by preserved T cells with alpha-tocopherol supplementation. ROS was measured by DCF fluorescence after intracellular hydrolysis and reacting with ROS. Results were normalised to the control, 0, 37 with background RFU subtracted. Graph shows the results with two temperatures of hypothermia. Control group did not include glutathione in the medium formulation. See Section 3.2.10 for the explanation of statistical analysis and consequent labelling. N = 6

4.3.10 Design of experiments model confirmation: discussion.

Comparison of the effects on metabolism and energy reserves were relatively straightforward in this study because of the lack of difference reported in cell viability, which was similar to the values shown in Table 4-2. Inclusion of ATOC in the preserving medium induced a short-term increase in T cell metabolic activity seen on day 0 and after day 1 of preservation at 4^{o}C compared to ATOC free controls. However, this effect disappeared with increased duration of preservation, as predicted by the DoE model. These effects of ATOC supplementation were confirmed to in an independent one factor trial. Unlike glucose supplementation, the metabolic effect of ATOC also produced a slightly increased ATP concentration on day 1 at 4^{o}C preservation. However, this effect decreased towards three days of preservation and was found to be insignificant at this longer duration of hypothermia. Therefore, stimulation of cell metabolism during long duration hypothermia does not increase available energy reserves after preservation. ATOC does improve the recovery after preservation due to slightly higher rates of cell metabolism and lower apoptosis on return to 37^{o}C, see Section 4.3.9. Huang et al. showed that ATOC supplementation affected AMPK signalling; increased markers of autophagy; and inhibited mTOR pathways, which led to anti-depressive effects on behavioural tests in mice [257]. An increase in AMPK signalling would increase cell metabolism. This was supported by the evidence in Section 4.3.9 of the increased T cell metabolism observed before and, to a somewhat limited degree, after hypothermia. The component ATOC has a noticeable effect on T cell metabolism and apoptosis related to hypothermia, and also has a previously known role in cell signalling.

In this study, supplementation of ATOC reduced the degree of apoptosis after exposure to 4^{o}C hypothermia. Avula and Hernandes have shown ATOC to decrease splenocyte apoptosis at the expense of increasing necrosis of the cells [258]. The results of Section 4.3.9 show that low temperatures favour the ability for ATOC to reduce apoptosis over increasing the amount of necrosis. ATOC supplementation was found to have some effect on preserved cell

metabolism during preservation, and it also reduced apoptosis under low temperatures, but these may not lead to a cell population with enough activity upon recovery.

This is the first study to report the amount of apoptosis that occurs under hypothermia in activated T cells. Apoptosis in T cells can mainly be avoided by hypothermic preservation at 4^{o}C. The amount of apoptosis was similar between cells preserved at 22^{o}C and those that had been cultured at 37^{o}C, both with antigens removed. The addition of antigens had no effect on apoptosis at either 4^{o}C or 22^{o}C hypothermia. This suggests that the increased apoptosis at 22^{o}C was not related to cytokine withdrawal induced cell death. Instead, it is suggested that this apoptosis may be related to glucose withdrawal. Section 3.3.4 showed that the inclusion of high phosphate concentration in culture medium inhibited glucose metabolism. Furthermore, Jelluma et al. showed that glucose withdrawal induced apoptosis in glycolytic glioblastoma cells [259]. In addition to this, activated T cells share a reliance on glycolysis with cancer cells [260]. Therefore, they may be as responsive to glucose withdrawal, which could continue at hypothermia of 22^{o}C. However, in the results of Section 4.3.5, the addition of glucose only increased the large amount of apoptosis at 22^{o}C. This suggests that the inhibition of glucose consumption by phosphate is also linked to an increased apoptosis when glucose is present.

The need to maintain 4^{o}C to successfully preserve T cells is contradictory to existing literature. Wang et al. showed that stem cell lines experienced less apoptosis when preserved at 22^{o}C compared to the lower temperature of 4^{o}C [160]. The high amounts of apoptosis found at 22^{o}C may be related to blood derived products used in this thesis. Sandlin et al. demonstrated that anti-apoptotic compounds were required for successful preservation of leukocytes in whole blood at ambient conditions [76]. It is suggested that different cell lines may experience different amounts of apoptosis in response to changes in temperature. Therefore, when attempting to preserve a new cell line in conjunction with low temperatures, the initial studies must take account of different temperatures because the optimum may not

be definable from previous studies. For T cells, the optimum temperature was 4^{o}C and apoptosis was further reduced by supplementation of the preserving medium with ATOC.

4.3.11 Glutathione supplementation improves cell metabolism on recovery to 37^{o}C but does not affect cell activity during hypothermia.

It was observed in Section 4.3.3 that supplementation of the modified UW medium with ATOC did not greatly affect T cell metabolism after application of hypothermia. This medium already contained a 3mM concentration of glutathione, another compound with antioxidant activity, which was shown to be beneficial during optimisation of the UW preservation medium for T cells in Chapter 3. Glutathione's mechanisms of action were discussed in Chapter 2. The effects related to concentration changes of glutathione were examined in greater detail in this section. Membrane integrity in response to concentrations of 0.1 to 10mM of glutathione are shown in Figure 4-14. Glutathione was shown to increase the viability slightly after 4^{o}C preservation. When combined with 4^{o}C hypothermia, there was significant increase in membrane integrity when the concentration of glutathione was increased from 0.5mM to 1mM. No change occurred when the concentration was further increased to 5mM, but there was an insignificant decrease in intact cells at 10mM glutathione. The 10mM concentration was, however, significantly lower than the day 0 control. Furthermore, at 22^{o}C, no effect was detected from changing glutathione concentration.

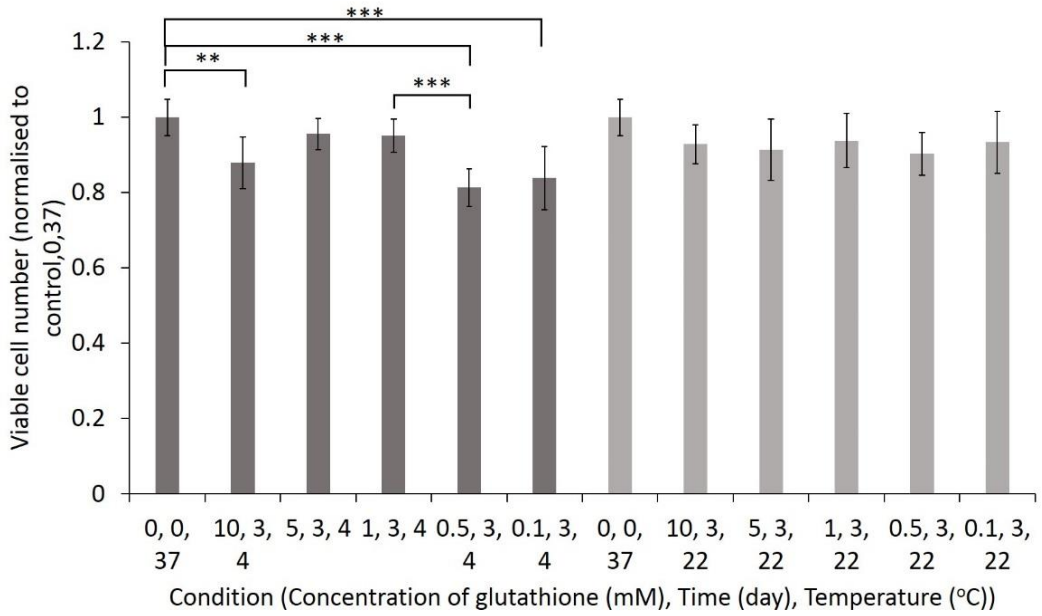

Figure 4-14 Viable cell count of hypothermic preserved T cells supplemented with different concentrations of glutathione. Viable cell count was measured total cell count, Draq 7 exclusion for membrane integrity, and total viable cells on day 0. Values were normalised to day 0 viable cell count. See Section 3.2.10 for the explanation of statistical analysis and consequent labelling. N = 5.

Glutathione concentration noticeably increased the cellular reduction of Alamar blue at 4°C, which indicated greater metabolic activity after recovery from preservation. The most important point was that all the metabolic activity after hypothermic preservation was significantly lower when compared to untreated T cells on day 0, to which the background corrected results were normalised, see Figure 4-15. Furthermore, 4°C overall returned a greater metabolic activity compared to 22°C where two-factor ANOVA calculated a P- value of 0.0371.

At 4°C, there was a step-increase in metabolic activity after preservation when the concentration of glutathione was increased from 0.5mM to 1mM. This was the same concentration change that produced a step change with membrane integrity shown in Figure 4-14. There was a slight decrease in metabolic activity to a normalised average value of 0.29 ± 0.09 from 0.38 ± 0.04 with glutathione concentration increased from 5mM to 10mM. Both activity levels were lower at 5mM and 10mM concentrations when compared to 1mM, which produced an activity of 0.047 ± 0.03. There was no statistically significant difference between all of these concentrations. The decreased metabolic activity with higher

glutathione concentration at a preservation temperature of 4°C, Figure 4-15, was a similar trend to that observed in membrane integrity, as shown in Figure 4-14.

Increased concentration of glutathione at 22°C was shown to have no effect on the cellular reduction of Alamar blue, which suggested that no change in metabolic activity had occurred. At 1mM of glutathione, the difference in metabolic activity between 4°C and 22°C was significant (P value = 0.0013) with average values of 0.475±0.031 found for 4°C and 0.253±0.072 for 22°C, suggesting there was a much greater effect of glutathione at the lower temperature.

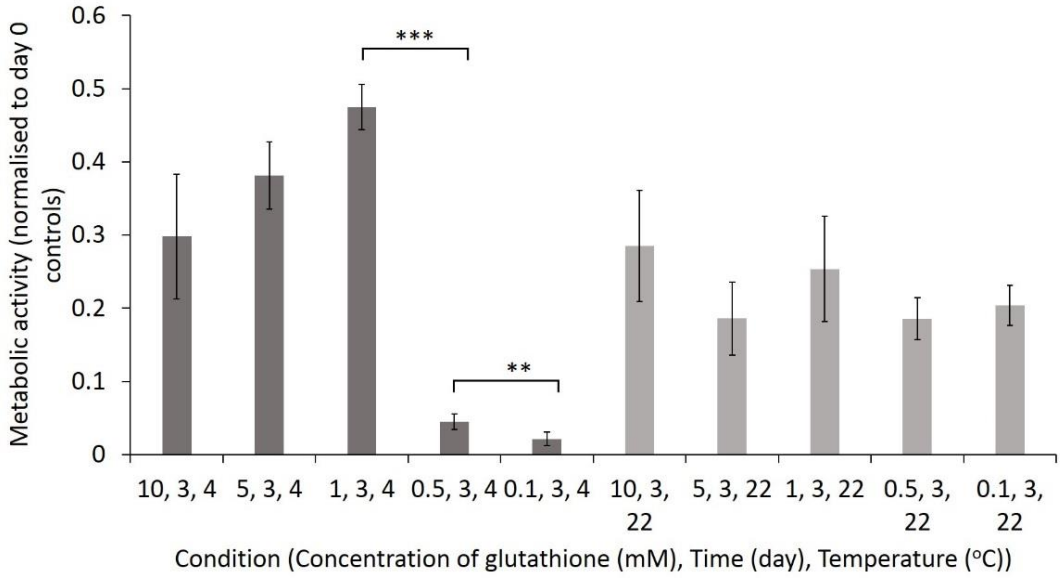

Figure 4-15 Alamar blue reduction of hypothermic preserved T cells with different concentrations of glutathione. Alamar blue reduction was interpreted as metabolic activity of T cells. Conditions are after hypothermic preservation at two temperatures, in combination with different concentrations of glutathione supplement. Normalisation accounted for different day 0 values observed for each concentration. See Section 3.2.10 for the explanation of statistical analysis and consequent labelling. N = 5.

Glutathione functioned as an antioxidant compound irrespective of temperature of preservation. A distinct reduction in DCF fluorescence was observed between 0.1 and 10mM of glutathione concentration on day 0 at 37°C, and after hypothermia at both 22°C and 4°C, see Figure 4-16. On day 0 at 37°C the difference in ROS activity produced by the range of glutathione concentrations was the smallest, and average values of normalised fluorescence decreased from 0.94 ± 0.09 at 0.1mM of glutathione to 0.76 ± 0.03 at 10mM. The largest

range in quantity of ROS produced was observed after preservation at 4°C. At low concentrations of glutathione at this temperature the amount of ROS produced exceeded the level observed on day 0, hence ratios greater than 1 were observed for 0.1mM and 0.5mM of glutathione. As concentration was increased further there was a significant step decrease in ROS production. This decrease left the production of ROS after supplementation with 10mM of glutathione and 4°C hypothermia lower than production at the same concentration on day 0. The effect of glutathione on ROS was smaller at 22°C, and no concentration tested produced ROS activity that was greater than that of untreated cells, to which the results were normalised. At 10mM of glutathione for 22°C preservation, the ROS production was the lowest with normalised average value of 0.63 ± 0.04. Overall, after preservation at 22°C, the ROS produced on recovery was significantly lower than compared to 4°C preservation.

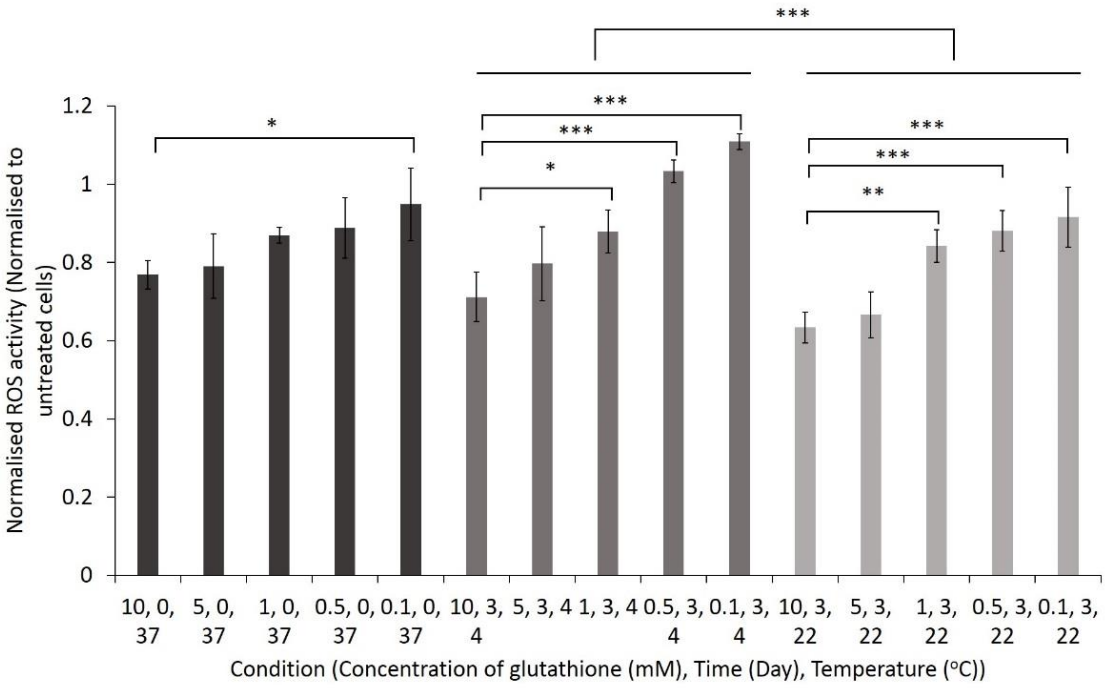

Figure 4-16 Reactive oxygen species activity of T cells at 37°C and after hypothermic preservation at two temperatures, all three temperatures were in combination with different concentrations of glutathione supplement. ROS activity was measured by DCF fluorescence upon reacting with ROS. Results were normalised to proliferating cells in complete medium without any supplementation. See Section 3.2.10 for the explanation of statistical analysis and consequent labelling. N=5.

Only low concentrations of lactate were present in the supernatant of preserved T cells. The absolute range was small, and values ranged from $3.61x10^{-10}$ to 4.5210^{-10} at 4°C preservation and 2.93 $x10^{-10}$ to 4.82 $x10^{-10}$ at 22°C over 3 days of hypothermia (all units

millimoles/cell/72hours). These small amounts indicate that very little cell metabolic activity occurred during either temperature of hypothermia, see Figure 4-17. Lactate production by T cells during hypothermia was slightly reduced when supplemented with increasing concentrations of glutathione. The reduction in lactate production in preservation medium supernatant at 4ºC had average values decreasing from 4.63±0.17 to 3.28±0.31 $x10^{-10}$ mmols/cell/72hours, at 0.1mM and 10mM of glutathione, respectively. This change did not hold significance. At 22ºC, the decrease of lactate production with increased concentration of glutathione was more noticeable but remained insignificant.

Only small differences were observed between the two temperatures examined. At a concentration of 0.5mM of glutathione, the average rate of lactate production at 4ºC hypothermia was 3.99 ± 0.28 $x10^{-10}$ mmols/cell/72hours compared to 4.06 ± 0.60 $x10^{10}$ mmols/cell/72hours for 22ºC. This suggested a slight increase in metabolism at 22ºC, however, two-way ANOVA and post-hoc Tukey tests confirmed that the difference between the two temperatures of preservation was insignificant. The largest difference was between the lactate produced by all the preserved cells and that produced by the activated T cells maintained under in-vitro conditions, shown by the significant differences between control at 37ºC and the groups of 4ºC or 22ºC hypothermia, see Figure 4-17.

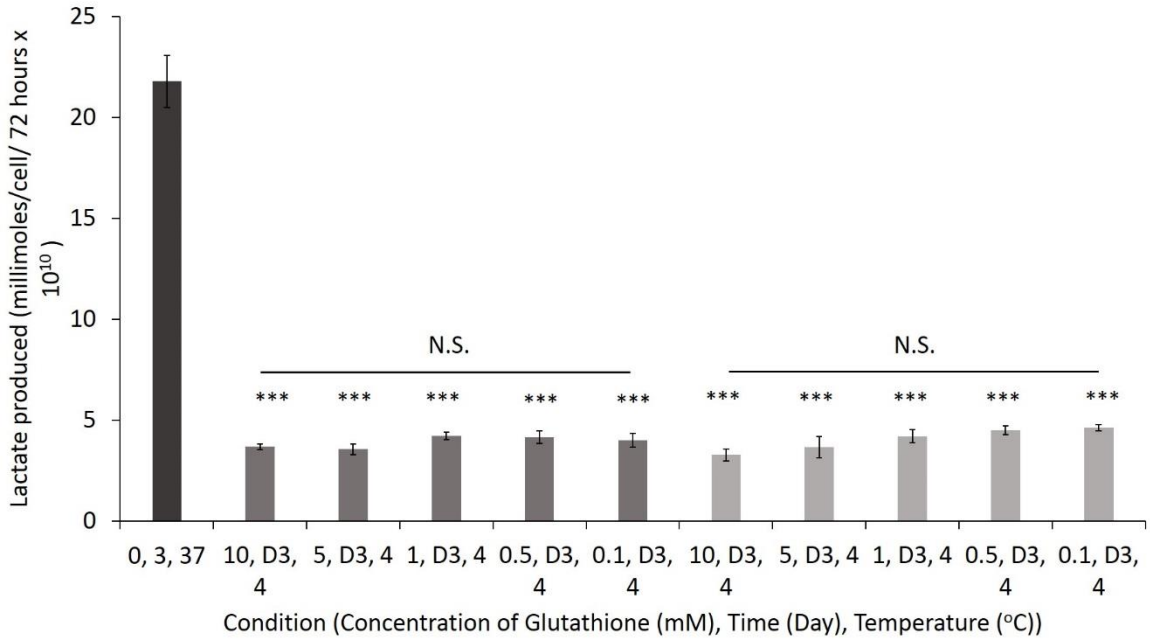

Figure 4-17 Lactate produced by T cells during incubation at normal conditions and hypothermia in conjunction with different concentrations of glutathione. 0,3,37 was the condition of 3 days of proliferative growth. The same duration was tested for hypothermic preservation at 4°C or 22°C in the presence of different concentrations of glutathione supplementation. N.S = No Significant difference. See Section 3.2.10 for the explanation of statistical analysis and consequent labelling. N=3.

Although there were differences in metabolic activity upon recovery of the T cells reported in Figure 4-15, the changes in glutathione concentration did not alter the mitochondrial membrane potential (Δψ). No significant differences in Δψ were reported within each temperature group of 4°C and 22°C. However, comparison between the two temperatures found a significant decrease in Δψ for 22°C preservation, see Figure 4-18. The Δψ at 22°C dropped significantly below the potential observed on day 0, whereas 4°C preservation showed no difference from the day 0 control.

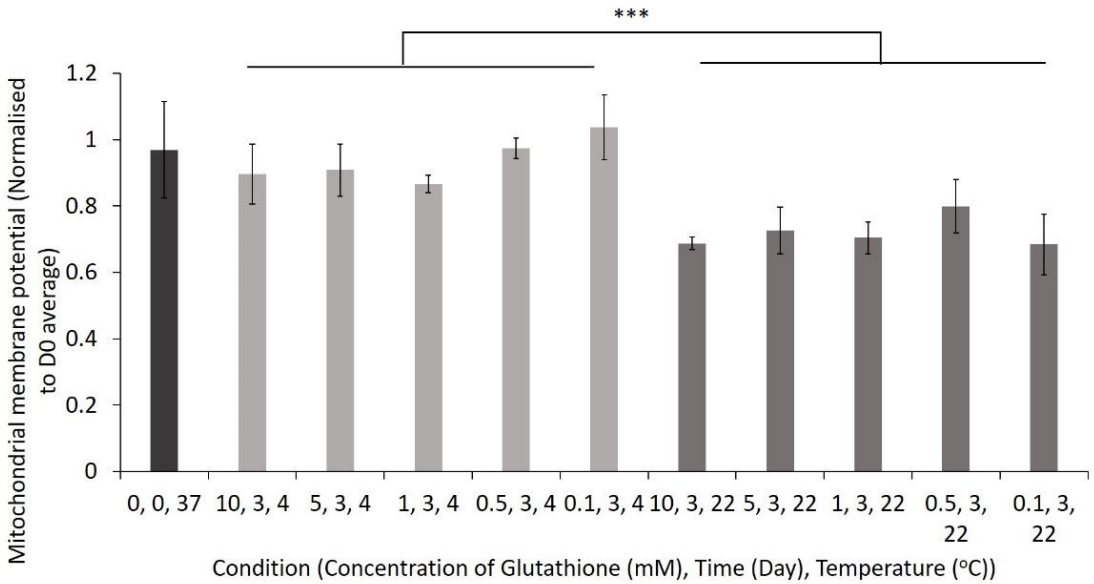

Figure 4-18 Mitochondrial membrane potential of T cells during proliferative culture and after 3 days of hypothermic preservation at 4°C or 22°C in the presence of different concentrations of glutathione supplementation. This potential was measured by commercial kit, the ratio of fluorescence values at two different wavelengths corresponds to a dimensionless measure of the potential. Values were normalised to the 0,0,37 control average of T cells before preservation. See Section 3.2.10 for the explanation of statistical analysis and consequent labelling. N=3.

Glutathione supplementation of the preserving medium was shown to have a profound effect on T cell metabolism after completing hypothermic preservation at 4°C. However, the results in Figure 4-19 show that supplementation with 1mM of glutathione was unable to avoid the depletion of ATP that occurred during hypothermic exposure. The concentration of intracellular ATP at both temperatures, 4°C and 22°C, showed no difference with and without glutathione. There was an effect of temperature on the amount of ATP, which was significantly lower for cells that had been preserved at 22°C compared to 4°C.

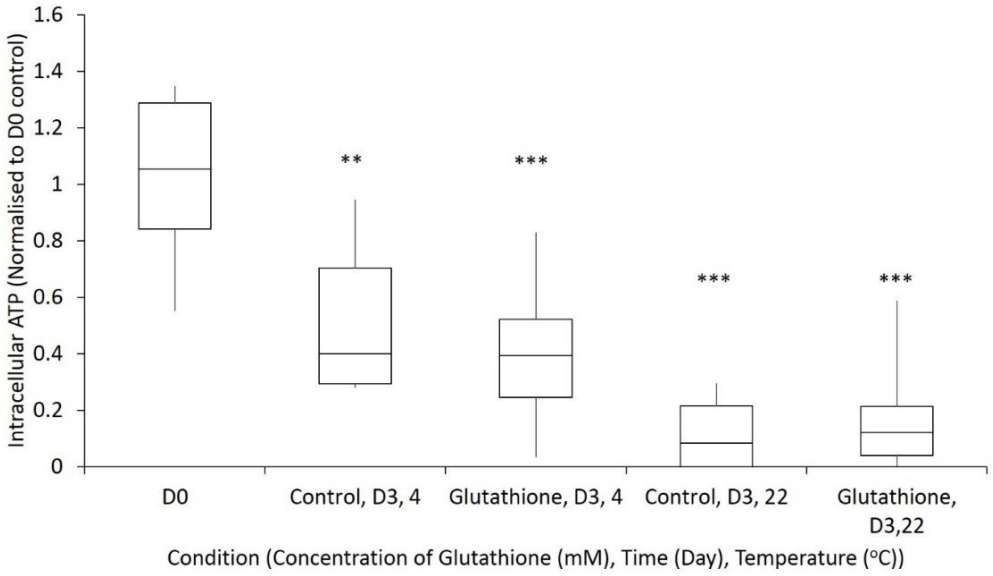

Figure 4-19 Intracellular ATP content of T cells preserved with hypothermia and supplemented with glutathione. ATP content was examined by extraction of ATP by boiling deionised water followed by analysis by luminescent firefly luciferase assay. The data points show how the application of two temperatures of hypothermia reduced ATP after preservation and the lack of affect of including 1mM of glutathione in the preservation medium. See Section 3.2.10 for the explanation of statistical analysis and consequent labelling. N = 6.

A large amount of the total reduction of Alamar blue resulted from the reduction potential of glutathione alone, with high fluorescence detected in the samples containing glutathione. Neufeld et al. recently reported Alamar blue chemical reduction by glutathione compound alone, although it was in concentrations of 100mM that caused significant deviation from controls [261]. The results presented in Figure 4-15 are corrected by removing the background reductions of the different glutathione concentrations in the medium. Therefore, the changing effect of glutathione concentration on Alamar blue reduction reported in Figure 4-15 was the result of biochemical reactions related to live cells. This means that glutathione had a large effect on cell metabolism after preservation.

Results in Figure 4-14 indicated that glutathione had a noticeable effect on the membrane integrity of T cells. Vreugdenhil et al. have shown that, if exogenous glutathione was present in the preservation medium, then rat hepatocytes retained a greater intracellular concentration of glutathione [222]. This suggests that the hepatocyte cell membrane was becoming permeable to the glutathione. In that study the concentration of intracellular glutathione, both with and without exogenous glutathione, fell well below the initial amount

present before induction of hypothermia. Exogenous glutathione also improved membrane integrity of the hepatocyte cell type. Hamilos et al. found that exogenous glutathione increased lymphocyte proliferation stimulated by phytohemagglutinin under in-vitro conditions [262]. That result was attributed to higher intracellular concentrations of glutathione being achieved by the inhibition of export by translocating enzymes. These two studies together suggest that extra-cellular glutathione can produce effects on the cell to maintain intracellular concentrations even if it is not clear whether the compound can cross the cell membrane.

Furthermore, the results of Figure 4-15 suggest that extracellular glutathione has an important role in altering activated T cell metabolism on recovery to normal temperatures after completing hypothermia. The mechanism behind this is proposed to be the avoidance of the hypothermic depletion of glutathione. Carilho-Torrao et al. showed that depleted glutathione increased the ROS level in T cells during activation [263]. The results of Figure 4-16 showed that very low levels of glutathione supplementation allowed ROS to increase activity above day 0 controls. The combinations of these results suggest that T cells were depleting their intracellular glutathione concentration during hypothermia.

At low concentrations of glutathione, the ROS activity exceeded day 0 controls and very little or no metabolic activity after preservation was observed. Increased ROS above normal levels during 37^{o}C recovery from preservation at 4^{o}C could negatively affect the outcome of T cell preservation. Hydrogen peroxide is known to induce DNA damage. Cerutti et al. found that cells transfected to upregulate peroxide scavenging enzymes then exhibited much less DNA damage after hydrogen peroxide exposure [264]. There is also a role of maintaining ROS levels in T cell proliferation being previously established [144,146]. The results of Figure 4-14 showed that the membrane integrity was compromised, and Figure 4-15 showed that metabolic activity was reduced, both after preservation, and when glutathione was included at a too low concentration during preservation at 4^{o}C. The established mechanism

of ROS damage occurred in the T cells exposed to hypothermia, but this was reduced by inclusion of glutathione.

High concentrations of glutathione also limited T cell metabolic activity after preservation. It is well established that some ROS species are used in cell signalling. Previte et al. found that the addition of the compound manganese metalloporphyrin inhibited ROS generation in splenocytes in response to stimulation with ionomycin [265]. Further to this, the compound arrested cells in the G0/G1 phase of the cell cycle, and prevented proliferation of T cells by preventing aerobic glycolysis and reducing mTOR signalling. The results in Figure 4-15 suggested that glutathione supplementation at 1mM concentration produced the maximum of overall T cell metabolism after preservation. This concentration maintained an ROS level slightly lower than that of untreated proliferating T cells. The Spearman's correlation between ROS activity and metabolic activity at glutathione concentrations above 1mM was reasonably strong at 0.741, with a significant P-value of 0.033, which confirms the notion that increasing concentration beyond 1mM decreased the metabolic activity recovered. Therefore, excessive inhibition of ROS by concentrations in excess of 1mM of glutathione led to the reduction of metabolic activity on recovery at the same concentrations. High glutathione concentrations inhibit the ROS required for cell signalling after preservation.

The glutathione concentration of 1mM maintained an ROS level slightly lower than that of untreated, proliferating T cells, whereas lower concentrations increased ROS activity on recovery and higher concentrations inhibited the ROS. Therefore, the results of Figure 4-14, Figure 4-15, and Figure 4-16 recommend that the concentration of glutathione in the preservation medium at a temperature of 4^{o}C can be reduced from 3mM in the standard UW medium to 1mM for optimised metabolic activity of T cells on recovery to normal temperatures. The requirement for different concentrations could be a result of cells having different intracellular concentrations of the glutathione. It is already known that hepatocytes that export the compound can contain 10mM of glutathione, whereas it is only present in

most other cells at 1-2mM [143]. Hepatocytes were the main cell type for which the UW preservation medium was originally designed [82]. It is already known that glutathione is permeable to the cell membrane during hypothermia [222]. Therefore, glutathione may be able to diffuse across the cell membranes if intra- and extra-cellular concentrations are different. The results of Figure 4-14 and Figure 4-15 confirm this mechanism of glutathione movement because there was an optimum concentration of glutathione to include in a T cell preservation medium. Therefore, it is important to adjust for the concentration of native antioxidants so that exogenous loading does not greatly change the intracellular concentration. However, the compound did not stimulate cell metabolism during the application of hypothermia, shown in Figure 4-16 by no difference in intracellular ATP concentration or lactate production.

The T cells at 22^{o}C responded with little metabolic activity recovered with any of the treatments applied, see Figure 4-15. This was most likely because of the large amounts of apoptosis that developed with cell preserved at this temperature. Pradelli et al. demonstrated that apoptosis increased caspase levels and subsequently decreased the glucose metabolism of proliferating cells [266]. The apoptosis that was indicated from the result of the lack of metabolic activity presented in Figure 4-15 could not be linked to withdrawal from the antigens used to culture the cells, see Section 4.3.9. Therefore, the mechanism causing the apoptosis at 22^{o}C must be investigated further to truly determine if T cells can be preserved under ambient conditions.

4.4 Summary.

The supplementation of a preservation medium with ATOC and glutathione as tested in this Chapter affected T cell metabolism before and after the application of hypothermia at 4^{o}C. However, at 22^{o}C the effect of antioxidant compounds did not go beyond reducing the amount of ROS activity in the cells upon recovery to normal temperatures. There were no differences observed in the ATP content of the T cells that had been treated with glutathione

or ATOC compounds compared to untreated T cells after 3 days of hypothermic preservation. This suggests that supplementation with either component could not drive the cell metabolism to produce more ATP than was consumed during preservation. A small quantity of T cell metabolism did persist under hypothermia, as evidenced by the production of lactate in the preservation medium, albeit at 10-fold lower concentrations than comparable proliferating cells. This low rate of metabolic activity was not affected by preservation medium supplementation. The low amounts of ATP and low concentrations of lactate produced under hypothermia suggest that the increased cell metabolism shown by Alamar blue reduction was an effect that only took place on recovery to 37^{o}C, and resulted from the T cells making use of the glutathione or ATOC on return to normal temperatures. ATP was reduced to low concentrations irrespective of the treatments. These factors led to the overall conclusion of Chapter 4 that compounds with antioxidant capability are capable of upregulating cell metabolism in the short-term, but this effect is lost with hypothermia and an increased preservation duration. This led to T cells that are depleted of ATP after completing 3 days of hypothermic preservation, which was greater at milder temperatures of preservation. Extra strategies are required to increase energy reserves of preserved cells, and these strategies are examined in Chapter 5.

5 Pre-treatment and Pre-conditioning of T cells to Improve Viability and Functionality After Hypothermic Preservation.

5.1 Introduction.

The methods tested in Chapter 3 were not successful in stimulating T cell metabolism to maintain ATP concentrations during hypothermic preservation. The loss of ATP could represent a major loss of therapy product quality, therefore a method to avoid the loss must be established.

The first topic that knowledge can be transferred from is the activity within organ preservation. Pre-conditioning organs with mild hypothermia is a common strategy for reducing the effects of ischemia/reperfusion injury, especially when considering hepatocytes and liver transplantation [111]. It is difficult to precondition whole organs without invasive measures. However, this difficulty was overcome in studies that attempted to produce the same effects with pharmacological methods of pre-treating the organ. One successful pharmacological method was the pre-treatment with compounds that react with adenosine receptors [101]: this pharmacological treatment produced a response similar to pre-conditioning whole rats with 10 minutes of ischemia and re-perfusion before attempting a full surgical procedure. That pharmacological study attributed the preconditioning result to adenosine levels and its receptors. It could also be related to the suppression of inflammation, for which adenosine and its receptors, in particular the A2A receptor, have a known role [102]. Adenosine monophosphate protein kinase (AMPK) has also been linked to the effect of pre-conditioning organs for transplantation due to its role in ATP sensing. The activation of AMPK signalling by a stimulant was shown to inhibit the decrease in the amount of ATP concentrated in rat liver cells, with a parallel reduction in glycolysis and in the subsequent accumulation of lactate [107]. Similar events were observed by preconditioning comparable tissue samples with physical ischemia [107,267]. This suggests that, as well as adenosine receptors affecting stress response, AMPK signalling within cells is also a mechanism

involved in the pre-conditioning of organs for successful transplantation. The manipulation of mechanisms related to energy generation and storage is concluded to be important to improve the results of hypothermic preservation.

The second topic for consideration is T cell anergy: the mechanisms of adenosine receptors and AMPK signalling are shared by T cell anergy. Activation of AMPK signalling was shown by Zheng et al. to inhibit IL-2 and IFN-Y production, but did not cause cell death, suggesting that a lack of ATP reserves will inhibit immunological function of the T cell [123,124]. T cells can be recovered from a state of anergy by adding exogenous IL-2 to the suspension, which will affect all the T cells in the population and will also increase lactate production [123,124]. Adenosine is a precursor to ATP, and extracellular concentrations of this compound can regulate naive T cell activity. This regulation was shown by Ohta et al. by differences in T cell homeostasis of mice models with, or without, the A2A receptor gene that relates to one adenosine receptor. Removing the effect of the gene resulted in a decline of naive but not memory T cells [141]. Stimulation of the A2A receptor resulted in a population of activated effector T cells that showed lower cytokine release, which suggested that, should extracellular adenosine be present, then immune function of activated T cells will be impaired [140].

From the results of the existing studies of T cell anergy and organ pre-conditioning it can be deduced that there is an interaction between previous pre-conditioning before hypothermic preservation and those mechanisms that would inhibit the function of adoptive transferred T cells. Therefore, alternative methods to pre-treat T cells to facilitate hypothermic preservation need to be identified. Chapter 5 investigated two potential alternatives to manipulate T cells before commencing hypothermic preservation.

Firstly, Chapter 5 investigated pre-conditioning methods that avoid interfering with the immunological function of T cells to be compatible with the processing of the cells. Cryopreservation already takes place in many cell therapy processes [13,268], so does not

represent an extra step that would need validation. This method involves exposure to a short cold-shock when performing controlled rate cooling and rapid thawing. In this study the effect of cryopreservation was examined to investigate if the hypothermic exposure of the process improved the survivability of CD3/28 activated T cells during hypothermic preservation.

Secondly, Chapter 5 investigated a biological process that consumes large quantities of ATP to identify the need for pre-treatment to reduce biochemical reaction rates. The largest single consumer of ATP in mammalian cells is protein synthesis, with DNA synthesis the second largest consumer [269]. Cell mitosis cycle progression was identified to be slowed but not stopped during hypothermia [270,271], whereas protein synthesis ceases during hypothermia [175]. Therefore, the method investigated in this study was to pre-treat the activated T cells by blocking the activity of DNA synthesis. This blocking was investigated by synchronising cells to the stationary G1 phase between mitosis and DNA synthesis [272]. Halting the mitosis cycle would stop a major ATP consuming reaction and cells should survive hypothermic preservation with greater energy reserves. Therefore, cells might retain greater function on recovery after preservation if they have more energy immediately available.

5.2 Materials and methods.

5.2.1 Immunostaining and flow cytometry to identify T cell phenotype.

$1x10^6$ cells were distributed for each sample to round-bottomed wells of plates and the cells pelleted by centrifugation at 300g at 4°C for 3 min. Antibodies and FACS Wash Buffer were added to 50ul per well to re-suspend the cells, and incubated for 20 minutes on ice, protected from light. Antibodies were purchased from BD Biosciences as follows: CD3-FITC: UCHT1 (555332); CD8-PE: RPA-T8 (555367); CD16 PE: B73.1 (561313); CD4-APC (55349); CD25-PE (555432); CD56-AP. After incubation, cells were washed two times with 200-µL of FACS Wash Buffer, then centrifuged at 300g for 3 minutes to pellet the cells. Samples

were then measured on a BD FACScalibur system, and analysis of samples was completed in Flowing Software 2 open source flow-cytometry software. Compensation between samples with two fluorochromes was achieved by labelling cell samples with the individual fluorochromes to produce data for a single label, which was then used to subtract spill-over into the channel of the second fluorochrome.

5.2.2 Thymidine block to synchronise cells to the G1 phase

A double Thymidine block was performed by re-suspending T cells to a live density of $1x10^6$ cells/ml. Thymidine, purchased from Sigma Aldrich UK, was made to a stock concentration of 100mM in PBS and filter sterilised with 0.22µm filter. Thymidine was added to the cell suspension to a final concentration of 2mM. The dosed suspension was incubated for 12 hours in a 37ºC incubator. Following this, cells were pelleted by centrifuging at 300g for 3 minutes and then washing in PBS: this process was repeated 3 times. After washing, cells were suspended in complete culture medium and returned to the incubator for 8 hours. 2mM thymidine was then dosed into the culture and incubated for a further 12 hours. Following incubation, cells were washed again with PBS and then used for further experiments.

5.2.3 Cell cycle analysis.

Cell suspensions of approximately $5x10^5$ cells were fixed in cold 70% (vol.) ethanol and deionised water for 30 minutes. Fixed suspensions were centrifuged and washed in PBS, repeating both steps twice. Ribonuclease was added to the suspension at a concentration of 100µg/ml, followed by DNA labelling with 50µg/ml of propidium iodide. Samples were measured on the same flow cytometry system and software as for phenotype, detailed above. Matlab software was used to determine the phases of the cell cycle by; counting the total amount of events; fitting binomial distributions to the G1 and G2/M phase peaks of the histogram; and, then subtracting the events in each of those peaks from the total to find cells in the S phase.

5.2.4 In-vitro cytotoxicity assay to identify therapeutic action of T cells.

In-vitro cytotoxicity assays followed the method described by Somanchi et al. [273]. Jurkat cells were cultured for 5 days, labelled with calcein-AM at 2µM for 30 minutes, washed, and then re-suspended in complete RPMI to a concentration of $2x10^5$ live cells/ml. T cells were re-suspended to a concentration of $1x10^6$ live cells/ ml. 100µL of each cell suspension were combined in a round bottom 96 well plate. This mixture of cells gave an effector to target ratio of 5:1. Cells were pelleted by centrifuging at 100xg for 1min. Settled mixtures were incubated at 37ºC for 4 hours. After this, cells were re-suspended and pelleted by centrifuge at 300xg for 3 mins. 150µL of supernatant was transferred to a flat bottom 96 plate and relative fluorescence was examined using the Spectramax system. To obtain accurate values for cytotoxicity of T cell samples, two corrections to sample values were made. Firstly, the value of a spontaneous release from labelled Jurkat cells alone was subtracted from each sample reading. Then, secondly, post-subtraction values were normalised to a maximum calcein release from Jurkat cells by complete lysis. The labelled Jurkat cells were lysed with 1% by volume of Triton X-100. These corrections were created for each medium formulation to remove any chemical effect on cell lysis. The remaining 50µL was re-suspended and imaged on the same system used to establish cell viability to confirm that no significantly different amount of incompletely lysed cells was found. This data is not presented.

5.2.5 Hierarchy of tasks for investigating combinations of pre-conditioning and pre-treating cells.

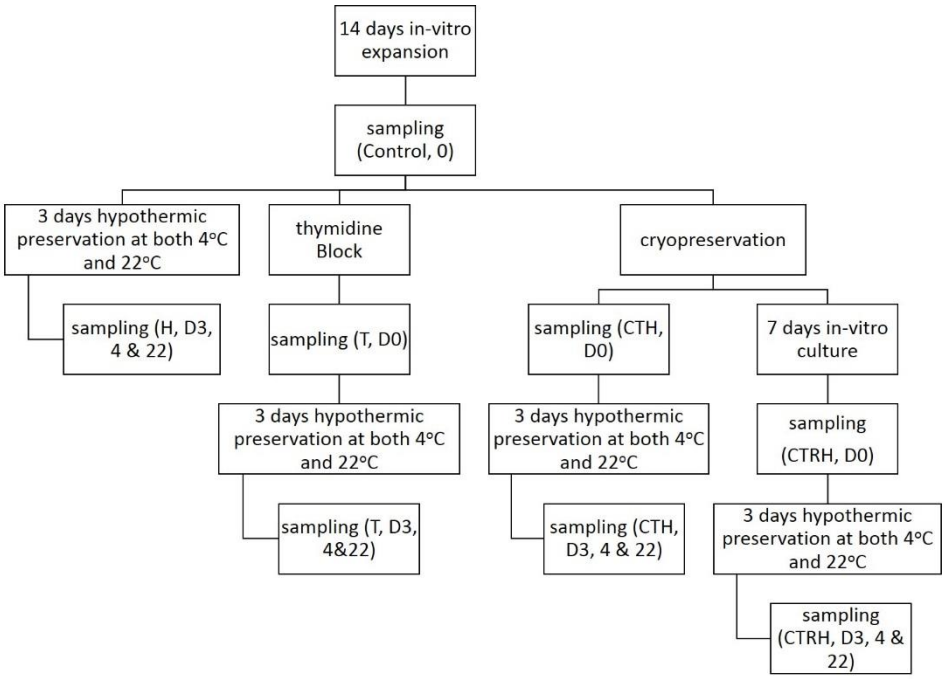

Figure 5-1 Hierarchy of culture, pre-treatment, and pre-conditioning of T cells before exposure to hypothermia. Brackets contain the key to labelling of following figures showing results.

The diagram in Figure 5-1 shows the hierarchy of actions performed to investigate pre-conditioning and pre-treating T cells. First, cells were cultured for 14 days before attempting any preservation, which increased to a maximum of 21 days for in-vitro culture with the addition of a cryopreservation and recovery step. This total duration is the same as the maximum culture duration investigated by Bonano et al. for the generation of CIK cells [16]. After 14 days, cells were either transferred directly to hypothermia, H category, or subjected to a thymidine block before hypothermia, T category. After cryopreservation and thawing, cells were subject to hypothermia immediately, CTH category, or allowed a period of recovery culture before commencing hypothermia, CTRH category. The diagram in Figure 5-1 also showed the investigation performed to define a suitable grade of hypothermia for preservation to occur at, with both 4°C and 22°C tested.

5.2.6 Previously used methods.

Detailed methods for the measurements of cell membrane integrity and viable cell count, metabolic activity, ATP concentration, and statistical analysis, can be found in Section 3.2 and Section 4.2. Cell culture, cryopreservation, and hypothermic preservation methods are also detailed in Section 3.2.1.

5.3 Results and discussion.

5.3.1 Pre-conditioning avoids necrosis in T cells preserved under hypothermia.

The first method to investigate if cryopreservation affected the hypothermic preservation of activated T cells was the analysis of viable cell count and membrane integrity. Rusotti et al. showed that pre-conditioning of cells by exposure to cold conditions before attempting a longer duration of hypothermic preservation minimised cold-shock induced cell death in isolated fibroblasts [112]. That result was shown by measurement of necrosis and was linked to heat-shock proteins (HSPs). This link between cold-shock and necrosis was used in the results below to detect the effect of pre-conditioning by measuring membrane integrity.

Direct transfer of thawed cryopreserved cells to hypothermic conditions was not capable of pre-conditioning cells to survive hypothermic preservation. However, the inclusion of a recovery step of in-vitro cell culture after thawing cryopreserved cells resulted in a cell population that did not display a significant loss of viable cell count (VCC) and membrane integrity after 3 days of hypothermic preservation. Cells thawed from cryopreservation and transferred straight to hypothermia, CTH, resulted in a large quantities of cell death after exposure to both 4^{o}C and 22^{o}C preservation temperatures. These conditions displayed the lowest VCC amongst those tested in Figure 5-2. The low normalised VCC for this direct transfer were medians of 0.43 for 4^{o}C preservation and 0.25 for 22^{o}C. The CTH cells stored at 4^{o}C demonstrated a significantly decreased VCC compared to cells that were not cryopreserved, H. The decrease in VCC at 22^{o}C with and without cryopreservation pre-treatment was small. Cells directly transferred from the first phase of cell expansion to

hypothermia, H, produced VCC that were slightly higher than the CTH category, Figure 5-2. The cells pre-conditioned by cryopreservation and recovery, followed by hypothermic preservation, CTRH, at both temperatures tested had insignificant probabilities of a difference compared to the VCC of untreated cultured cells, Control, 0, see Figure 5-2. The probability values were 0.631 at 4°C and 0.267 for 22°C. The membrane integrity after completing the recovery step was insignificantly lower than the Control, 0, 37 category. The normalised median VCC to consider were 0.981 on the day 0 control compared to 0.996 at 4°C and 0.904 for 22°C. This lack of difference demonstrated that there was no change in membrane integrities between pre- and post- hypothermic preserved cells when a recovery step was introduced.

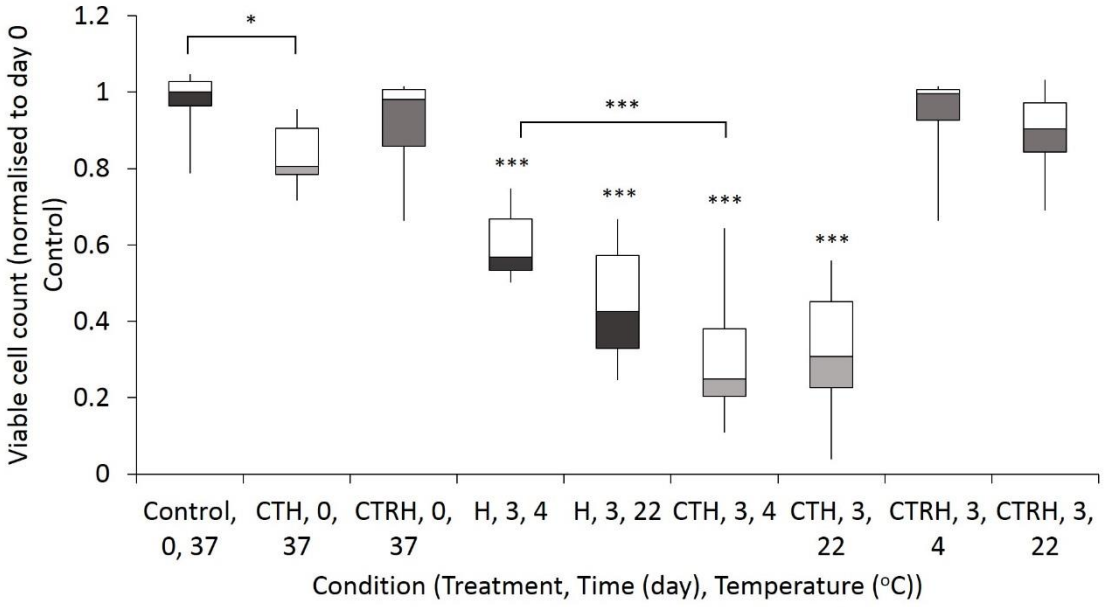

Figure 5-2 Viable cell count of T cells after pre-conditioning with cryopreservation prior to hypothermia. Hypothermic preservation was completed over a period of 3 days. Refer to Figure 5-1 for key to X axis labels. Viable cell count was examined by counting total number of cells, membrane integrity by exclusion of Draq 7, and comparison to day 0 control viable cell count. Kruskal-Wallis testing follow by pairwise Mann-Whitney tests were used because of non-normal populations. See Section 3.2.10 for the explanation of statistical analysis and consequent labelling. N=6.

These results showed that pre-conditioning activated T cells with a cold-shock before a long duration of hypothermia was vital to maintain viable cell count after completing the whole preservation process. An excessive amount of necrosis would stop the preserved cell population from being used in downstream applications. Galipeau reasoned that a reduced

number of viable cells after attempting cryopreservation to deliver stem cells to treat graft-versus-host disease (gvhd) was the reason that the outcome of the clinical trial was not successful [69]. The small differences in VCC between 4°C and 22°C of untreated cells in Figure 5-2 meant that no difference in necrosis occurred. The lack of difference between the two temperatures was unexpected. Wang et al. showed robust cell lines survive with lower necrosis and apoptosis only at the warmer temperature of 22°C [160]. The robust cell lines used in that study were reported to continue with proliferation at 22°C, hence suggesting that this was the more suitable temperature to store and ship cells. The primary CD3/28 activated T cells used in the results presented in Figure 5-2 suffered from slightly larger amounts of necrosis at the 22°C temperature. The cell line itself could, therefore, be the reason for the contrast. It could be possible that robust cell lines that have been frequently manipulated in-vitro are capable of surviving deviations from growth conditions.

Pre-conditioning T cells with a cold-shock by cryopreservation in this study, before attempting longer duration hypothermic preservation, can have both a positive and negative effect on the final cell membrane integrity after hypothermic preservation. The positive result was dependent on the addition of a recovery step between the two processes. Cryopreservation alone reduced the membrane integrity after completing hypothermic exposure. This was mitigated by a duration of in-vitro culture that allowed the T cells to recover from the initial cold-shock to produce the positive effect on membrane integrity during the application of hypothermia for preservation. Therefore, the cold-shock can be provided by a process that already occurs in many cell therapy processing chains, which could reduce the complexity of the process for pre-conditioning the cells.

Pre-conditioning has been previously documented to improve quality of biological systems other than T cells after a further stressing condition. Existing studies describe pre-conditioning by pre-stressing cells or tissue by the processes of hyperthermia [274], hypothermia [275,276], and ischemia [96]. The further stressing condition can be the same

as the preconditioning stress, or different in some cases. The difficulty with existing studies was that the pre-conditioning step was an extra deviation from existing cell therapy protocols. Cryopreservation investigated in the results of Figure 5-2 already takes place in cell therapy processing. Therefore, it does not represent a deviation or extra processing steps that require validation [13,268]. Cryopreservation-stressed cells maintained membrane integrity more effectively after exposure to hypothermia compared to unconditioned cells. Therefore, the combination of cryopreservation, recovery culture and the hypothermic preservation is an effective method that maintains the membrane integrity of T cells during short-term preservation.

5.3.2 Pre-conditioned viable preserved cells move through the cell cycle but do not complete mitosis.

This study aimed to investigate the effect of limiting cell cycle movement during preservation. Therefore, the effect of pre-conditioning with cryopreservation on the cell cycle distribution during hypothermic preservation was investigated. Cell cycle movement was investigated by intracellular propidium iodide staining of DNA followed by flow cytometry to quantify the changing amount of fluorescence within a single cell that relates to the quantity of DNA within. This investigation suggested that recovered cells progressed forward through the mitotic cycle, whereas untreated cells appeared to collect in the G1 phase, see Figure 5-3A.

Untreated cells that were exposed directly to hypothermia, H group, appeared not to progress through the cell cycle, and collected in the G1 phase of the cell cycle. The S phase of T cells treated in this way was relatively stable. The most noticeable result about the distribution amongst the cell cycle was the significant decrease in G2/M cells of the H group.

After the normothermic, 37^{o}C, recovery period for cryopreserved cells, but before exposure to hypothermia, termed CTRH day 0 cells, there was a small shift towards a population with more resting cells, demonstrated by increased frequency of G1 cells, see Figure 5-3A. The

increased G1 phase was accompanied by decreased S and G2/M phase cells compared to control cultured-only cells, termed control Day 0 cells, see Figure 5-3B and Figure 5-3C for S and G2/M phases, respectively. During hypothermic exposure there was some evidence of the cell population progressing through the cell cycle for CTRH cells. This can be seen through a decrease in the G1 population with corresponding increases in S phase, and particularly G2/M phase cells.

It is suggested from these results that G2/M phase cells were susceptible to cold-shock. This was because high cell membrane integrity was maintained by CTRH cells in conjunction with a decreased percentage of G1 cells and increased S and G2/M population, indicating progression through the cell cycle. Whereas, unconditioned cells showed a significantly decreased viability and a significant decrease in the G2/M population.

Pre-treatment by a thymidine block to synchronise cells to the G1 population on day 0 showed an increase of this population to an average of 93.6±1.3% compared to a population of 71.4%±5.0% in cultured cells, see Figure 5-3A. Cells treated with a thymidine block showed much less progression through the cell cycle for CTRH day 3 cells after preservation at both temperatures. Some cells were released from the G1 phase into the S phase, but there was no change in the G2/M phase cells, see Figure 5-3C. All changes in the cell cycle distribution of thymidine-treated cells during hypothermia were insignificant.

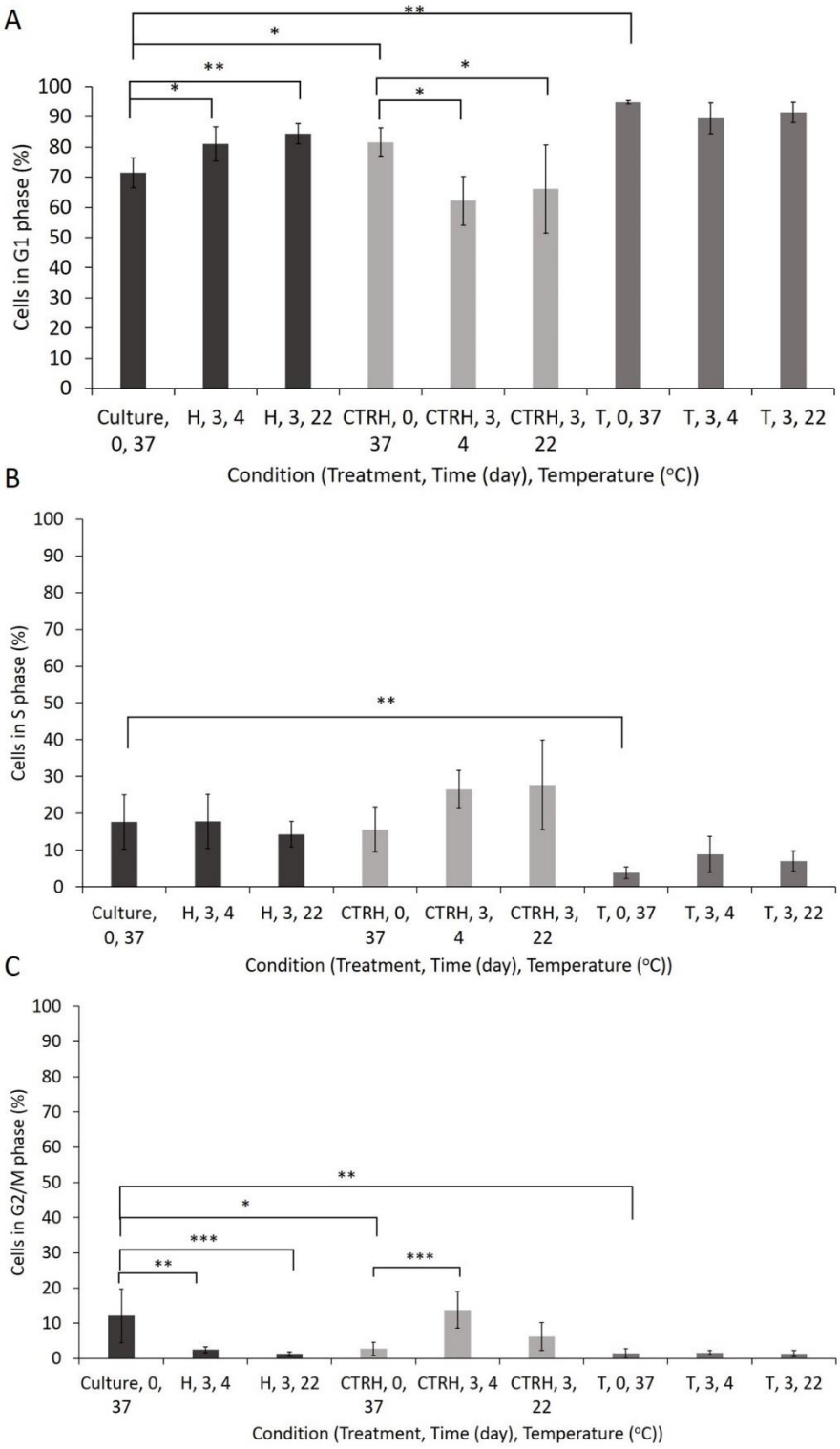

Figure 5-3 Cell cycle distribution of pre-conditioned and pre-treated T cells in combination hypothermic preservation. Hypothermic preservation was performed over a period of 3 days. Cell cycle distribution was assessed by intracellular PI staining and flow cytometry. Histograms of PI fluorescence per individual cell were assessed in Matlab for phases by fitting distributions to peaks of different phases. A) G1 phase cells, B) S phase cells C) G2/M phase cells. Refer to Figure 5-1 for key to X axis labels. Original data satisfied assumptions for ANOVA test. See Section 3.2.10 for the explanation of statistical analysis and consequent labelling. N=6.

The population of CTRH T cells moved forward through the cell cycle from the G1 phase with increased frequency of cells in the S and G2/M phase cells. These cells did not complete mitosis because the cytometry involved with viability data in Figure 5-2 did not report an increase in cell number. The quantity of cells was considered in the viable cell count in Equation 3-1 and Equation 3-2. Untreated cells saw increased G1 cells, without an increase in number of cells present, whereas pre-conditioned cells moved towards the G2/M phase. Two effects on cell cycle were previously reported by other studies, but both were from robust cell lines. Firstly, Rieder and Cole observed that the cell cycle progression during hypothermia collected cells in the G2/M phase when adenocarcinoma and immortalised human epithelial cell lines were cooled to 19^{o}C for 48 hours [271]. That study did not condition the cells in a particular way and no increase in cell number was found. Secondly, Marchant et al. found an apparent increased population of G1 phase cells when Chinese hamster ovary cells were cooled to a higher temperature of 27^{o}C [277]. In this second study, an increase in live cell number was reported, suggesting that some cells types are capable of completing replication under hypothermia. Both of these studies show that these robust cell lines in an untreated state are capable of moving through the cell cycle. Results of Figure 5-3 showed that T cells also progress through the cell cycle once pre-conditioned with a cold-shock. Without pre-conditioning, the G2/M phase of T cells significantly decreased in frequency, suggesting an apparent backwards movement because of no detected increase in VCC. Therefore, the results shown in Figure 5-2 and Figure 5-3 add new knowledge that methods of pre-conditioning primary cells can make the cell cycle behave similarly to robust cell lines during hypothermia.

However, this movement through the cell cycle may not be ideal because it indicated continued cell activity as discussed in the introduction to this Chapter. Addition of a thymidine-block to synchronize T cells to a G1 phase before the application of hypothermia adequately maintains the cells in this phase during the exposure to reduced temperatures.

This suggested that the application of hypothermia to preserve CD3/28 activated T cells without further intervention does not limit progression through the cell cycle. Matijasevic et al. and Roobol et al. reported that hypothermia causes an arrest of the cell cycle in human fibroblasts and Chinese hamster ovary cells, which was related to the expression of the cell cycle controlling gene p53 [270,278]. This natural regulation of the cell cycle under hypothermia may explain why these cells are more capable of surviving hypothermia with no requirement for interventions to regulate the cell cycle. Therefore, it can be suggested that activated T cells may require interventions to limit cell cycle progression during exposure to hypothermia.

5.3.3 Cryopreservation reduces intracellular ATP and thawed recovered cells adapt to these lower ATP concentrations.

This study was conducted to investigate how pre-conditioning could affect the ATP depletion of T cells during hypothermia. Therefore, intracellular ATP was measured at different time points within the preserved T cells. This was completed by extracting ATP from the cells and then using a commercial luminescence luciferase assay to convert ATP into a measurable quantity.

A complete cryopreservation and thawing process led to a reduced amount of ATP immediately after cell thawing, see Figure 5-4. This did not recover to the quantity present before cryopreservation, even with continued in-vitro culture. Immediately after thawing the ATP present was reduced to a normalised average of 0.37 ± 0.14 of the quantity in the cells after finishing the first 14 days of in-vitro cell culture. After in-vitro recovery, the quantity of ATP present in CTRH Day 0 cells was an average of 0.29 ± 0.09 of the quantity in untreated control cells. Therefore, ATP did not change with the recovery step. Elements of the cryopreservation that reduced the ATP content of the cells could be the cryopreservation agent loading (CPA), 1^{o}C per minute cooling, storage at liquid nitrogen temperatures, or rapid thawing back to 37^{o}C.

Pre-conditioning T cells with cryopreservation, followed by immediate hypothermic preservation, termed CTH cells, resulted in T cells that were exhausted of ATP after completing both steps of preservation, see Figure 5-4. T cells preserved at 4^{o}C, with no recovery between cryopreservation and hypothermic preservation, showed an average 0.06 ± 0.03 of the ATP present compared to the control condition without cryopreservation after preservation (CTH, D3, 4 and Control, 0, 37, Figure 5-4). At 22^{o}C preservation, the quantity of ATP present in cells after completing hypothermic preservation had a depleted value similar to the condition without cryopreservation (CTH, D3, 22 and Control, 0, 37, Figure 5-4). This ATP was lower than the quantity present after 4^{o}C preservation.

The inclusion of recovery after cryopreservation limited the relative drop in quantity of intracellular ATP during hypothermic preservation, comparing the day 0 CTRH category with day 3, 4^{o}C, CTRH and day 3, 22^{o}C, CTRH, see Figure 5-4. There was more ATP detected for these cells that experienced a recovery step compared to the untreated control after hypothermia. For 4^{o}C preservation, there was a 1.85 fold increase over the cryopreservation-free cells (CTRH, D3, 4, and H, D3, 4, Figure 5-4). A 1.51 fold increase in ATP present for cells stored at 22^{o}C was also detected (CTRH, D3, 4 and H, D3, Figure 5-4). But 22^{o}C hypothermia remained lower than the cells preserved at 4^{o}C. Neither of these increases was significant, but the probability of difference from the corresponding day 0 group was reduced.

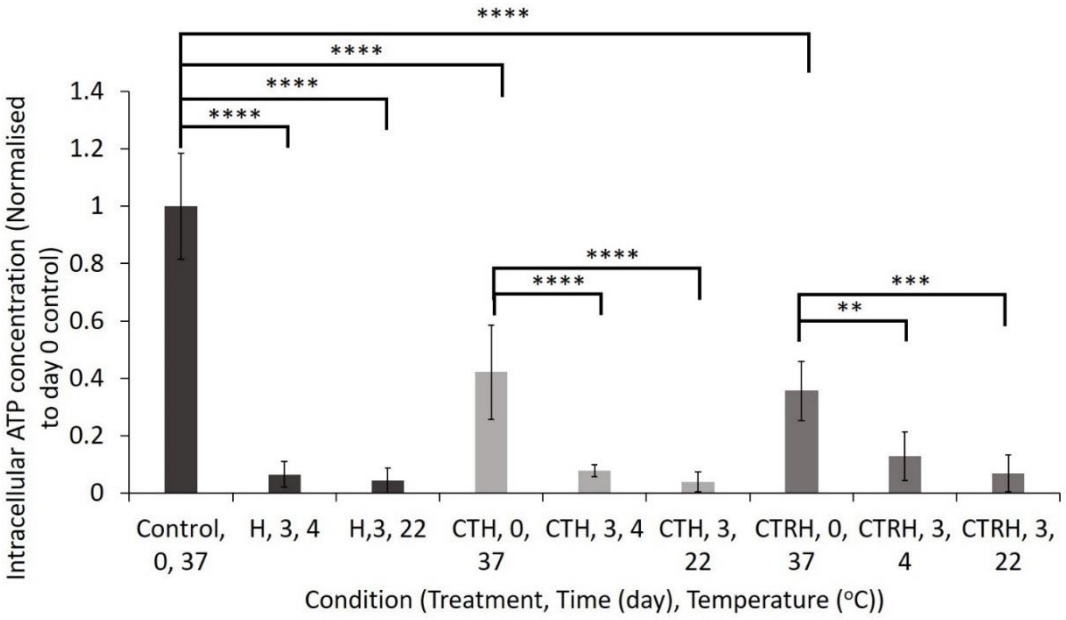

Figure 5-4 Intracellular ATP content of T cells after pre-conditioning cells with cryopreservation and then exposing to hypothermia. Hypothermic preservation was completed over 3 days. Intracellular ATP was assessed by lysing cells and examining lysate for ATP with commercial kit using firefly luciferase. Refer to Figure 5-1 for key to X axis labels, conditions include hypothermia, cryopreservation-hypothermia, and cryopreservation-recovery-hypothermia. Results are presented normalised to day 0 control at 37°C. Log_{10} transforms were used to validate ANOVA assumption for analysis. See Section 3.2.10 for the explanation of statistical analysis and consequent labelling. N=6.

It was not clear which part of the cryopreservation process caused the reduction of ATP shown in the results of Figure 5-4. A similar reduction in ATP after cryopreservation was reported by Sadeghi et al. in primary T cells [22]. In that study, the cryopreservation of fresh, untreated T cells displayed a small decrease in ATP content after completing the process. With activation and in-vitro culture included in the processing, the ATP loss after cryopreservation was increased. The specific part of cryopreservation that caused the change was not explained. Bajerski et al. found no change in ATP content in a leukaemic T cell line, Jurl-mk1, during the separate stages of cryopreservation [279]. In that same study, plant and fungal cells lost ATP content after treatment with 10 % (vol.) dimethyl sulfoxide before completing a freezing programme. Future cryopreservation studies may seek to investigate this apparent decrease in ATP to improve the cryopreservation process.

The culture of activated T cells after cryopreservation did not recover ATP levels, shown in the results of Figure 5-4. Sadeghi et al. found that after treating T cells with IL-2 there was a drop in intracellular ATP content [22]. They also found an increase in ATP concentration was detected after recovery without additional stimulation, whereas, a decrease in ATP concentration in T cells was observed when the cells were treated with IL-2 only. This previous study could explain why Figure 5-4 showed no change to ATP concentrations after thawing cells and then recovering with IL-2 supplementation.

Pre-conditioning by cryopreservation alone was not capable of reducing the consumption of ATP during hypothermic preservation and, because these cells display less available ATP after thawing, after completing the subsequent hypothermic preservation, ATP was almost completely consumed by the end of the processing. This appeared to be another cause of necrotic cell death due to the decreased membrane integrity shown in Figure 5-2. Tripmacher et al. showed that T cells will survive conditions of low intracellular ATP [280]. The results of Figure 5-2 and Figure 5-4 confirm not only the T cell survival but also that they can continue to proliferate with low ATP concentrations. These results also show that necrosis took place once all ATP was depleted, when the low ATP cells were exposed to the ATP depleting condition of hypothermia. Conversely, cells that had been recovered after cryopreservation retained a slightly higher amount of ATP after recovery so had a slower rate of consumption during hypothermic preservation. Recovery may have reduced the biochemical reaction rates to maintain more ATP during hypothermia.

Figure 5-2 showed that even though the cell cultures increased viability with recovery, they remain challenged by a much lower level of ATP than before cryopreservation. There are mechanisms in cells that can sense reduced energy states. AMPK is known to detect ATP:ADP ratio and then inhibit ATP consuming reactions [267,281]. This mechanism is also known to exist within T cells [123]. It is suggested that cryopreservation and recovery were activating a low energy state in primary T cells, indicated by a change in the ATP:ADP

ratio. This is shown by the lack of change of ATP during recovery and the smaller decrease in ATP during the application of hypothermia in Figure 5-4.

There was a requirement for a recovery step between the first (cryopreservation) and second (hypothermia) shock shown in the results of Figure 5-2 and Figure 5-4. This requirement agreed well with a prediction that can be made from extrapolating the results of Russotti et al. [112]. Russotti et al. showed that HSP70 and HSP27 were produced to maximum concentrations after 5 hours of normothermic recovery. Therefore, some recovery time is required to mature the benefit of protection offered by pre-conditioning in T cells, as confirmed in Figure 5-4 by the decreased ATP concentration of direct transfer after thawing to hypothermic preservation. More investigation would be required in the case of T cells to determine the optimum duration after commencing recovery to improve hypothermic preservation. The 7-day recovery tested in Figure 5-4 puts the T cells beyond the 20 hours at which Rusotti et al. found that the HSPs started to decline in concentration in their human fibroblast study [112]. However, several alternative mechanisms to HSPs were discussed in the introduction of this Chapter. A future study might aim to consider the different identified mechanisms related to cold-shock to determine the optimum point at which cells should be moved from recovery to preservation. Such a study of mechanisms might include HSP synthesis, AMPK signalling, along with investigation of autophagic markers, all of which have been discussed so far in this Chapter [130,171].

Chung et al. showed that pharmacological induction of HSP production increased glucose consumption in the cell metabolism in a rat cell model [282]. Therefore, the mechanism that cryopreservation may have in preconditioning the T cells to hypothermia may be further identified by investigating their metabolism.

5.3.4 Preconditioned cells lose metabolic activity with recovery, but this has no affect after hypothermia.

It was shown in Section 5.3.3 that there was lower ATP consumption during hypothermia for T cells that had undergone pre-conditioning with cryopreservation and recovery. There are many mechanisms that have been investigated related to the shock response from pre-conditioning cells, and some of these mechanisms are known to affect cell metabolism. Also, Chapter 3 showed that mUW maintained slightly higher metabolic activity of T cells recovered from hypothermic preservation but still demonstrated a significant decrease from pre-preservation controls. That Chapter also showed that cryopreservation appeared to affect the ATP content of the T cells. Therefore, cell metabolism in response to cryopreservation and subsequent hypothermia was investigated in this current Section.

Immediately after thawing from cryogenic temperatures, T cells exhibited a slight increase of metabolic activity compared to the level of cell culture alone, with a normalised median of 1.10. Thawed cells could be suggested to continue cell activity at a similar rate as before cryopreservation. This continuation resulted in cells that have less activity after exposure to hypothermia, CTH D0 and D3, with normalised medians of 0.07 and 0.05 for 4^{o}C and 22^{o}C, respectively, see Figure 5-5. The untreated controls showed that ATP utilisation continued during hypothermia and depleted the energy store, see Figure 5-4. Therefore, normal levels of cell metabolic activity and reduced energy stores can be suggested to result in necrotic cell death during hypothermia, see Figure 5-2.

Membrane integrity remained high after a recovery period of the T cells, CTRH, 0, 37, see Figure 5-2. Cells treated in that way displayed a level of metabolic activity that was lower than that of the cultured only cells, with a normalised median reducing to 0.58, see CTRH D0 Figure 5-5. CTRH cells lost a large quantity of metabolic activity on recovery after hypothermic preservation, with medians reduced to 0.09 and 0.04 for 4^{o}C and 22^{o}C,

respectively. This loss was slightly greater than that lost by cells that had been preserved by hypothermia without pre conditioning, H cells, see Figure 5-5, and comparable to CTH cells. The first cold-shock (cryopreservation) reduced the cell metabolic activity, but did not prevent a further reduction during a second hypothermic stress.

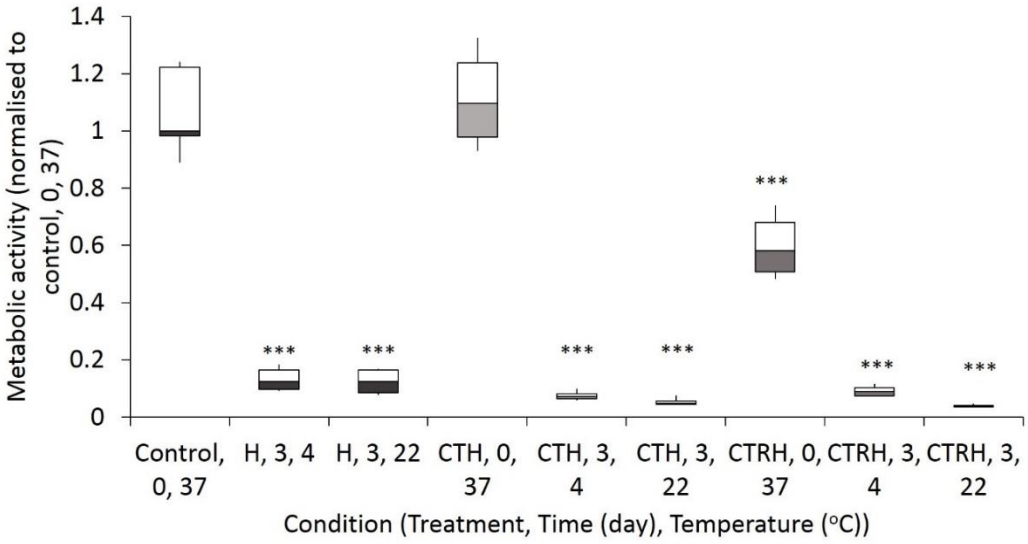

Figure 5-5 Alamar blue reduction of hypothermic preserved T cells in combination with pre-conditioning by cryopreservation. Alamar blue was interpreted as an indicator of metabolic activity of T cells after pre-conditioning cells with cryopreservation and then completing 3 days of hypothermia at two temperatures. Refer to Figure 5-1 for key to X axis labels. Kruskal-Wallis testing followed by pairwise Mann-Whitney tests were used for statistical analysis. See Section 3.2.10 for the explanation of statistical analysis and consequent labelling. N = 6.

The results presented in this study question whether human cells retain original properties after the cryopreservation process when using established protocols of a 10% ME_2SO cryo-protectant in high serum medium. Viability was recovered to high levels, but there were changes in intracellular ATP and a reduction of metabolic activity after cryopreservation, which suggested there were some biochemical changes to the cell.

Pre-conditioning of T cells by cryopreservation avoided cell death of T cells during subsequent hypothermia, and was shown to cause a change to intracellular ATP concentrations, and a consequent decrease in cell metabolic activity after recovery, along with an increase in cells in the resting G1 phase of the cell cycle. Pre-conditioning did not stop the loss of cell activity experienced during hypothermic preservation. The recovery step was shown to provide the cells with a chance to respond to the lack of ATP, which

manifested as decreased metabolism before the application of hypothermia. This change in metabolism confirms a role of AMPK signalling, rather than HSPs that increase metabolic activity [282]. AMPK signalling is known to regulate glucose metabolism and cell growth [283,284], and the activation of this signalling causes inhibition of proliferation by cell cycle arrest and prevents apoptosis [285]. Chai et al. showed that AMPK activators reduced cold-induced damage in a rat liver model through the reduction of LDH release [286]. Therefore, the activation of the AMPK signalling by cryopreservation, shown by the results of Sections 5.3.2 through to this current Section, would explain the avoidance of T cell death during hypothermia shown in Section 5.3.1.

However, despite this preconditioning of cell metabolism before the application of hypothermia, there was a large drop in Alamar blue reduction observed in Figure 5-5 throughout all conditions of hypothermia. This suggested that there was an importance to managing cell activity that goes beyond the administration of cold-shock pre-conditioning that cryopreservation was shown to supply. The pre-conditioning step was only capable of mitigating the necrotic cell death that occurred during hypothermia.

It was shown in Section 5.3.2 that the pre-conditioned, recovered cells were progressing through the mitotic cell cycle, and even unconditioned cells displayed changes to the location in the mitotic cycle. Since the aim of this thesis was to identify methods to preserve T cells, this would imply that a sufficient quantity of cells for a therapeutic dose has already been achieved. Therefore, progression through the cell cycle is no longer required to produce a higher quantity of cells. Synchronising cells to the G1 phase limited the quantity of DNA to that of G1 phase cells, which should limit the amount of DNA synthesis precursors required in the cell and reduce the activity of the cell to produce these components.

5.3.5 Synchronised cells and its effect on membrane integrity.

The previous sections of this study suggested that T cells could change their position in the cell cycle during application of hypothermia. This was not desirable because the number of

cells for a dose had been previously achieved by in-vitro culture. Therefore, methods to limit progression through the cell cycle were investigated in the following section. The effect of pre-treating on viable cell count (VCC) after hypothermic preservation was investigated to understand if it alleviated T cell death.

Pre-treatment by thymidine block did not significantly reduce the VCC when performing the omnibus testing across the data points presented, see Figure 5-6. The thymidine block displayed a reduction in normalised median viable cell count to 0.97. The slightly decreased membrane integrity produced by thymidine treatment may be results of a cytotoxic effect of prolonged thymidine exposure. This could be explained by the imbalance in nucleotide pools, which is the known mechanism for thymidine inhibition of DNA synthesis [287]. Cell division from unbalanced nucleotide pools is known to cause DNA damage and P53 activation [288]. This division could have occurred during the second step of the synchronisation protocol, when cells were cultured in the absence of thymidine before the second application of the compound. The synchronisation protocol exhibited no great effect on VCC on day 0 comparisons, so the effect of the technique was examined with cells exposed to hypothermia, shown in Figure 5-6.

The synchronisation protocol had a varying effect, both in magnitude and positivity, on VCC after hypothermic exposure. After preservation at 4^{o}C, the membrane integrity from synchronisation using a thymidine block was minimally increased. There was a large decrease in VCC of cells preserved by direct transfer from culture to 22^{o}C, see Figure 5-6. In optimised preservation medium mUW, the difference in post-preservation VCC between the two levels of hypothermia was noticeable and significant, with normalised medians of 0.57 and 0.43 for 4^{o}C and 22^{o}C, respectively, with *P*-value = 0.0012. The effect of the cell cycle synchronisation protocol at 22^{o}C showed significant improvement over hypothermia alone. The synchronisation effect was much stronger and positive at 22^{o}C on VCC. It is

suggested that implementing a cell synchronisation protocol before preservation makes moderate hypothermia a possible strategy for preservation.

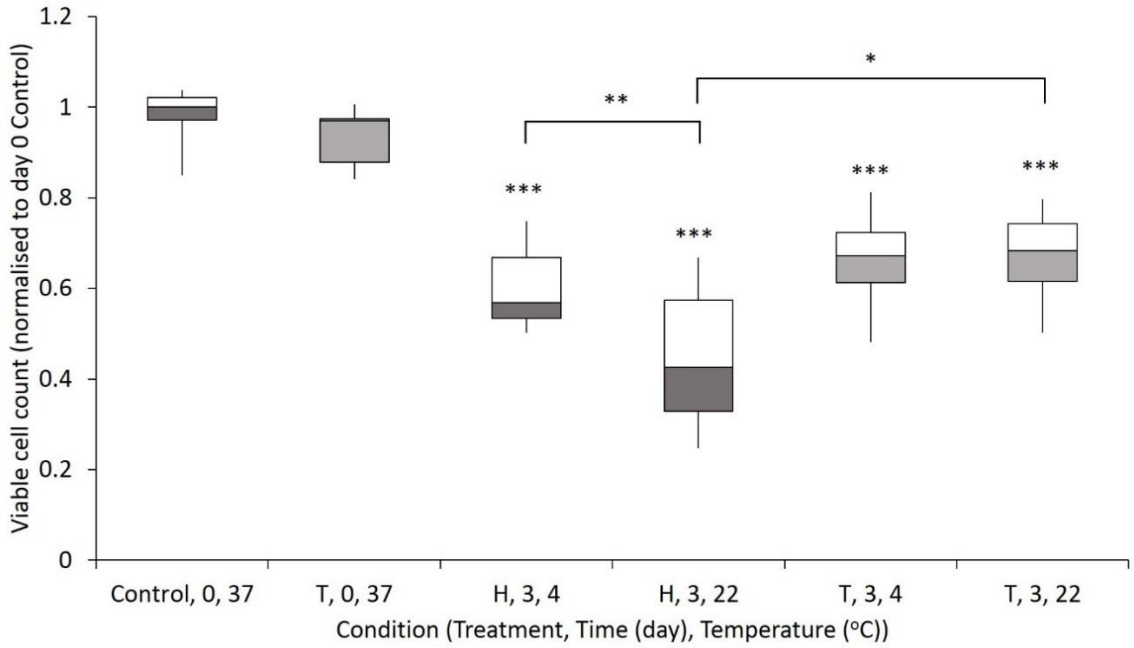

Figure 5-6 Viable cell count after in-vitro pre-treatment and post hypothermic preservation. Pre-treatment was by thymidine block to synchronise cells to the G1 phase. Viable cell count was calculated by total cell number, membrane integrity assessed by exclusion of Draq 7 stain, and total viable cell number in control samples. Indicated on the figure is that all viabilities with hypothermia were significantly lower than day 0 viabilities, with the largest P-value of the analysis, shown by indicators without brackets. Kruskal-Wallis omnibus testing followed by pairwise Mann-Whitney tests were used due to non-normal data. Refer to Figure 5-1 for key to X axis labels. See Section 3.2.10 for the explanation of statistical analysis and consequent labelling. N = 6.

These viable cell count results suggested that the application of cell synchronisation methods did not have a great effect on the membrane integrity of T cells. At 4ºC hypothermia, the synchronisation did not greatly affect viable cell counts, whereas, at 22ºC the effect was significant. This could suggest that the proportion of the untreated cells that lost viability during hypothermia at 22ºC was related to movement through the cell cycle. Interestingly, once mitosis was blocked, the amount of cell death was the same between 4 ºC and 22 ºC, which could suggest that the remaining stress causing the cell death at the two temperatures could be the same. There were small differences in VCC between 4 ºC and 22 ºC observed in the results of Figure 5-6. This was similar to the results of Section 5.3.1, and the reasoning is discussed in that Section.

5.3.6 Thymidine block reduces metabolism of T cells, and subsequently increases metabolic activity on recovery after hypothermic preservation.

This study hypothesised that inhibiting cell cycle would affect ATP consumption, therefore, metabolic activity and energy reserves in relation to inhibiting cell cycle progression were investigated. Neither of these measures were adequately maintained by pre-conditioning the cells with cryopreservation. Metabolic activity was assessed by the ability of preserved cells to reduce Alamar blue, and the results are presented below.

Direct transfer from in-vitro cell culture to hypothermia for preservation was not successful in maintaining the metabolic activity of the T cells when recovered to 37^{o}C after preservation. The decrease in metabolic activity was significant and both grades of hypothermia were around 10-fold lower compared to the day 0 metabolic activity, see Figure 5-7. A small, non-zero amount of cell activity remained above a negative control for both grades of hypothermia. Therefore, some of the preserved cell population may be recoverable after hypothermic preservation because some metabolic capacity remained.

Application of the thymidine block reduced T cell metabolic activity after completing the protocol and before the application of hypothermia, which was significant compared to the day 0 post culture samples. The metabolic activity of cells treated in this way displayed a reduction of median value to 0.70 that of the untreated cells. The effect of applying the synchronisation protocol was beyond bringing cells to the G1 phase, see Figure 5-3. The results presented in Figure 5-7 demonstrated that cell cycle synchronisation also reduced the metabolic activity when the cell population was almost completely in the resting phase of the cycle. Synchronisation could have benefits to hypothermic preservation beyond maintaining membrane integrity.

Reducing the metabolism of T cells before attempting preservation led to an increase in metabolism of cells after preservation when recovering to in-vitro conditions. This increased metabolic activity was greatest at profound hypothermia, 4^{o}C. At this low temperature, the

thymidine treated cells showed a 5.3-fold increase in median activity after completing preservation, compared to untreated cells transferred straight from culture to hypothermia. The metabolic activity was still decreased over the control day 0 values. The effect of synchronisation on maintaining metabolic activity was not as great at a hypothermic temperature of 22ºC. After 22 ºC preservation, thymidine treated cells presented a 1.5-fold increase over the untreated control. Restricting DNA synthesis and 22 ºC preservation did not allow recovery of cell activity after hypothermia.

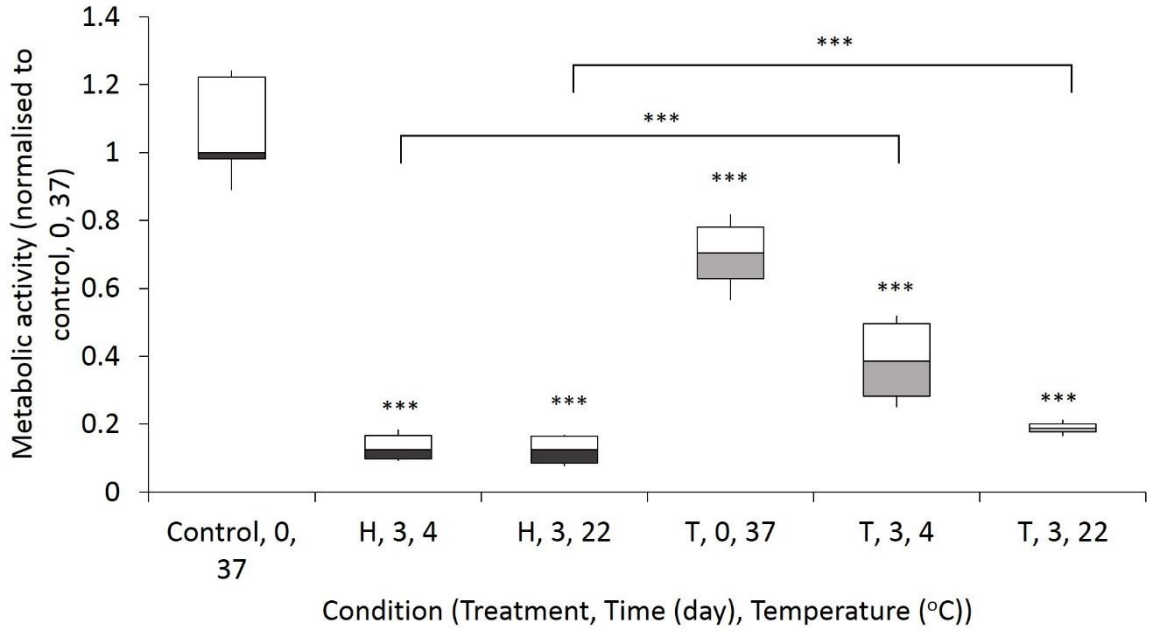

Figure 5-7 Alamar blue reduction of hypothermic preserved T cells that were pre-treated to synchronise cells to the G1 phase of the cell cycle. Alamar blue was interpreted as an indicator of metabolic activity of T cells. In the T condition T cells were pre-treated with a thymidine block and then completing 3 days of exposure to hypothermia at two temperatures. Refer to Figure 5-1 for key to X axis labels. Kruskal-Wallis tests followed by pairwise Mann-Whitney tests were used for statistical analysis. See Section 3.2.10 for the explanation of statistical analysis and consequent labelling. N=6.

By synchronising cells to the G1 phase of the cell cycle, metabolic activity was decreased before commencing hypothermia, but these treated cells could have increased metabolic activity on return to normal conditions after completing preservation when compared to untreated cell populations. It is suggested that hypothermia treatment alone was insufficient for preserving the T cells. Extra measures must be taken to pause T cells before attempting

hypothermic preservation. Further interventions to limit processes on top of limiting DNA synthesis will be required to improve preservation at 22^{o}C.

For cell types other than T cells there are alternative methods to increase cell activity after preservation. Mathew et al. showed that a specialised medium for hypothermic preservation with water soluble vitamin E and caspase inhibitors in an appropriate vehicle increased the amount of Alamar blue reduction of a range of other cells after completing 5 days of hypothermic preservation at 4 ^{o}C[21,289]. Those compounds led to further increased activity after continuing in-vitro culture of these cells, but those studies lack information about how well the renal, muscle, endothelial and hepatic cells regain function after recovery. Vogelaar et al. showed that another variant of antioxidant, a novel chromanol compound, increased activity of stem cells after 1 hour exposure before starting hypothermic preservation, which led to increased viability after 48 hours of refrigerated 4 ^{o}C preservation [202]. These additive components appear to have a biochemical effect and would be present at the time of infusion, hence would represent an additional action of the therapy in combination with the cells, which might require extra checks on complete formulation functionality. Wheeler et al. showed that supplementation of glucose and fructose increased cell activity and ATP production during short term hypothermia, but this activity reduced back to similar levels of un-supplemented conditions at 48 hours of 4^{o}C hypothermia [180]. In that study, the eventual decrease of activity suggested that supplementing cells to maintain activity did not produce lasting preservative effects. Both these stimulating components and metabolites were tested in Chapter 4 and did not have such a profound effect on T cell hypothermic preservation. Instead, results in Figure 5-7 showed that T cell metabolic activity after recovery of cells from preservation was greatly increased by blocking mitosis through cell cycle synchronisation before commencing hypothermic preservation. The synchronisation lasted through hypothermia without the presence of the inhibiting compound and was shown to result in T cells with more metabolic activity after 3 days of hypothermia. This provides

evidence that blocking T cell activity was a more effective method for prolonging hypothermic preservation compared to supplementing cell metabolism. A future study might directly compare strategies of restriction and supplementation using the same biological model and the same medium throughout, which could affect cell function, to establish which is the best method for extending preservation beyond 3 days.

5.3.7 Inhibiting DNA synthesis before hypothermia increases intracellular ATP concentration after Hypothermia.

ATP represents the energy reserves of the cell. Therefore, if cells are successfully preserved, they should retain initial ATP concentrations to avoid changes in cell function on return to normothermic conditions, whether in-vitro or in-vivo. Initial ATP concentrations were not maintained by pre-conditioning activated T cells with cryopreservation, although the recovered cells with lower initial metabolic activity did retain slightly more ATP. Furthermore, inhibiting DNA synthesis greatly improved metabolic activity on recovery. To continue investigating the effect of this inhibition, intracellular ATP concentration of the synchronised cells was investigated in conjunction with hypothermia.

Direct transfer from culture to both grades of hypothermia (4 ^{o}C or 22^{o}C) allowed untreated cells to consume most of the ATP present before starting preservation. The decrease in detected normalised average ATP was to 0.06 ± 0.04 and 0.04 ± 0.03 of the initial intracellular concentration available before preservation for 4^{o}C and 22^{o}C, respectively. This suggested that those cell processes that consume ATP continued during preservation, and the cells were incapable of producing a new supply in the conditions tested, as shown by the low reduction of Alamar blue of the untreated controls, see Figure 5-6.

Synchronising cells to the G1 phase increased the amount of ATP detected per cell before commencing hypothermic preservation. This synchronisation continued to greatly increased ATP concentrations detected after hypothermic preservation, see Figure 5-8. Thymidine treatment resulted in a significant 1.5-fold increase in detected ATP over the control. The

main result presented in Figure 5-8 was the greatly increased intracellular ATP after hypothermic preservation that resulted from thymidine treated cells preserved under 4ºC hypothermia. Thymidine treated cells at 4ºC, T, 3, 4 maintained an intracellular concentration of ATP that was insignificantly different compared to cells before any preservation, Control, 0, 37. The T, 3, 4 cells had 8.6-fold higher ATP compared to the H, 3, 4 cells that were untreated when exposed to hypothermia, see Figure 5-8. The same large increase was not found after preservation at 22ºC with the addition of thymidine treatment. At 22ºC, ATP was significantly reduced compared to day 0 cells and only a 3.5-fold increase was found over untreated controls for this temperature.

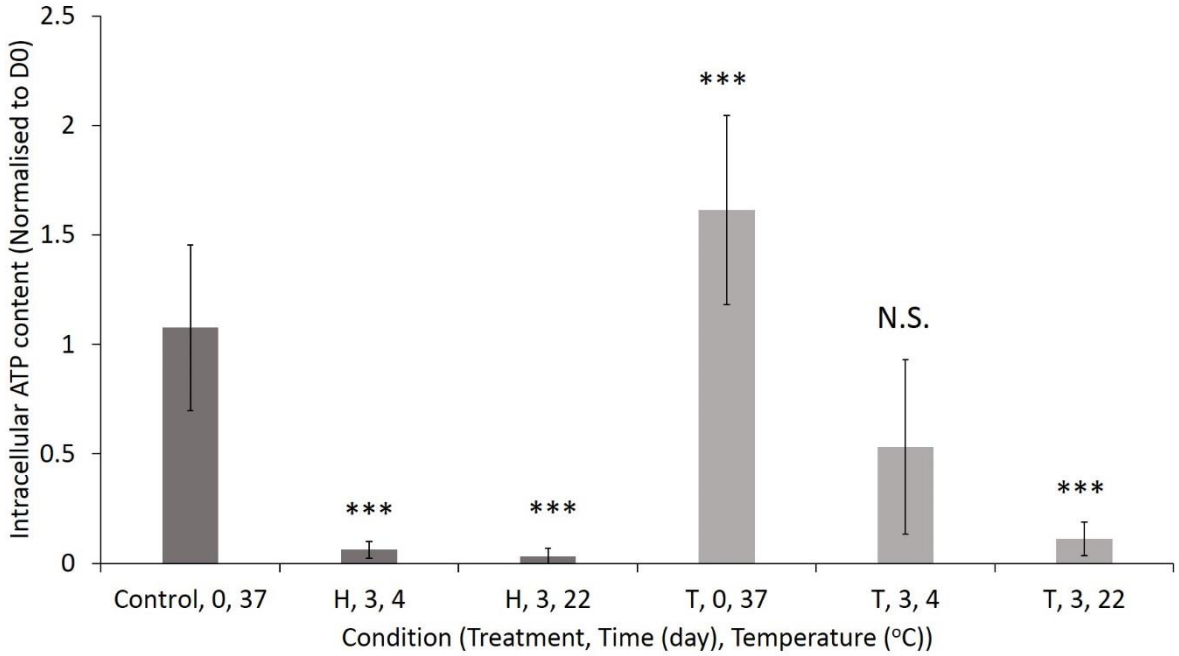

Figure 5-8 Intracellular ATP content of T cells after pre-treating T cells with a thymidine block and then completing 3 days of exposure to hypothermia at two temperatures. ATP content was measured by luciferase assay. Refer to Figure 5-1 for key to X axis labels. Results are presented normalised to the ATP content of the control on day 0 at 37ºC. N.S. = not significant compared to day 0 concentration. A $\log_{10}$ transform satisfied assumptions for ANOVA testing. See Section 3.2.10 for the explanation of statistical analysis and consequent labelling. N=6.

The decrease in intracellular ATP measured after hypothermic preservation irrespective of treatment, shown in Figure 5-4 and Figure 5-8, agreed with what is generally known about hypothermia. For example, Fremes et al. have also reported that, when isolated myocardial cells were preserved in hypothermic conditions, ATP was found to be decreased [290].

Furthermore, Bagul et al. showed that rabbit renal tubules had a reduced energy state after 48 hour of refrigerated hypothermia and required time to recover with normothermic re-perfusion [291]. Additional recovery would be difficult to achieve with a CGT at a clinic. The application of a thymidine block as a pre-treatment before commencing preservation may be a readily implementable method to increase cell metabolism and ATP content of the cells over control levels after completing exposure to hypothermia. Pre-pausing the T cells to prevent a significant loss of ATP may avoid further upregulation of the AMPK signalling discussed previously, which subsequently avoids a decline in metabolic activity on recovery from hypothermic preservation. Pre-treatment did not greatly improve the cells' membrane integrity.

The strategy investigated in Sections 5.3.5 to this current Section of limiting cell activity by inhibiting the cell mitosis cycle led to an increase in cell metabolic activity after completing preservation of activated T cells. Limiting cell activity is suggested to be a necessary extra pausing-step for hypothermic preservation of T cells. However, preconditioning the T cells with a cold-stress was required to induce a low metabolic state to avoid cold-shock related injury. A combination of pre-conditioning and pre-treatment to pause cells could provide a population of T cells that functions effectively after short-term preservation.

5.3.8 A combination of pre-conditioning and pre-treatment delivers T cells that retain the intended therapeutic action.

The peripheral blood mononuclear cells (PBMC) were cultured in-vitro to produce a cell population known as cytokine induced killer (CIK) cells. This culture should produce a cell therapy product that displays some cytotoxicity to leukemic cell lines [16]. If hypothermic cell preservation was to be successful, and ultimately a useful tool, then preserved cells should display the same intended therapeutic effect before and after the preservation step. Tripmacher et al. have reported that impaired oxidative phosphorylation and inhibited glycolysis reduced the ATP content of the T cell but these cells did maintain some

immunological responses [280]. In Sections 5.3.1 to 5.3.7, progress was made on methods to avoid changes in the ATP and metabolic activity of T cells as a result of preservation. However, this investigation has not yet examined whether cells retain their intended function upon recovery. The following section evaluates if preserved T cells maintain the same original phenotype.

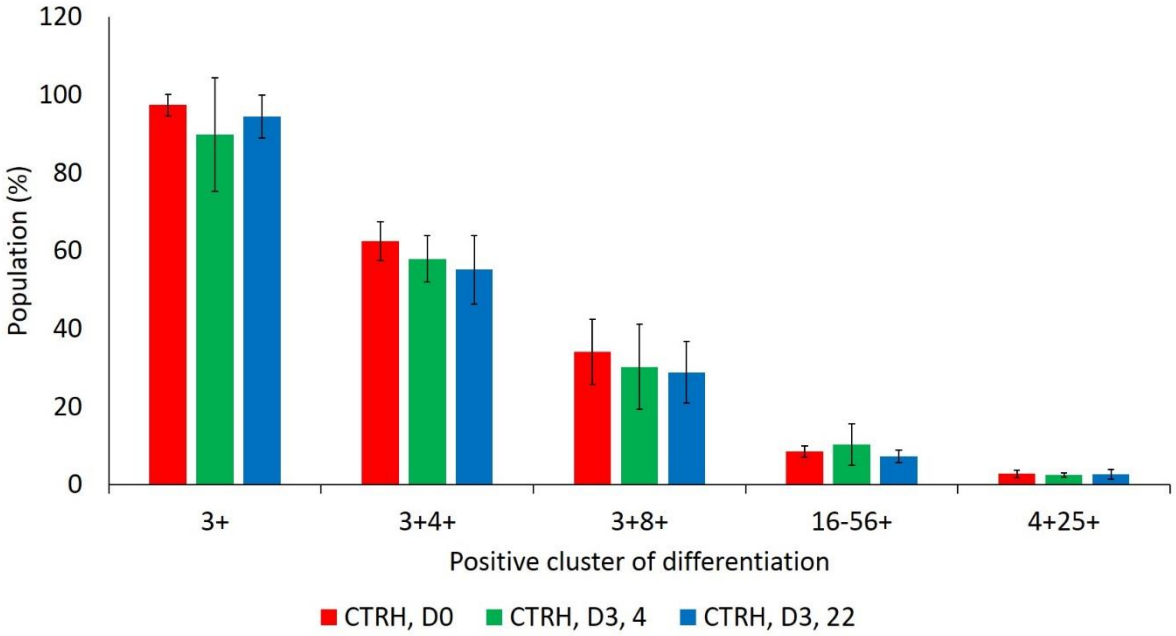

Figure 5-9 Phenotype of T cells pre-conditioned with cryopreservation. Graph shows population before and after three days of exposure to hypothermia at two difference temperatures. Samples were loaded with fluorochrome loaded antibodies for each CD. Data was acquired by flow cytometry and then assessed in Flowing software 2. Refer to Figure 5-1 for key to category labels. Statistical analysis was performed by Mann-Whitney pairwise tests compared to the control on day 0 before preservation for individual combinations of CDs examined. No significant differences were found. See Section 3.2.10 for the explanation of statistical analysis and consequent labelling. N=3.

The first parameter of cell functionality evaluated was that of the phenotype of the T cell population. Almost no change was reported between the surface proteins examined in Figure 5-9. There was a small decline in T helper cell population, CD3+4+, from 62.5% of all cells to 57.9% for cells preserved at 4°C, and 55.14% for 22°C, but this was not significant. T cytotoxic cells, CD3+8+, also demonstrated a similarly insignificant decrease. These small losses match up well with the small decrease in viability of pre-conditioned cells in Figure 5-2. The CIK cell population, CD16-56+, started off at a lower percentage of total population but remained stable. The small changes that occurred during the 3 days of preservation could

suggest that a possible susceptibility of differentiated T cells to hypothermia cannot be overlooked and could prove a challenge if the preservation duration was extended. The stability of CD4+ and CD8+ T cells could become an issue during hypothermic preservation longer than three days if the slight decline shown here continues.

Application of the thymidine block did not induce or increase the amount of apoptosis detected above untreated controls after subjecting the cells to preservation with hypothermia. After applying a thymidine block, apoptosis detected by phosphatidylserine exposure remained low at an average of 8.1% ± 4.0% of the cell population, see Figure 5-10. After hypothermia, there was an increase in apoptosis to 17.2% ± 2.2% for cells treated with a thymidine block and 4°C hypothermia, and to 38.0% ± 3.4% for 22°C also with a thymidine block. This small increase in apoptosis was shared with untreated cells. Two-way ANOVA between the CTRH and T conditions on day 3 showed that application of the thymidine block reduced apoptosis that occurred during preservation, see Figure 5-10. However, the incidence at 22°C remained significantly higher. Much like the drift in phenotype, the small increase in apoptosis could be an issue for cells stored longer than 3 days.

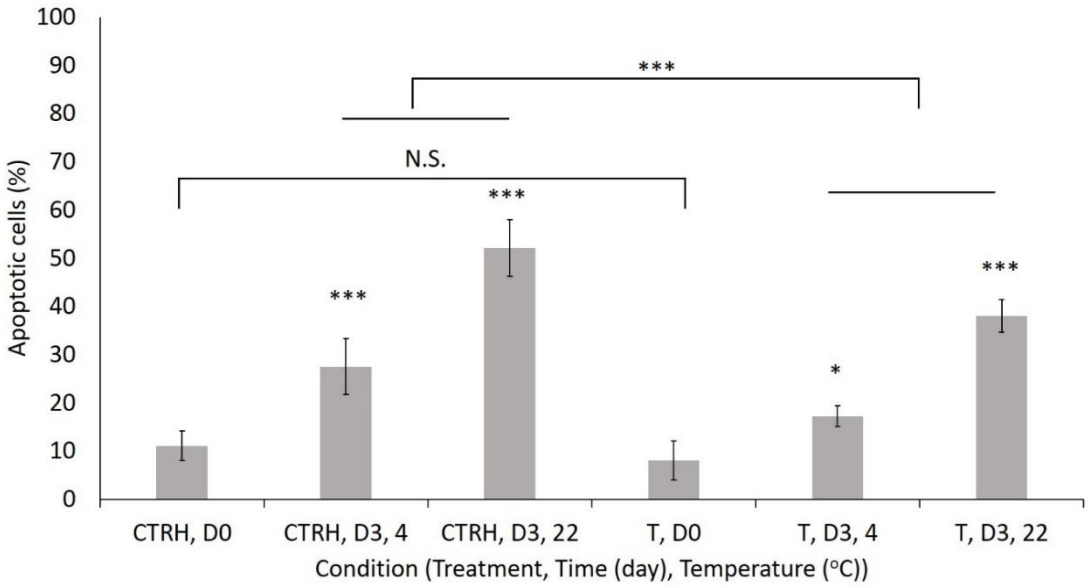

Figure 5-10 Apoptosis of T cells with pre-conditioning and pre-treatment. Y axis shows the percentage of cells that were negative for phosphatidylserine exposure. The conditions on the X axis were firstly, with pre-conditioning alone and secondly, in combination with thymidine treatment, refer to Figure 5-1 for key to category labels. See Section 3.2.10 for the explanation of statistical analysis and consequent labelling. N=6.

The current study focussed on 3 days preservation. This duration represents a 2 day logistical buffer over the 1 day prediction for shipping a cell therapy product from a remote site to clinic and administration [268]. Good results in terms of viability, metabolic activity, phenotype, and low apoptosis were shown, suggesting that hypothermic preservation of activated T cells was possible over a duration of 3 days. Luo et al. found only small changes in CD3, 4, and 8 cell phenotypes in antigen supplemented T cell cultures after thawing the cells from cryopreservation and then completing a total of 14 days cell culture [23]. They found that the introduction of cryopreservation to those T cells increased the ratio of CD3+CD4+ to CD3+CD8+ cells. A similar small increase in the CD4:CD8 ratio of T cells after cryopreservation was reported in Figure 5-10. Following the application of hypothermia presented in Figure 5-10, the results suggest that the CD 4 and 8 phenotypes were relatively stable under hypothermic preservation conditions with the application of cryopreservation as a pre-conditioning event to hypothermia. Figure 5-2 showed an average of only 10% CD16-56+ cells at 21 days total culture, which was different to the high amount of the same set produced by methods of Bonano et al. Those methods achieved a median of 64% CIK cells of the whole population produced from a continuous 21 day culture [16]. This discrepancy in frequency could be a consequence of including cryopreservation in the processing. Liang et al. reported that PBMCs cryopreserved after isolation and then cultured to generate CIK cells produced a population that contained about 27.3% CD3+CD56+ of the whole population of cells [292]. This low value matches with the lower value shown in Figure 5-2. Therefore, it is suggested that the CD3+CD56+ phenotype does not survive cryopreservation, hence other methods of pre-conditioning T cells to hypothermia are required. However, Sections 5.3.1 to 5.3.4 showed that pre-conditioning is vital to maintain membrane integrity during hypothermic preservation. Overall, it appears that excessive processing can affect the phenotype of T cells intended for therapeutic purposes. Hypothermia provides some stability to phenotype over short durations. However, a slight

decrease in CD4+ and CD8+ phenotypes and slight increases in apoptosis may become a challenge if T cells are preserved for longer. Preservation times beyond 3 days would require further study, which is investigated in Chapter 7.

5.3.9 T cells with metabolic activity after preservation are required to retain cytotoxic activity against leukemic cells.

Pre-conditioning to cold-shock by cryopreservation meant that a high cell viability was retained after hypothermic preservation. However, these cells lost a great deal of cytotoxicity against the target cell line. This population of unsynchronised cells suffered a significant loss of ability to induce necrosis in the target cell line, with cells preserved at 4^{o}C displaying only an average of 7.4% of the initial day 0 cytotoxicity, and an even lower 4.4% of the initial activity for cells preserved at 22^{o}C, see Figure 5-11 for results. The membrane integrity was high in these cell populations, see Figure 5-2, and these cells did not display the largest fall in ATP, see Figure 5-4, but they did display a significant loss of metabolic activity upon recovery to in-vitro conditions, see Figure 5-5. Therefore, it is reasoned that if cells lose metabolic activity upon recovery, then they also lose high order functions such as recognition of target cell lines and cytotoxic capabilities.

Thymidine block was shown to produce a cell population that retained a larger amount of the initial proliferative activity after hypothermic preservation, see Figure 5-7. The more active cells from this paused population should produce cells with a much higher cytotoxic activity upon recovery. Higher cytotoxic activity was verified for this condition by the results in Figure 5-11. After 4^{o}C preservation, the pre-treated cells produced a cytotoxic effect that killed an average 87.3% of the quantity of cells killed by control cultures before hypothermic preservation. This decrease in cytotoxicity showed no difference at all with a *P*-value of 0.9623. Cells preserved in a similar way at 22^{o}C displayed a larger drop that was significant compared to the day 0 activity. However, the drop was not as great as compared to the loss of activity in the untreated condition at 22^{o}C. This showed that pausing cell mitosis before

hypothermia resulted in better preservation at 22 ^{o}C because some cytotoxic activity was retained. Therefore, to maintain the full function of T cells after preservation, the cells must be paused in addition to hypothermia. This suggests that hypothermia alone does not provide an effective preservation method.

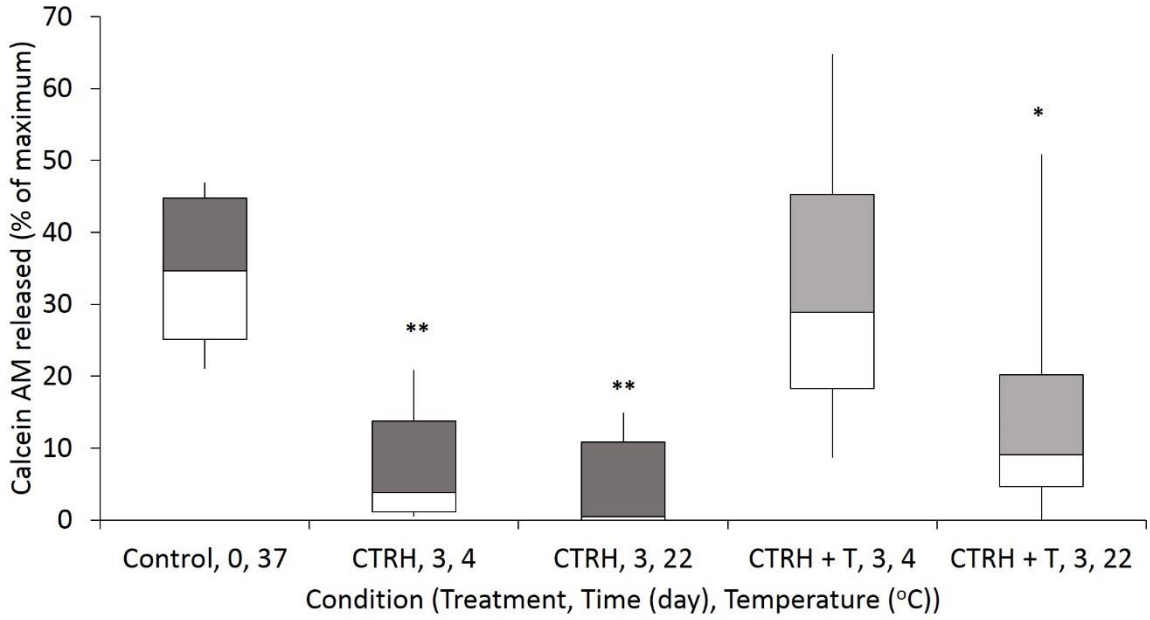

Figure 5-11 In-vitro cytotoxicity activity of T cells on calcein AM labelled Jurkat leukemic cells. calcein is released from labelled cells that are killed by necrosis as a result of interacting with T cells, however release can also be spontaneous. Y axis is the percentage of calcein released compared to a maximum produced by chemical lysis and corrected by subtraction of spontaneous release conditions. The conditions on the X axis were 1), cells before preservation, 2) with pre-conditioning alone and, 3) in combination with thymidine pre-treatment, refer to Figure 5-1 for key to category labels. Statistical analysis used Kruskal-Wallis tests followed by pairwise Mann-Whitney tests. See Section 3.2.10 for the explanation of statistical analysis and consequent labelling. N=6.

The T cell population that displayed very little metabolic activity after hypothermic preservation, see Figure 5-5, also displayed very little cytotoxicity against the Jurkat cell line, see Figure 5-11. Zeng et al. showed that when T cells were anergic with reduced immunological function, then these T cells also displayed a reduced metabolism [123]. That study also identified that T cells, which had their metabolism impaired by induced AMPK signalling, had an impaired immune response through restriction of IL-2 and IFN-y production. The results in Section 5.3.6 confirmed that hypothermic preservation decreased the metabolic activity of activated T cells, which also led to a decreased immunological function observed by a reduction in cytotoxicity against a Jurkat cell line, shown in the

results of Figure 5-11. If this state of reduced response was similar to T cell anergy, it could be possible to recover the activity of the T cells by addition of exogenous IL-2 to re-stimulate the cells [124]. However, this would require administration of an inflammatory cytokine along with the cell containing therapy product. The requirement of additional cytokine could exacerbate the cytokine release syndrome associated with adoptive transfer of T cells [2]. It was discussed earlier that T cells are able survive restricted ATP conditions. However, with extremely restricted ATP after preservation (Section 5.3.3), the untreated T cells preserved in hypothermia lost most of their therapeutic action (Figure 5-11). Hypothermia alone does not preserve T cell qualities and cells preserved in this way would require additional processing before use.

Pre-conditioned and thymidine pre-treated T cells showed no difference in cytotoxic activity between day 0 and day 3 when the cells were preserved at 4^{o}C. This result demonstrated that it was possible to preserve activated T cells with hypothermia with suitable clinical qualities retained. The 3-day duration achieved in this present Chapter would allow for transportation and short-term storage at the clinic with simple refrigeration equipment. This refrigeration would avoid the specialist cryogenic equipment required with the current transportation of T cell therapies [268]. The results of this current Chapter show that the pausing of ATP consuming biochemical reactions is required in order to achieve this. The results of this Chapter also produced no evidence to suggest that the use of cold chain (2-8^{o}C) could be replaced with moderate hypothermia (22^{o}C) because the latter had the characteristics of cells continuing with a number of biochemical reactions, depleted energy stores, and less activity on recovery to 37^{o}C. The improvement of 2-8 ^{o}C, and slight improvement to 22 ^{o}C preservation, by pre-treating the cells and pre-pausing mitosis could

suggest that, to move beyond the cold chain, more biochemical reactions rates must be reduced by interventions and hypothermia to maintain all cell function on recovery.

5.4 Summary.

This Chapter has shown that it is critical to apply pre-conditioning to maintain membrane integrity of primary, activated T cells during hypothermia. However, maintaining viability did not produce a preserved cell population that had therapeutic action. Transfer directly between the two conditions resulted in a cell population with depleted ATP and low levels of metabolic activity on recovery. There was also a great need to manage the transfer between antigen loaded in-vitro cell culture and hypothermic preservation. The effect of pre-treatment by synchronisation on the cell mitosis cycle of the T cells was significant after initial application on day 0 and maintained a stable population during the application of hypothermia. The inhibition also had significant effects on cell activity when recovering T cells from hypothermia. Inhibited, preserved T cells had improved intracellular ATP content; recovered metabolic activity more effectively; and, most importantly, therapeutic cell function was maintained. Inhibiting other ATP-consuming biochemical processes in combination with mitosis may further improve preservation where temperature is reduced below normal in-vitro conditions. A vital process to achieve hypothermic preservation of primary, human, CD3/28 activated T cells is to manipulate cells to a resting phase of the cell cycle before the application of hypothermia.

6 Cell Sedimentation and its Effect on Preservation Outcome.

6.1 Introduction.

Physical properties of the preserving medium are known to have effects on maintaining the quality of a range of cells during exposure to hypothermia. An existing study has identified gelatin to be important in maintaining viability during hypothermic preservation of hepatocytes [212]. These studies found that gelatin set to a solid hydrogel, and that inclusion of this mechanism then prevented the physical occurrence of cytoplasm outside of the hepatocyte cells. When this movement of cytoplasm occurred in the control conditions there was also a reduction cell viability [212]. The drawback of these gelatin studies was that they did not identify how well the cells recovered on return to normal conditions. In another study, encapsulation within alginate hydrogel increased the recoverability of mesenchymal stem cells after hypothermic preservation [293], which adds further evidence to the need for avoiding cell sedimentation during hypothermic storage. A further study by Yang et al. demonstrated that alginate microparticles, when compacted together with a cell suspension, mitigated sedimentation and improved the longevity of preservation over liquid controls [213]. In their study, it was reasoned that the encapsulation separated cells from each other and stopped adhesion, keeping the cell lines tested in a quiescent state. Encapsulation can improve the hypothermic preservation of a range of cell lines, but the mechanism of how the encapsulation benefits cell preservation is unclear.

Furthermore, one study on whole blood found that polymerised sucrose, Ficoll® PM70 (ficoll), physically stabilised red blood cells (RBC) during preservation through the avoidance of aggregation by mitigating electrostatic interaction [294]. The addition of ficoll was shown to increase the viscosity of the preservation media in that study. However, the increase was small and would have a minimal effect on the physical stabilisation of a cell type that does not aggregate in a similar way to RBC. Wong et al. also presented that the polysaccharide treatment improved leukocyte viability after preservation under hypothermic

conditions, but no direct mechanism was proposed to explain the improvement of the secondary target of leukocyte preservation [294]. The role of physical property changes does not fit the result of the improved preservation of the leukocytes well. This could suggest that additives to influence physical properties of the preservation media also have other effects that are not yet identified.

The main objective of this study was to investigate methods of preserving activated T cells without the use of sub-zero temperatures. Therefore, given the existing positive results of hydrogel solidification and polysaccharide addition on the preservation of different cell types discussed above, it could be beneficial to understand if altering the physical properties of the preservation medium improved the hypothermic preservation of T cells.

The first consideration was to quantify the settling velocity of T cells to understand how quickly a cell suspension may sediment out, which could then be used to understand how to reduce the rate at which cells would sediment during preservation. Secondly, additive materials were chosen in an attempt to either reduce the rate at which cells settle or to completely suspend cells during hypothermia, when compared to the effect of a control medium with no additives. The success of these methods was judged through cell viability and cell function after preservation.

The hypothesis of this study was that if non-adherent cells form a sediment in static preservation medium during storage then it could lead to negative effects on cell viability and metabolism during preservation. Cells at a relatively high cell density would collect with gravity at the base of the container holding them during preservation, which would produce localised high concentrations of the metabolic waste compounds. This waste would continue to be produced at a low rate due to the low temperatures of hypothermic exposure and may accumulate locally around the settled cells due to lower diffusion rates because of the reduced temperature. This was examined by investigating the effects that the materials added

to alter physical properties have on the metabolic activity of T cells during hypothermic preservation.

6.2 Materials and methods.

6.2.1 Quantitative assessment of settling velocity.

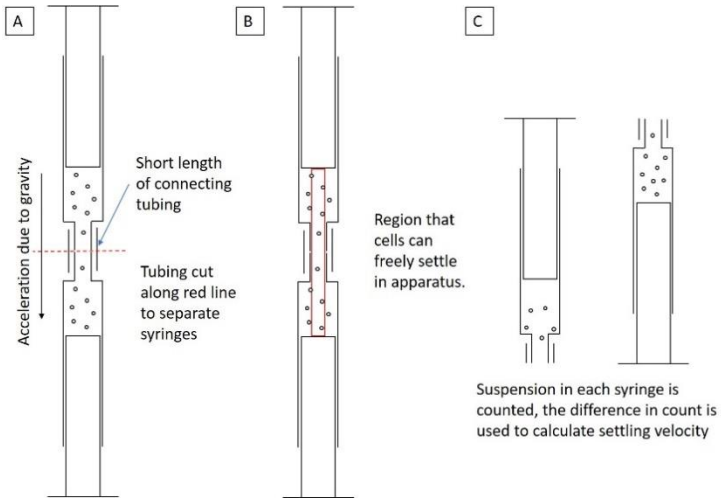

Figure 6-1 Diagrams that show the layout of components used to assess the settling velocity of cells suspended in a liquid. A) Complete structure with indications of; direction of acceleration due to gravity; placement of connecting tubing; and the place where the tubing is cut to separate syringes. C) Complete apparatus with an indication of the region in which cells can freely settle: this region was used to define the dimension used in Equation 6-1. C) Syringes after separation: in this state the cells can be re-suspended and the liquid recovered to assess the difference in cell number.

Settling velocity was measured using a method of counting cell number in two different compartments after allowing a period of time for cells to settle through the suspending liquid. Initial cell suspensions were counted by a Thermofisher Countess automated cell counter, and then either concentrated or diluted by a culture medium to obtain the final cell suspension of required concentration. After adjustment, the cell suspensions were recounted to ensure accuracy. The final cell suspensions were individually loaded into Terumo® 1ml luer slip syringes. Each loaded syringe was connected to a similar empty syringe, nozzle to nozzle, using a short section of 3.2mm internal diameter silicone tubing, then the cell suspension was evenly distributed between the two syringes. This assembly was then suspended vertically from a retort stand and left for 90 minutes for cells to settle. Following this, the two syringes were separated, the cells re-suspended in each, and the resulting

suspension re-counted as described above. The apparatus used in this assessment method is shown in Figure 6-1.

6.2.2 Ficoll and gelatin containing preservation medium preparations.

Ficoll PM70 (ficoll) was purchased from VWR and gelatin was purchased from Sigma Aldrich. Ficoll and gelatin were individually sterilised by autoclaving a measured mass of each dry material in loosely capped 50ml centrifuge tubes and then adding a fixed volume of the basal preservation medium to each tube after slight cooling. The gelatin was then dissolved by warming the tube to 70^{o}C with intermittent mixing by vortexing until there were no visible signs of gelatin crystals present. The ficoll solutions were left at room temperature with occasional vortexing. A fixed density of cells, 10x10^{6} cells/ml, was re-suspended into the liquid forms of the compounds. The gelatin was warmed to 37^{o}C to liquefy. These suspensions were transferred into multi-well plates, and sealed against moisture loss, and then exposed to hypothermia by method described in Section 3.2.1. No other method was used to set the gelatin containing medium. Preserved cells were recovered from gelatin after preservation by re-warming the gel to 37^{o}C to liquefy.

6.2.3 Previously used methods

The methods of cell culture, hypothermic preservation, high throughput cell counting and membrane integrity assay, high throughput metabolic activity assays, ATP measurement, and enzymatic measurement of glucose and lactate compounds used the same methods described in Section 3.2 and Section 4.2.

6.3 Results and discussion.

6.3.1 Characterising the sedimentation of T cells.

Wang and Belovich have previously explained that the settling velocity, v, can be calculated from measuring cell density changes in a column [295]. Initially, the cell concentration in the whole suspension, X_o, in a column is measured before sedimentation. Then a period of

time, t, is allowed for cell movement to occur. Following this time, the resulting increased concentration, X_f, of cells that collected in the lower part of said column due to gravity is measured. The column has height, h. The equation that relates settling velocity to cell numbers is shown in Equation 6-1:

$$v = \frac{h(X_f - X_0)}{tX_0}$$

Equation 6-1

This theory was originally applied by Wang and Belovich to a macroscale column that required a volume of 32ml to fill [295]. There was no term related to volume or cross sectional area in Equation 6-1, because the term cancels out when considering the mass balance of the cells settling with gravity. Therefore, a small modification of the design was made to use two 1ml syringes joined by tubing to replace the large custom-made column. The mathematics still applied when considering the column of liquid within the syringe that would allow cells to freely settle through the nozzle into the lower syringe, thus the increase in cell number measured in the lower syringe would be those cells that settled from one syringe to the other. The term, h, was the height that cells would travel though including the nozzle length and barrel of the syringe.

The use of small volume syringes significantly reduced the quantity of cells that were required, allowing settling velocity to be investigated with a lower requirement of scarce consumable equipment without introducing negative effects. Firstly, Equation 6-1 was still applicable because it was a direct measure of settling cells and not related to laminar or turbulent flows. Secondly, Terumo® syringes were used in the apparatus because they have shoulders, which connect the barrel to the nozzle, that form a surface perpendicular to acceleration due to gravity, see depiction in Figure 6-1. This means that the cell suspension above these shoulders should settle and collect on their surface, and not interfere with the results of settling through the region shown in Figure 6-1B. This collection of cells was confirmed by the formation of a visually observable sediment on the shoulders during the

experiments that produced the results shown in Figure 6-2. Thirdly, interactions due to the inner surface of the syringe nozzle would have a limited effect because the value of the denominator in Equation 6-3 was calculated to be 1.007 with the geometry shown in Figure 6-1. This was based on a T cell diameter of 7µm and a syringe nozzle diameter measured at 2.1mm. Hence, the diameter of this small-scale apparatus would avoid surface interactions when using it to assess the settling velocity of T cells. The three arguments of; direct measurement of settled cells; lack of effect from upper syringe shoulders; and very small effects from syringe walls support the reliability of the apparatus shown in Figure 6-1. Further validation based on the results collected are presented after Figure 6-2.

The results in Figure 6-2 showed that the settling velocity of T cells increased with increase in live cell density at an incubation temperature of 37ºC. Cells used throughout this dataset all had similar viabilities so this factor should not have affected the settling velocity results. The correlation between cell density and settling velocity was strong with a Spearman's rank of 0.7919, and a significant *P*-value of 0.0002. Regression fit trend lines were less accurate. An exponential fit, see Figure 6-2A, was slightly better than a linear trend, see Figure 6-2B, for the range of data points. This shows that the result of settling velocity with increasing cell density is a strong correlation rather than exact mathematical relationship.

Use of the slightly more accurate exponential trend suggested that cells could settle out completely in a liquid phase preservation medium in 107.3 minutes, with a settling velocity of 0.093mm/min. This calculation was based on the dimensions of a typical cell culture bag where the volume occupies a recommended 10mm depth, and on a cell density of 10 $x10^6$ cells per millilitre of medium, along with the assumption that viscosity of a liquid preservation medium does not differ greatly from that of cell culture medium. The calculated settling time suggested that sedimentation of non-adherent T cells could occur rapidly during preservation conditions and that interventions to mitigate a sediment layer of cells at the lower surface of a preservation container should be investigated. These results also suggested

that sedimentation may be more of a concern at higher cell densities where cell suspensions would quickly sediment in to one much higher cell density layer.

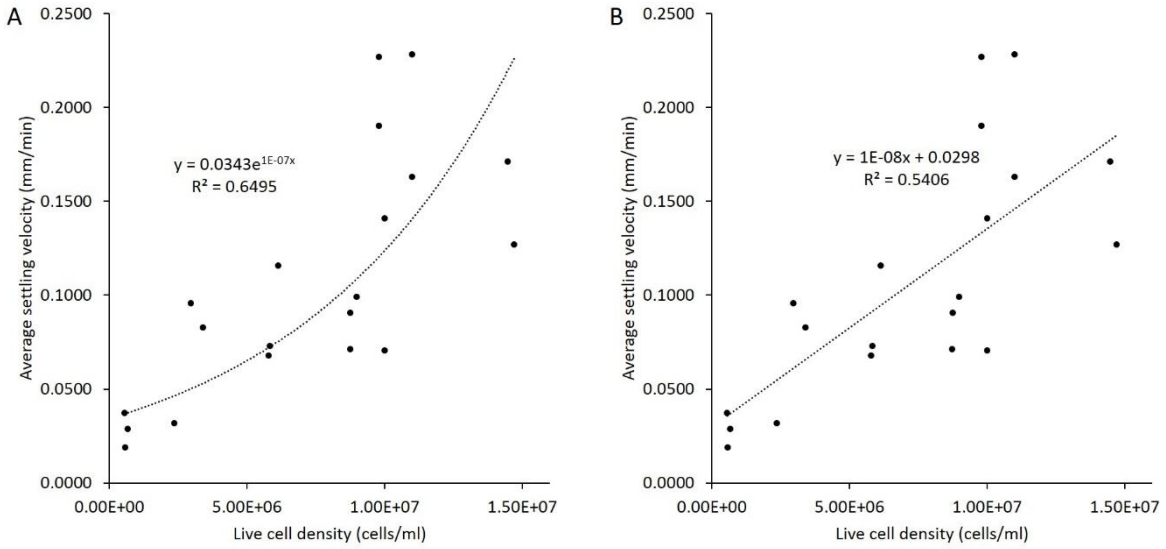

Figure 6-2 The effect of cell density in suspension on the settling velocity due to gravity. Settling velocity was measured directly through the two-syringe method and calculated by Equation 6-1. A) Data points fitted with exponential trend. B) Data points fitted with a linear trend.

To further validate the results, calculations were performed to find the Reynolds number in Equation 6-2:

$$Re = \frac{\rho u d}{\mu}$$

Equation 6-2

produced Re = 0.00133, which showed the movement to be laminar, hence Stokes' law would still be valid for calculating other properties of the medium using cells as falling sphere viscometers. Values used in calculating Reynolds number were: a viscosity of 0.94mPas [296], medium density of 1926 kg/m^3, and T cell size of 7µm. The laminar flow regime of settling cells validates that it was acceptable to use syringes with shoulders because there would be no mixing perpendicular to the flow of settling cells. Hence, the calculation of the cell-settling velocity through the specific region shown in Figure 6-2 would produce an accurate result.

There was a strong cell density effect in this study that increased the actual settling velocity, which would need to be corrected for before applying Stokes' law. This study found that

increased cell density increased the settling velocity of T cells, which shows that T cells behave in an opposite way to the correction usually applied to correct for particle density (c) in Stokes' law. The usual correction is shown within the brackets of the numerator in Equation 6-3:

$$v_{corrected} = \frac{v_{stokes}((1-c)^n)}{\left(1 + 2.1\left(\frac{d_{particle}}{d_{vessel}}\right)\right)}$$

Equation 6-3

Settling-particle diameter is represented by $d_{particle}$ and vessel diameter is represented by d_{vessel}. Since n in Equation 6-3 is always a positive integer, this suggests that increased density decreases the corrected settling velocity, whereas this study found that there was a strong correlation that suggested increased cell density increased settling velocity.

Wong et al. showed that Ficoll® PM70 (ficoll) greatly improved the preservation of whole blood, particularly the red blood cells [294]. They suggested this to be by the prevention of aggregation of this cell type and the subsequent avoidance of sedimentation of the clusters. They also showed that leukocytes in the whole blood benefitted with greater viability after preservation. Results of Figure 6-3 showed that the application of the ficoll compound reduced the settling velocity of activated T cells significantly up to a concentration of 10% w/v. This reduction had a progressively reduced rate, and only a slight overall reduction beyond 10% w/v that was not significant, see Figure 6-3. The average settling velocity of 10% (w/v) of ficoll suggested that a suspension of T cells would sediment in 136.9 minutes using the same assumptions as the previous calculation. Over the duration of 72 hours of preservation, the addition of ficoll would not greatly increase the time that cells were maintained in suspension during the application of static hypothermia.

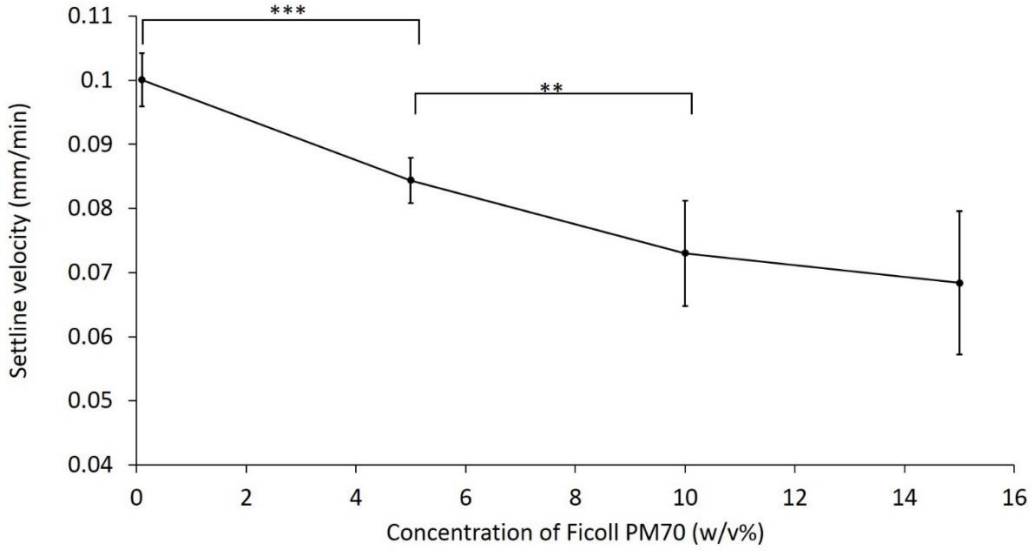

Figure 6-3 The effect of ficoll concentration on the settling velocity of activated T cells. The settling velocity was measured using the same technique used in Figure 6-2. Cell density was held at a fixed concentration of $1x10^7$ live cells/ml. See Section 3.2.10 for the explanation of statistical analysis and consequent labelling. N = 6.

With no difference between 10 and 15 wt. / vol. % of ficoll on settling velocity and cell aggregation, a 10% concentration of ficoll was considered for further investigation in combination with hypothermic preservation. Large concentrations of ficoll were calculated to produce cell sedimentation, therefore an extra condition of gelatin hydrogels was investigated to establish if complete immobilisation provided protective effects during hypothermia.

Gelatin would prevent sedimentation through solidification to a gel, but the hydrogel nature meant that the settling velocity of cells in the gel could not be investigated quantitatively as in the previous section. The images shown in Figure 6-4 suggested that gelatin immobilised the T cells throughout the height of a solidified preservation medium, with cells present at both the extremes of the height.

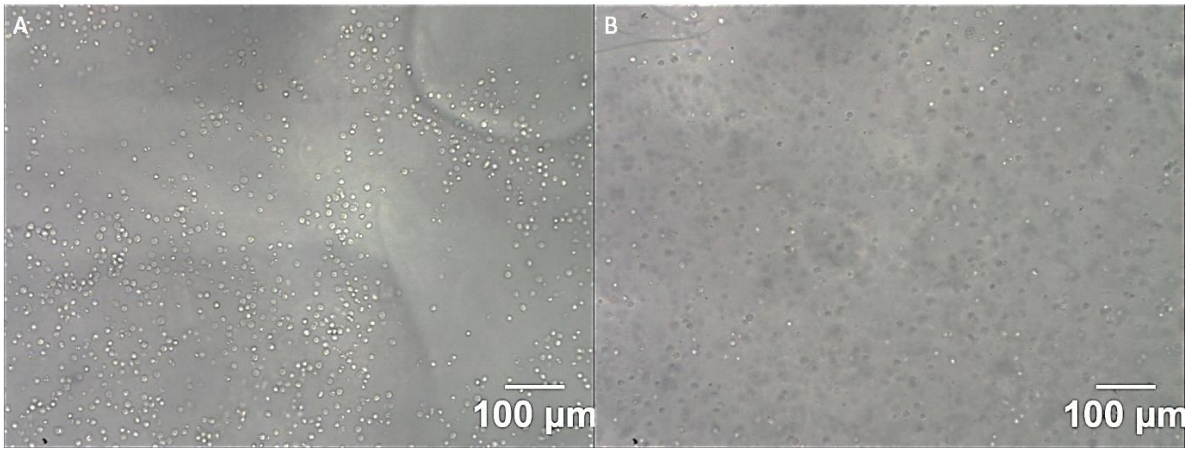

Figure 6-4 Qualitative assessment of how T cells were suspended throughout the preservation medium with 2.5% (w/v) gelatin present. A) Image captured from surface of plate. B) Image captured from the surface of the gel. Both panels shows that cells were present, indicated by the white spheres in each image.

Examination of settling velocities showed that T cells would sediment out quickly when exposed to preservation conditions. This could result in a high cell density monolayer which, although reduced temperature will reduce the metabolic activity of the cells, could result in a local environment around the cells where metabolic products accumulate due to reduced diffusion coefficients with reduced temperature. It is well established that temperature affects diffusion coefficients [297]. The settling velocity data may also be of use in bioreactor design to optimise between the need to agitate a vessel to provide metabolite exchange, against the need for a static environment that enables T cells to interact with antigens and form immunological synapses required for activation. The data generated in Figure 6-2 showed that high cell densities will quickly complete sedimentation during static preservation. It was confirmed that both the control preservation medium and the medium with ficoll added allowed the cells to sediment into a monolayer. This discussion shows ficoll does not avoid sedimentation, therefore, it could affect cell preservation through other mechanisms.

The diminishing effect of increased ficoll concentration on settling velocity shown in Figure 6-3 is suggested to be related to the osmotic effect of increased concentrations of ficoll. The product datasheet was consulted and showed an exponentially increasing osmolality through

a concentration range of 0 to 15 % (w/v) ficoll. At a concentration of 5% w/v, osmolality was approximately 2.5% mOsm/kg H_2O; at 10% it was approximately 10 mOsm/kg H_2O; and 15% it was approximately 27 mOsm/kg H_2O [298]. Increased osmolality would draw more water out of the cell and thus decrease the cell radius which, according to Stokes' law, would decrease settling velocity with a second order relationship. The change in osmolality explained why there was no difference observed between 10 and 15% (w/v) ficoll. Gelatin at the tested concentration inhibited sedimentation throughout the 3 days of preservation, which was assessed qualitatively.

6.3.2 Ficoll increases cell activity after hypothermia, gelatin does not.

Ficoll only slightly reduced cell sedimentation, results are shown in Section 6.3.1. Wang et al. showed that ficoll had a positive effect on the preservation of whole blood, reasoned to be due to physical effects [294]. Gelatin was shown to avoid cell settling in Section 6.3.1. Evans showed that encapsulation in gelatin avoided physical degradation of hepatocytes [212]. Both ficoll and gelation were tested in Section 6.3.1 but only gelatin had a noticeable physical stabilisation affect. The mechanism of action for these two materials during preservation requires further investigation. In Chapter 5, it was shown that pre-treating T cells had a profound effect on maintaining T cell activity during preservation. Therefore, similar assays were used to investigate the effect of the two compounds, initially included to affect cell sedimentation during hypothermic preservation, on cell activity during and after hypothermic preservation.

Viable cell count was investigated to examine if either of the additive materials affected the recovery of cell numbers after hypothermic preservation. After a preservation temperature of 4^{o}C was applied for 3 days, the results in Figure 6-5 showed that no difference occurred between the control medium and the gelatin- or ficoll-containing medium. There was a small

decrease in viability observed for all three components of this data set compared to the day 0 control.

Application of 22°C hypothermia in combination with additive modifications produced some effects on cell membrane integrity when compared to the day 0 control. The untreated control at 22°C had slightly lower viability than day 0 samples, which was almost the same viability as that of the control at 4°C. At a temperature of 22°C, gelatin reduced the viable cell count slightly of the recovered cells, which was shown by the increased likelihood of difference from day 0. The addition of ficoll improved preservation at 22°C with an insignificant decrease in viable cell count compared to the day 0 viability, *P*-value = 0.249.

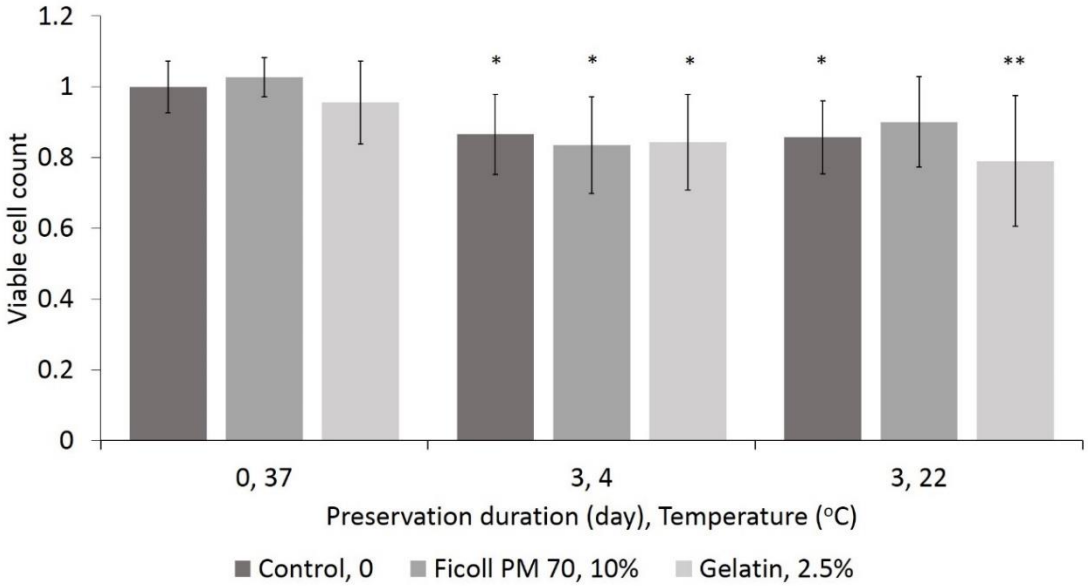

Figure 6-5 Membrane integrity of T cells with preservation in control preservation medium and medium with gelatin and ficoll additives. Hypothermic preservation was completed at two temperatures, each for a period of 3 days. Viable cell count was measured by imaging cytometry and the exclusion of Draq 7. The day 0 controls were taken directly from unpreserved cells after resuspension in to the media investigated. Results were normalised to day 0 viable cell count of control medium. After preservation, the gelatin samples were incubated at 37°C to liquefy the medium before performing the count to examine if any cells were lost in the process of recovery. See Section 3.2.10 for the explanation of statistical analysis and consequent labelling. N = 6.

Figure 6-5 showed that the addition of ficoll or gelatin to the preserving medium had a small effect on T cell membrane integrity at 22°C. The results in Figure 6-6 suggested that there was no observable difference between the metabolic activities of these cells upon recovery to 37°C after completing preservation at 22°C. There was a large drop in the activity after

preservation of the cells at 22°C irrespective of additive compound, suggesting that the compounds tested here did not improve recoverability of cell metabolism after preservation at this temperature. At 4°C, there was a significant difference amongst T cell metabolism on recovery after completing 3 days of preservation, see Figure 6-6. Cell metabolic activity remained significantly lower compared to day 0 controls for all three of tested conditions at this temperature. Differences were detected amongst the three conditions, even compared to the decline between day 0 and day 3. The main difference was that supplementation of the preservation medium with 10% (w/v) ficoll significantly increased the recovered metabolic activity compared to that of both the un-supplemented control and gelatin preservation medium. Significantly higher metabolic activity of the 4°C group remained when compared to the 22°C group examined by two-way ANOVA with post-hoc tests.

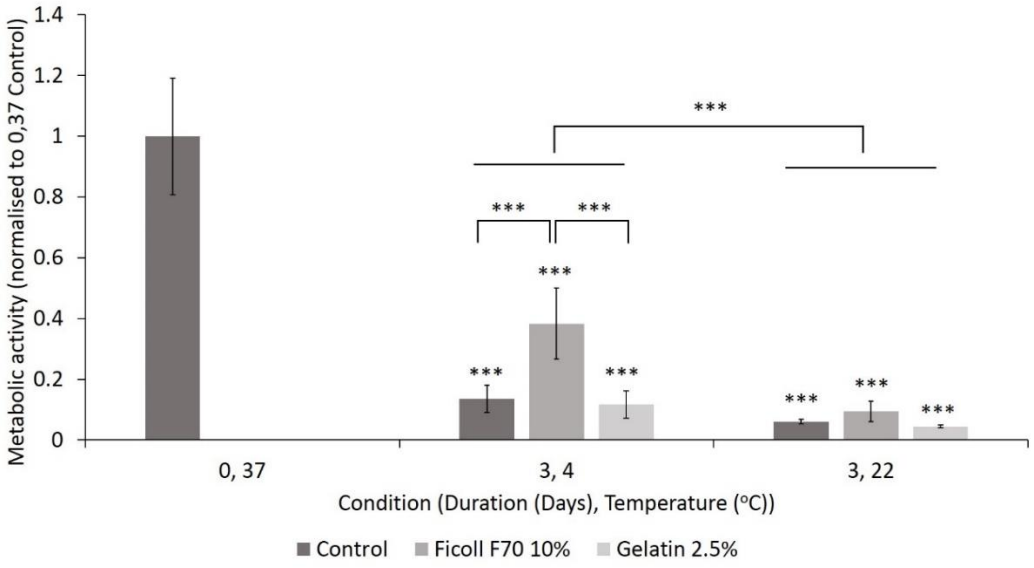

Figure 6-6 Alamar blue reduction of T cells after hypothermic preservation at 4°C and 22°C with ficoll and gelatin additives to the preservation medium. Hypothermic preservation was performed for a 3-day duration. Alamar blue reduction was interpreted as metabolic activity. Log_{10} transformations were used to validate the assumptions for ANOVA testing. See Section 3.2.10 for the explanation of statistical analysis and consequent labelling. N = 6.

The addition of either ficoll or gelatin to the preservation medium increased the amount of intracellular ATP found in T cells preserved at temperatures of both 4°C and 22 °C, the results are shown in Figure 6-7. This effect was more noticeable at 4°C where both ficoll and gelatin addition resulted in no significant difference from the quantity of ATP detected from

day 0 control samples, showing that both ficoll and gelatin maintain ATP at pre-preservation concentrations. However, at 22°C, there was a decreased effect of the additives on maintaining ATP. There was a lower likelihood of difference from the day 0 control compared to that of the un-supplemented control medium at 22°C, which was the largest significant decrease compared to day 0 results in the data shown in Figure 6-7.

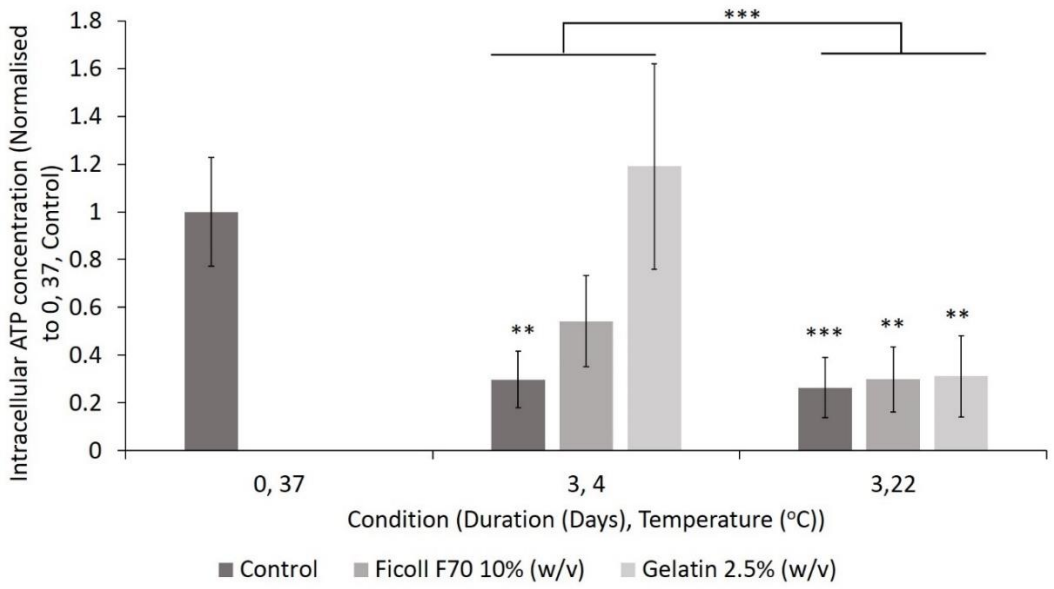

Figure 6-7 Intracellular ATP concentration of T cells after hypothermic preservation with ficoll and gelatin supplementation. Hypothermic preservation was performed over a 3-day period at two temperatures of 4°C and 22°C. ATP presence was measured by luciferase assay after extraction from the cells. ATP was normalised to the day 0 control average. The control indicates the case of no additive materials, comprised of the mUW formulation. Log_{10} transformations were used to validate the assumptions for ANOVA testing. See Section 3.2.10 for the explanation of statistical analysis and consequent labelling. N = 6.

The supplementation of the preservation medium was also suggested to increase cell metabolism during hypothermia. Supplementation of the preservation medium with ficoll was shown to significantly increase the lactate produced by the T cells during both 4°C and 22°C preservation, as shown in Figure 6-8. The increase was greatest at 22°C with much more lactate produced compared to 4°C supplementation. The addition of gelatin also slightly increased the amount of lactate produced by T cells during preservation but this held no significant difference compared to the un-supplemented control. The difference between lactate produced at each temperature was much smaller for gelatin supplementation compared to ficoll. These results suggest that the addition of ficoll to the preservation

medium was stimulating some form of cell metabolism during hypothermia because of the increased lactate production. However, the effect of gelatin on lactate production was much less, but not completely absent.

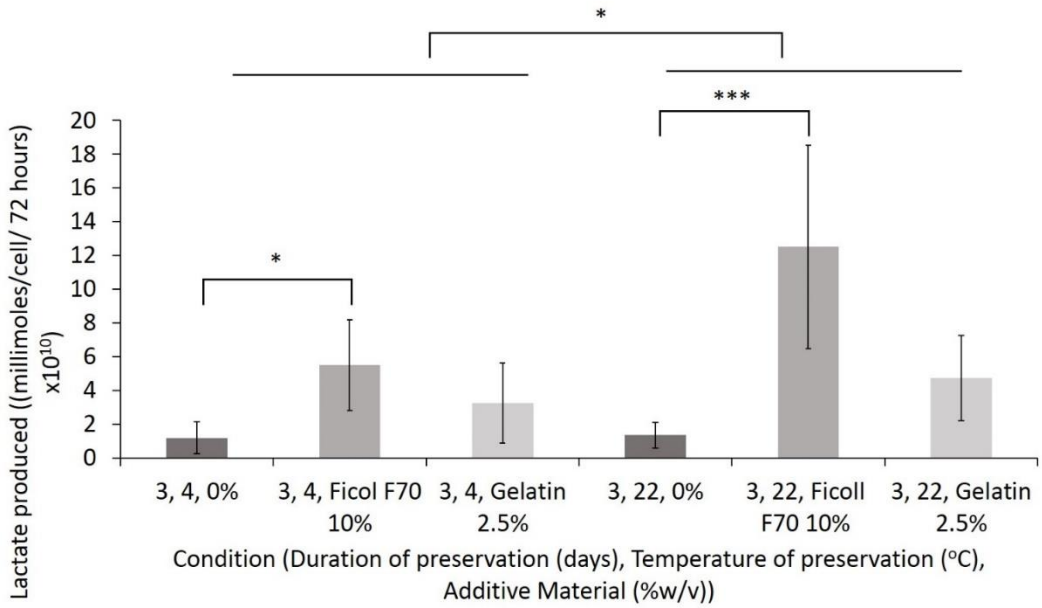

Figure 6-8 Rate of lactate production by T cells during the application of hypothermia with gelatin and ficoll supplemented preservation medium. These supplementations were compared to mUW control, indicated by the 0% additive medium condition. Two temperatures of 4°C and 22°C were assessed for hypothermic preservation. Hypothermic preservation was completed for 3 days. Lactate was measured from supernatant obtained from the preservation medium by enzymatic assay. Day 0 samples with only additives present Log_{10} transformations were used to validate the assumptions for ANOVA testing. See Section 3.2.10 for the explanation of statistical analysis and consequent labelling. N = 6.

The addition of either ficoll or gelatin did not affect the quantity of apoptotic cells after preservation at a temperature of 4°C. There was no statistical difference in apoptotic cells between these conditions after completing hypothermia for 3 days, see Figure 6-9. Three days of hypothermia at 22°C resulted in a large increase of apoptosis compared to the group after 4°C preservation. Gelatin produced no effect on the amount of apoptotic cells at 22°C, whereas ficoll reduced the amount of apoptosis slightly, as judged by the decrease in probability of difference compared to day 0. However, even with this slight reduction in apoptosis resulting from ficoll at 22°C, occurrence of apoptosis remained much higher than cells preserved at 4°C.

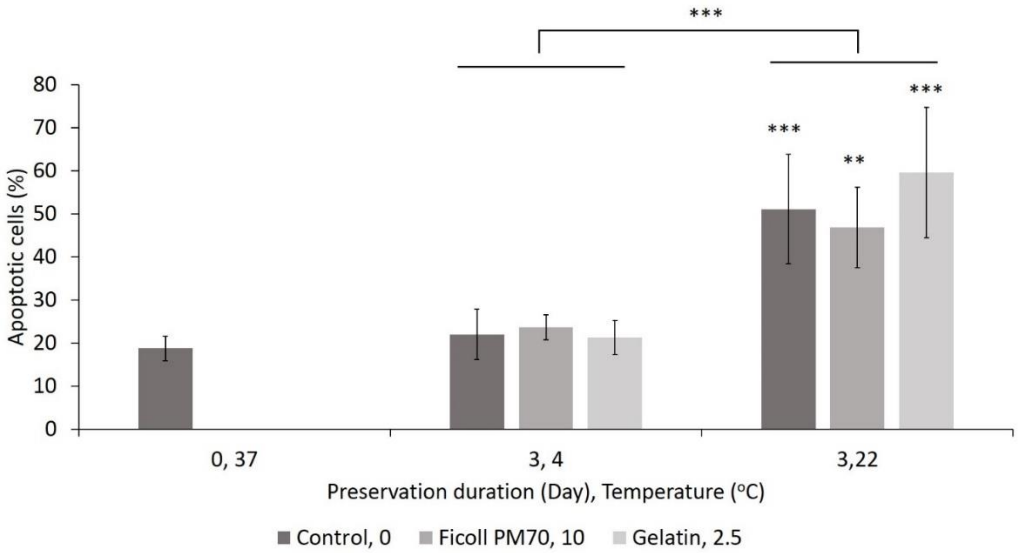

Figure 6-9 Amount of apoptotic cells after hypothermic preservation with ficoll and gelatin additives. Two temperatures of hypothermia at 4°C and 22°C were tested. Results after 3 days of hypothermic preservation were compared to a day 0 untreated control. Control indicates no additive medium. Ficoll was supplemented at 10% (w/v) and gelatin at 2.5% (w/v). Apoptosis measured by phosphatidylserine exposure on cell membrane, binding of Apopxin green, and then measurement by flow cytometry. See Section 3.2.10 for the explanation of statistical analysis and consequent labelling. N = 6.

An in-vitro cytotoxicity assay revealed that the addition of ficoll to the preservation medium increased the amount of calcein-AM released by labelled Jurkat cells as a result of co-culture with the activated T cells, and therefore cytotoxic activity, compared to control cells. Results relating to cytotoxic activity are shown in Figure 6-10. The cytotoxic activity of the T cells preserved at 4°C with ficoll added was indistinguishable from that of the day 0 activity before any treatment. Ficoll addition produced much greater activity compared to the control- and gelatin- preserved cells at 4°C. The addition of gelatin was suggested to slightly inhibit the cytotoxic activity because there was a slight reduction in the average calcein-AM released compared to the control. Overall, 4°C preservation produced significantly higher cytotoxic activity from the T cells after completing preservation compared to the application of 22°C hypothermia when all three of the compositions were considered.

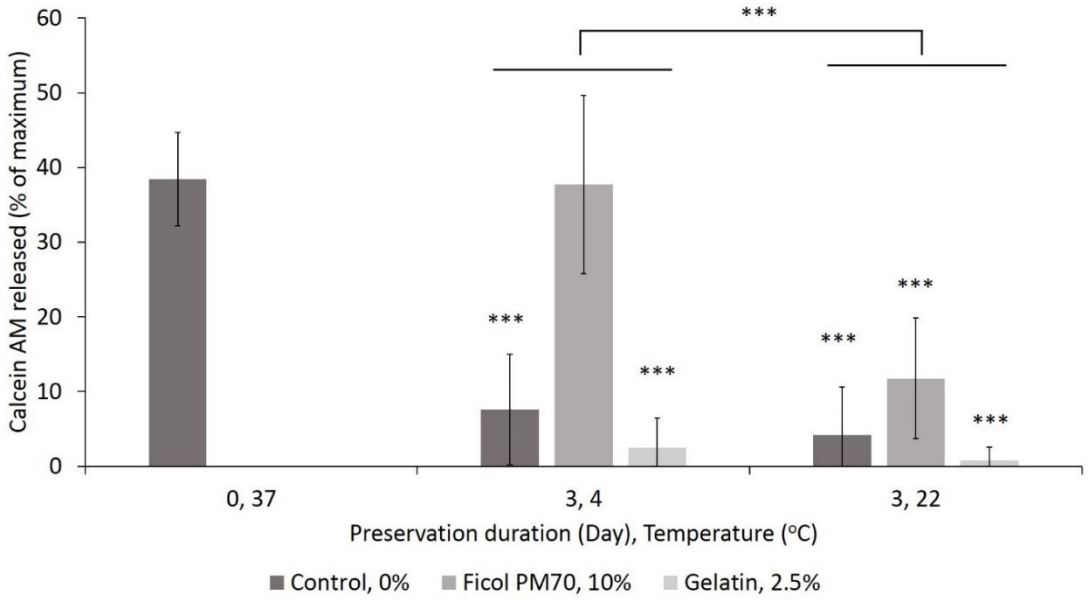

Figure 6-10 Cytotoxic activity of preserved T cells after preservation with ficoll, gelatin, or mUW liquid control. Cytotoxic activity was assessed by co-culture of T cells with calcein-am labelled Jurkat cells. The release of fluorescent calcein into the supernatant was interpreted as necrotic jurkat cell death. Spontaneous and maximum amounts released were used to correct readings to obtain percentage of maximum released. Two temperatures of hypothermia at 4°C and 22°C were tested. Preservation duration was 3 days. Ficoll was supplemented at 10% (w/v) and gelatin at 2.5% (w/v). Log_{10} transformations were sufficient to satisfy the assumptions of ANOVA testing. See Section 3.2.10 for the explanation of statistical analysis and consequent labelling. N = 6.

Two components were tested to investigate the effect of settling during hypothermic preservation. Firstly, ficoll was shown to reduce the settling velocity up to a concentration of 10% (w/v), although this was calculated to allow complete sedimentation of cells within the 3 days of preservation and did not provide much of a delay of the sedimentation compared to the un-supplemented control medium. Secondly, gelatin at 2.5wt%, which was known to form a solid gel [299], was tested to investigate if complete alleviation of settling would produce an observable effect on non-adherent cell preservation. Ficoll was suggested to support some form of metabolism, whereas complete suspension in a gelled medium can be suggested to be inhibitory on cell activity.

Ficoll greatly increased the amount of lactate present in the preservation medium after preservation compared to the control condition. This was accompanied by a slight increase in intracellular ATP concentration detected after preservation compared to the control. These two increases show that T cell metabolism was taking place because of ficoll

supplementation in the preservation medium. The continuation of metabolism was sufficient to sustain the T cells over 3 days of hypothermic preservation at 4^{o}C and led to increased metabolic activity on recovery to 37^{o}C. The increased metabolism on recovery to 37^{o}C after 4^{o}C preservation was also shown to coincide with an increased cytotoxic activity of the preserved T cells with ficoll supplementation. This showed that the stimulation provided by the ficoll component maintained the T cell mediated cytotoxicity on recovery.

Unlike the addition of antioxidant components or glucose shown in Chapter 4, the addition of ficoll was shown to increase cell metabolism sufficiently during hypothermia to sustain a higher concentration of intracellular ATP that was insignificantly reduced from day 0 concentrations. The physical stabilisation provided by ficoll was minimal. The reduced settling velocity meant that T cells would spend the majority of preservation in a sediment at the bottom of the container. Therefore, rather than physically stabilising the T cells in suspension, ficoll acts a metabolic stimulant during the exposure to hypothermia and was sufficient for the recovery of the cells after the application of hypothermia.

Ficoll significantly increased the cell metabolism during preservation at 22^{o}C, which was accompanied by a small decrease in apoptosis observed in T cells preserved under this condition, but ATP was still depleted. This suggested that the apoptosis that occurred at this temperature was partially related to metabolic activity of the T cells. The large reduction in ATP detected at 22^{o}C despite increased cell activity during the application of hypothermia could be due to the greater amounts of apoptosis that occur at 22^{o}C. Martin et al. showed that phosphatidylserine exposure was related to apoptosis [300]. Reutz and Gros showed that transport of lipids across the membrane is an active process that is ATP dependent [301]. Pradelli et al. showed that apoptosis affects intracellular ATP concentrations through impairment of glycolysis pathways by caspase activity [266]. That evidence shows that there are links between apoptosis and cell metabolism. However, the results of Figure 6-6 showed that metabolic activity stimulated by ficoll did not avoid a significant amount of apoptosis

at 22^{o}C. Therefore, the majority of apoptosis that occurs in T cells preserved at 22^{o}C is not related to maintaining cell metabolism during preservation at this temperature.

The addition of gelatin to the preservation medium allowed complete avoidance of sedimentation of the cells by gelation of the medium, compared to the reduced settling velocity produced by the addition of ficoll. The encapsulation in gelatin produced a desirable preservation property of maintaining ATP concentrations identical to the day 0 control cells. Despite the high quantities of ATP, there were only slightly increased concentrations of lactate produced by cell metabolism detected in the post-preservation medium supernatant when gelatin was added at both preservation temperatures. Therefore, the complete encapsulation of T cells in gelatin at 4^{o}C achieved preservation by a reduction in cell activity during the application of hypothermia.

Although gelatin achieved preservation, metabolic activity on recovery was compromised in a similar way as the control cells. Also, the cytotoxicity of the T cells preserved in gelatin was slightly reduced to even lower levels compared to the control cells. This was not an issue of physical recovery of the T cells, which was straightforward and evidenced by similar viable cell count compared to the control medium observed after all preservation. Therefore, the gelatin itself had an inhibitory effect on cell metabolism. The recovery included a dilution step of 1:10, suggesting that even in lower concentration and in liquid form, gelatin was inhibitory on T cell activity after preservation. It would be difficult to separate the gelatin and the cells in a clinical setting. Therefore, this study did not investigate separation of the T cells from gelatin as a method to improve recovery of T cell activity. When gelatin remained in the medium with the T cells after preservation there was continued inhibition of the cell activity that was observed during preservation.

Evans' study showed that gelation of the preservation medium was required for the successful hypothermic preservation of hepatocytes [212]. In that study, a 1.5% gelatin in

Lebovitz L-15 medium stabilised the membrane integrity of the cells and prevented "haloing", a mechanism identified in the study of hepatocytes that formed cytoplasm around the cell in addition to membrane blebbing, showing that gelation was required for hypothermic preservation. That study did not test any biological activities of the cells so it would be difficult to comment whether the cells were fully recoverable. A study by Swioklo et al. agreed that complete encapsulation in an alginate hydrogel improved the hypothermic preservation of cells, in that case adipose derived stem cells [211]. In that study, the cells were recovered from the hydrogel by dissolving the medium in a citrate buffer followed by centrifugation and resuspension in culture medium alone. Those recovered cells were able to attach to culture plates, proliferate, and maintain differentiation potential after storage. The removal of the gelling material appears to be required to recover cell functionality.

Therefore, all these points combined show that complete immobilisation of T cells in a hydrogel preservation medium benefited the cells during exposure to the hypothermia by an additional pausing of cell activity. However, gelatin did not allow recovery of cell activity after preservation. It would be necessary to remove the immobilising compound to recover the cell activity after preservation. Ultimately, the requirement for further processing will increase complexity and may make the method unsuitable for clinical application.

6.3.3 Ficoll addition to 10% (w/v) is inhibitory on cell growth and glucose metabolism at normal temperature in-vitro conditions.

The results in Figure 6-8 showed that ficoll supplementation of the preservation medium, in combination with hypothermia, increased the lactate production during preservation at 4^{o}C and 22^{o}C. The results in Figure 6-7 showed that the compound also limited the decline of ATP during preservation at 4^{o}C: the decrease in ATP was greater at 22^{o}C. These two facts suggested that ficoll was stimulating rather than suppressing metabolism at hypothermic

temperatures. Therefore, the effect of ficoll on cell metabolism was investigated under in-vitro conditions with temperatures of 37°C.

The results in Figure 6-11 showed that ficoll addition to 10% (w/v) under normal temperatures was inhibitory on cell proliferation at 37°C. Control cells free of the compound continued to expand in quantity unlike that of ficoll treated cells, which remained at the same cell density compared to the condition of day 0 control.

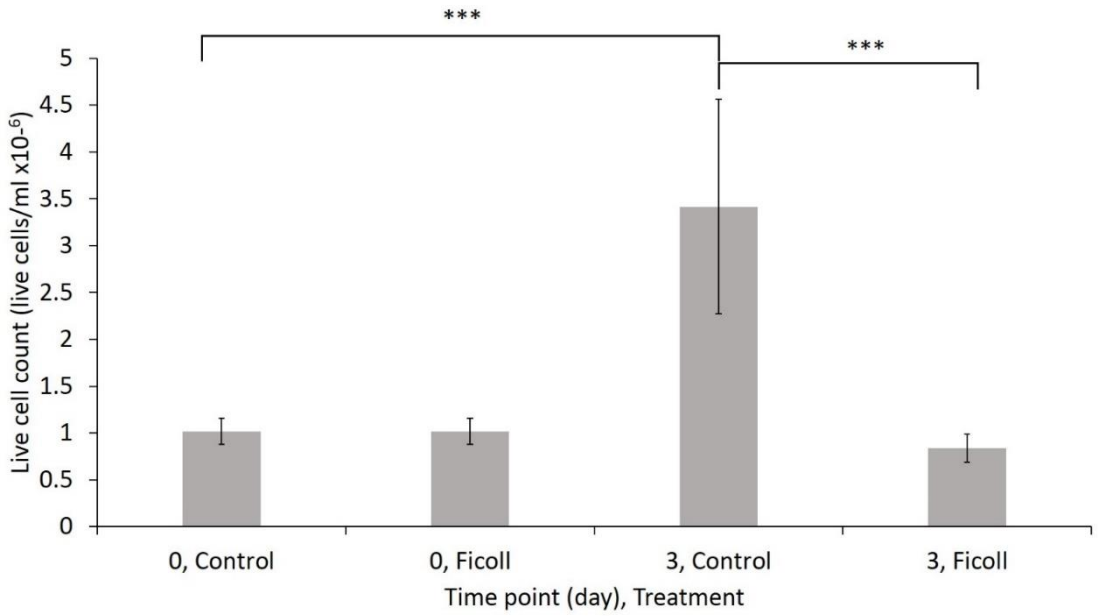

Figure 6-11 Quantity of live cells after normothermic incubation with 10% (w/v) ficoll compared to standard culture control. Live cell count was performed on a Countess automated cell counter with live cell number identified by trypan blue dye exclusion. Incubation at 37°C was implemented over 3 days. Cells were previously cultured as normal, and re-suspended into fresh medium supplmented with IL-2, and IL-2+ ficoll. See Section 3.2.10 for the explanation of statistical analysis and consequent labelling. N = 6.

The results in Figure 6-12 showed that the addition of ficoll to 10% (w/v) reduced glucose uptake under normal temperatures compared to the control condition. With ficoll present in the culture medium, the glucose consumption was almost completely inhibited, with an average consumption of $0.06 \pm 0.15 \times 10^{-9}$ millimoles/cell/72hours. Lactate continued to be produced by cell culture populations treated with ficoll additive at a low average rate of $0.38 \pm 0.20 \times 10^{-9}$ millimoles/cell/72hours, which was significantly lower than the control population. ATP was also depleted by T cells supplemented with ficoll, as shown in Figure 6-13. The control cells were shown to increase ATP with continued culture three days

beyond that of the seven day recovery from cryopreservation. That seven day recovery period did not increase the amount of intracellular ATP present after completing a rapid thaw from cryopreservation, shown in Chapter 5.

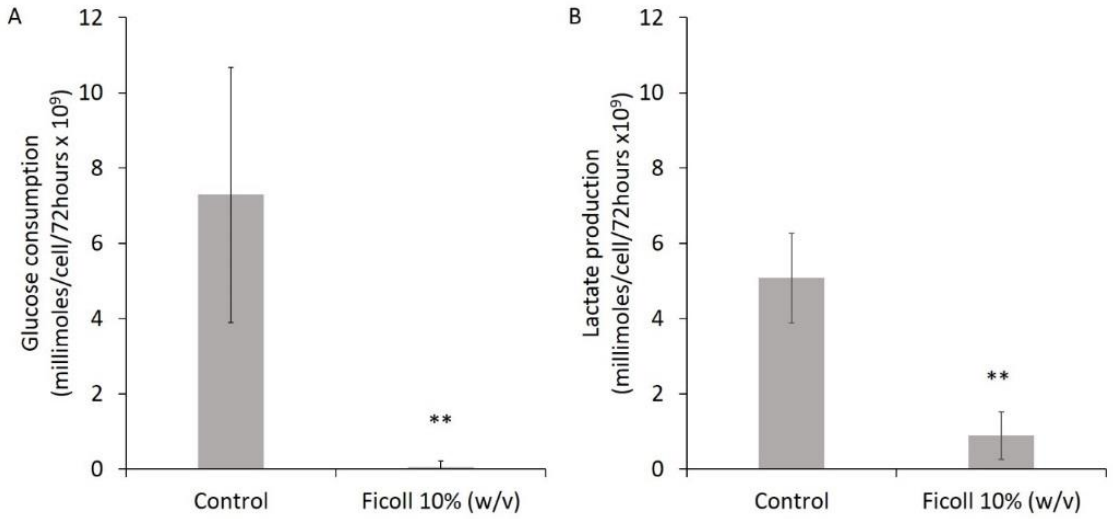

Figure 6-12 The effect of ficoll addition on glucose and lactate concentrations after 3 days of normothermic in-vitro culture. Normothermic temperature was at 37ºC. A) Glucose consumption from the culture medium. B) Lactate produced in the supernatant. Both compounds were assessed for concentration in the culture supernatant by enzymatic assay. Pairwise T tests assuming unequal variances were used for statistical analysis. See Section 3.2.10 for the explanation of statistical analysis and consequent labelling. N = 6.

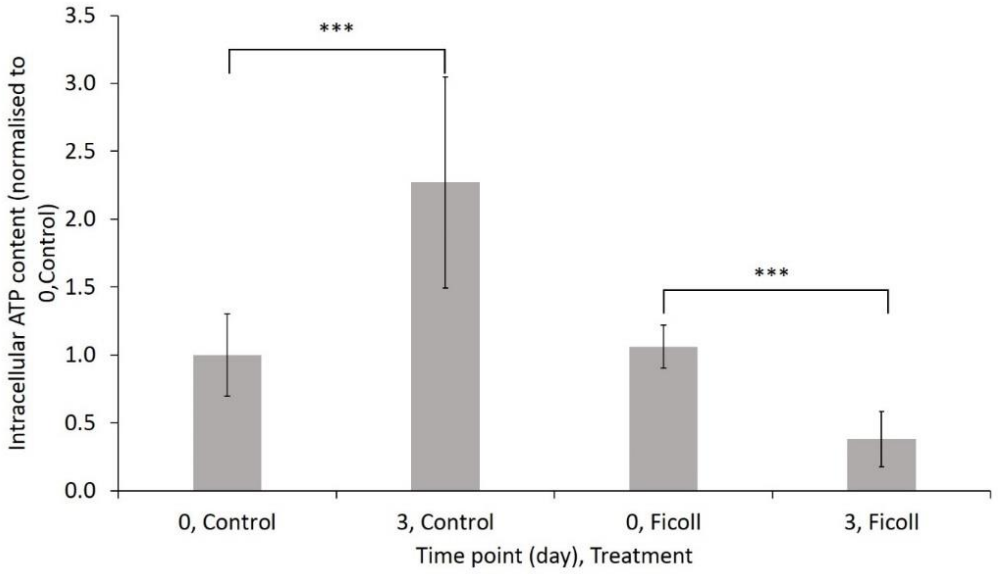

Figure 6-13 Intracellular ATP concentration of T cells after 3 days of in-vitro culture at 37ºC with 10% (w/v) ficoll in the culture medium, or without ficoll as a control. ATP concentration was measured by luciferase assay after extraction from the cells by lysing in boiling in deionised water. ATP was normalised to the day 0 control average. Log_{10} transformations were suitable to satisfy ANOVA assumptions. N = 6.

The results in Figure 6-14 showed that ficoll decreased the reduction in Alamar blue observed after three days of incubation. Therefore, that result confirmed that the compound was suppressing overall cell metabolism after extended exposure at 37°C. However, the cells treated with ficoll showed a small increase in metabolic activity after a short-term 4 hours incubation with the compound. Control cells displayed an increased average normalised Alamar blue reduction of 3.5 ± 0.8, which was almost the same increase in live cell quantity shown in Figure 6-11.

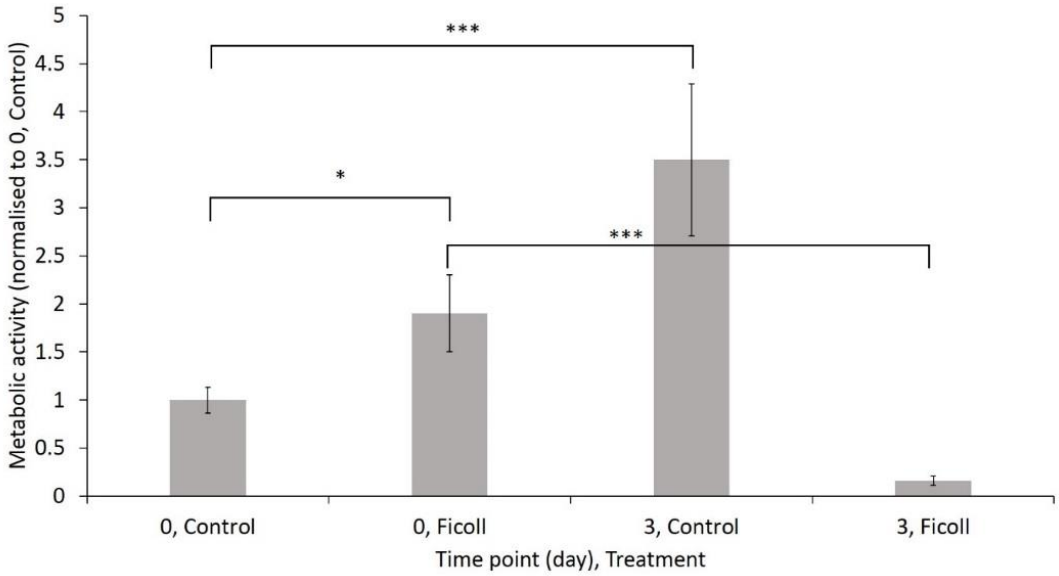

Figure 6-14 Alamar blue reduction of T cells after 3 days of in-vitro culture at 37°C with 10% (w/v) ficoll in the culture medium, or without ficoll as a control. Alamar blue reduction was interpreted as metabolic activity. Day 0 values were obtained after re-suspending T cells into the test medium and incubating for 4 hours in the presence of Alamar blue compound. After 3 days of culture, Alamar blue was added to treated cell samples and incubated for 4 hours before examining fluorescence. Results were normalised to the day 0 control, which was without ficoll supplementation. See Section 3.2.10 for the explanation of statistical analysis and consequent labelling. N = 6.

6.3.4 Sterilisation by autoclaving does not affect the chemical structure of ficoll.

Terminal sterilisation of the ficoll powder before addition to the preservation medium was achieved through autoclaving. The avoidance of breakdown of the ficoll chemical structure into its constituent saccharides was confirmed by the inability of the enzymatic glucose assay to detect concentrations of glucose different to normal culture medium when ficoll was added. The final results that showed no change in initial glucose concentrations with supplementation are shown in Figure 6-15. With no change in structure of ficoll reported, it

could be suggested that T cells are able to interact with the poly-sucrose compound, but with positive or negative effect depending on the temperature of incubation.

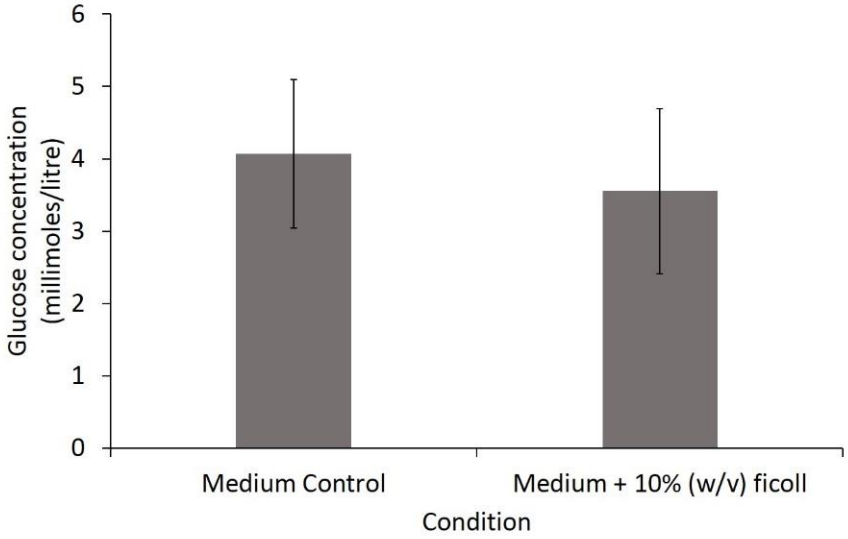

Figure 6-15 Glucose concentration in control medium, and a medium supplemented with 10% w/v ficoll. Glucose concentration was measured in the medium + 10% (w/v) ficoll sample after dissolving autoclaved ficoll powder in the same medium as the control group. Glucose concentration was measured by enzymatic assay kit. There was no significant difference reported between the concentrations detected in each sample. See Section 3.2.10 for the explanation of statistical analysis and consequent labelling. N = 6.

6.3.5 The role of sucrose is temperature dependent.

Continued exposure of T cell cultures to 10% (w/v) ficoll suppressed the cell proliferation and metabolism in 37^{o}C in-vitro culture but did not negatively affect the quantity of live cells present. At hypothermic temperatures of 22^{o}C and 4^{o}C, the addition of ficoll did not decrease viable cell count but stimulated cell metabolism. A mechanism for the alternating results between physiological and hypothermic temperatures needs to be explained.

Ficoll is a polysaccharide of polymerised sucrose [298]. Sucrose is a disaccharide composed of glucose and fructose, therefore, it contains the components used in cell metabolism. Dietary sucrose led to increased glucose circulating in the blood stream in whole mouse models [302]. A whole animal body is likely to possess the enzymes within the digestive system that break down complex sugars into basic components so that they can be absorbed in to the body through the intestinal wall. The preservation media developed in this thesis does not contain such enzymes that would break the sucrose down in to its constituent

saccharides. Isolated mammalian cells are unlikely to be able to process sucrose either. Besterman et al. showed that human fibroblast and macrophages are both capable of endocytosing radiolabelled sucrose when extracellular concentrations were presented to the cells [303]. Those cells loaded with sucrose were found to release sucrose intact when the compound was removed from the extracellular medium. The released quantity had constant levels of radioactivity from the sucrose, which indicated that none had been lost to the cell metabolism of the product. Meyer et al. have established that mammalian cells do have a sucrose membrane transporter [304]. This confirms the idea that endo- and exocytosis is possible. Leong et al. recently established that mammalian cell cultures used in biopharmaceutical production are capable of using certain disaccharides to sustain proliferation: although, it was only maltose that was found to do so in that study, which also included sucrose [305]. The ficoll, polymerised sucrose, is not a usable metabolite while present in its complete form for T cells during hypothermic preservation.

Section 6.3.4 established that the processing of the ficoll before addition to the preservation medium would not alter the glucose concentration initially present within. Hydrolysis can break the glyosidic bond within sucrose, but this is a slow process and requires catalysis by acidic conditions and also high temperatures to increase the rate of the reaction [306]. It is suggested that decomposition of the ficoll will not happen at a rate sufficient to provide a nutrient source that explains the metabolic stimulation shown in Section 6.3.2 for the T cells during preservation. In addition, Section 4.3.6 showed that glucose itself was unable to produce a noticeable effect on T cell metabolism during hypothermia. Therefore, the small quantities of ficoll that degrade in to glucose and fructose would be unable to have an effect on T cell metabolism during hypothermia.

These pieces of evidence establish that chemical decomposition of the polymerised sucrose is unlikely under the hypothermic preservation conditions tested in this thesis, nor is the stimulation a result of direct metabolism. It is understood that the result of T cell metabolism

being both stimulated and inhibited by ficoll with change in temperature is not related to direct metabolism of ficoll or its constituent saccharides.

If direct metabolism of ficoll or its constituent components are not freely available to the T cell, then this may suggest that mechanism of ficoll is related to biochemical signalling. Evans' study found that the addition of sucrose at high concentrations of 200mM to 250mM benefited the hypothermic preservation of hepatocytes [212]. That investigation did not examine further than membrane integrity, but the result from sucrose addition was hypothesised to be related to an extracellular mechanism. The results presented in Section 6.3.2 show a similar benefit from ficoll to hypothermic preservation of T cells but do suggest that the effect of ficoll was intracellular because of the noticeable change in cell metabolism. Bestermen et al. established that sucrose can be endocytosed even if it is not metabolised [303]. Therefore, sucrose may be able to react with biochemical processes other than cell metabolism. Chen et al. have identified that high molecular weight saccharides; trehalose, sucrose, and raffinose induce autophagy in human cells, which was linked to AMPK signalling [83]. The results of Section 6.3.2 and Section 6.3.3 showed that the response of T cell metabolism to ficoll changes between being stimulated during hypothermia and being suppressed at 37^{o}C. Since autophagy and AMPK signalling are likely to be affected by the inclusion of ficoll, this may explain why ficoll can have both a stimulatory and suppressive action depending on temperature. At 37^{o}C, autophagy and AMPK signalling would reduce cell activity, which the results of Section 6.3.3 confirm. At lower temperatures, autophagy and AMPK signalling could limit T cell activity so that the cells do not exhaust ATP during preservation. Section 6.3.2 demonstrates clear continuation of T cell metabolism, which is difficult to reason from the result of direct metabolism of the polymerised sucrose or its constituent components. Since the involvement of intracellular signalling rather than metabolic reaction is the most reasonable explanation behind the behaviour of ficoll, this would present further evidence, in addition to Chapter 5, that manipulating biochemical

processes to reduce cell activity beyond the reduction produced by reduced temperatures is required to successfully preserve T cells in hypothermia.

6.4 Summary.

It was shown that the sedimentation of cells at 4^{o}C has no adverse effect during the preservation of activated T cells at a concentration of $10x10^{6}$ live cells/ml, when preserving them in container geometries that are of the same dimensions as those that are used for routine cell culture. It has also been shown that T cells respond metabolically to the poly-sucrose compound ficoll, which was an inhibitory reaction at 37^{o}C yet stimulated cell metabolism at lower temperatures. The exact mechanism of the ficoll effect could not be explained fully but is thought to be related to biochemical signalling rather than metabolism. Therefore, the effect of high order saccharides on human cells may be considered for more investigation in the future. The complete encapsulation in gel, when combined with the application of hypothermia, appeared to pause metabolic activity in cells. However, despite the reversible nature of gelatin gels to liquid form with the application of 37^{o}C temperatures, the T cells did not recover metabolic activities after preservation and removal from the gelatin gels.

7 Oxygen Supply and Management During Hypothermia for Extended Preservation.

7.1 Introduction

Some cell activity will continue with reduced temperature [20]. The oxygen uptake rate in isolated mitochondria from kidney cortex followed an Arrhenius relationship, where the reaction rate is exponentially related to temperature [158]. Jorjani and Ozturk agree that oxygen consumption follows an Arrhenius relationship when testing other cell lines, but also conclude that cell types have different consumption rates [70]. The difference in consumption rates indicated that different cell types may require a different amount of oxygen during storage. Oxygen uptake is one biochemical reaction that can be taken to represent the general drop in metabolic rates with temperature due to the lack of intracellular oxygen store [20]. Oxygen is accepted as being poorly soluble in a water based medium. Continued oxygen consumption and poor solubility mean that it could be important to consider the concentration of oxygen available in the medium during hypothermic preservation. If the cells are still consuming oxygen with reduced metabolism, then a high cell density therapy stored in a sealed container for a long time may require some method of oxygenating the cells to avoid its depletion.

Static cold storage remains the standard method to preserve organs because of its simplicity, cost effectiveness, and compliance with the current requirements for functioning organs after transplantation. Oxygenation may not be required during hypothermic machine perfusion of some organs because of their reduced metabolism during hypothermia, and a study without additional oxygenation produced acceptable results [307]. However, hypothermic machine perfusion with oxygenation benefits a range of organs. In the case of a kidney transplant in a rodent model, oxygenation of the preservation medium by inclusion of a perfluorocarbon compound to increase oxygen solubility benefitted the hypothermic preservation of kidneys [308]. This benefit was conveyed by reduced oxidative stress and lower levels of damage associated molecular patterns, which was measured through release of HMGB-1 into the

perfusate. A similarly positive response was reported for rodent fatty liver by oxygenation of the perfusate with hollow-fibre gas exchange devices [87]. The 1-hour oxygenated perfusion followed a period of 12 hours of static cold storage and was still shown to reduce oxidative stress after implantation. The main point from these studies on machine perfusion oxygenation was that maintenance of physiological oxygen tensions or slight hyperoxia benefit organ preservation [309]. These studies were conducted on organs where there is less consideration for the oxygen partial pressure (pO_2) that individual cells within the organ are exposed to. It would be important to re-focus oxygenation studies to an individual cell level when considering the preservation of cell products, because excessive oxygenation may result in excessive hyperoxia when tissue is not present to inhibit diffusion.

The response of T cells to hypoxia is not wholly negative when consideration of definitions of hypoxia are taken into account [310]. A study by Atkuri et al. showed that 5% pO_2 improved T cell activation compared to atmospheric 20% pO_2 because cells at 5% pO_2 had more intracellular glutathione [311]. Glutathione was discussed in Chapter 2 to be important for T cell expansion. The results of Chapter 4 showed that the compound was important to achieve metabolic activity of T cells after hypothermic preservation. 2.5% pO_2 was shown to produce CD8+ T cells that had a greater lytic capacity on a target cell type measured through chromium release. Caldwell et al. showed that 2.5% pO_2 reduced proliferation compared to cells cultured under atmospheric pO2 [312]. Another study by Silly et al. reported increased cytokine release by T cells but reduced proliferation at 5% pO_2 [313]. The response of T cells to hypoxia is not completely negative but appears to affect the therapeutic action of the cells. T cells may have to be sustained at the same pO_2 level as they were cultured at during preservation to avoid the hypoxic responses described above.

The studies above establish that oxygen will be consumed during hypothermic preservation, and that T cells will respond to decreases in pO_2, but it is not known how quickly pO_2 may change during preservation. Mathematical models could be a straightforward method for

investigating how oxygen tension is maintained during extended hypothermic preservation. This method would identify if there is a region of the cell suspension that experiences a change in pO_2. If there is a requirement for external oxygenation, this can also be modelled to evaluate if there is improvement before attempting experimental validation. Avoiding changes in pO_2 would allow the previously developed hypothermic preservation protocols presented in Chapter 5 and Chapter 6 to be tested for an extended duration without decreased pO_2 affecting the results. An increase in duration would allow extra CGT product processing tasks, in addition to transportation, to take place while the cells are in a preserved state. Evaluating the same quality parameters as used in the previous Chapters would identify how long the developed protocols are effective for during the extended preservation.

7.2 Mathematical modelling.

7.2.1 Layout of the oxygenating source.

Mathematical modelling was completed to identify suitable methods for oxygenating a T cell suspension during preservation. Oxygenation may be important when preserving metabolically depressed cells at high density despite a temperature reduction decreasing the activity. The initial method investigated was the use of a gas permeable container such as commercially available cell culture bags, which can be made of fluorinated ethylene polypropylene, a polymer that allows gas diffusion through it. These have been tested for the culture of T cells in in-vitro conditions producing successful results[16]. However, a mathematical modelling study by Avgoustiniatos et al. suggested that such gas permeable bags may have allowed an anoxic region to develop around three-dimensional islets during 22^{o}C hypothermic preservation and concluded that devices with silicone membranes may be required instead to provide higher oxygen flux through the membrane [314]. Therefore, a new study presented in this Chapter was conducted to investigate the specific case of activated T cells. The layout of the model is shown in Figure 7-1. The assumptions made in developing the physical model were:

- The initial concentrations are at saturated levels, which is reasonable because it assumes that cells are placed into medium that has been equilibrated with air.
- The air on the external surface of the bag would be sufficient to maintain saturated concentration of oxygen in the bag material at the outer boundary throughout the time period investigated because the concentration of oxygen in air is reasonably high.
- The diffusion through the bag would be axis symmetric about the centre of the bag because there are two sides made of the gas permeable material.

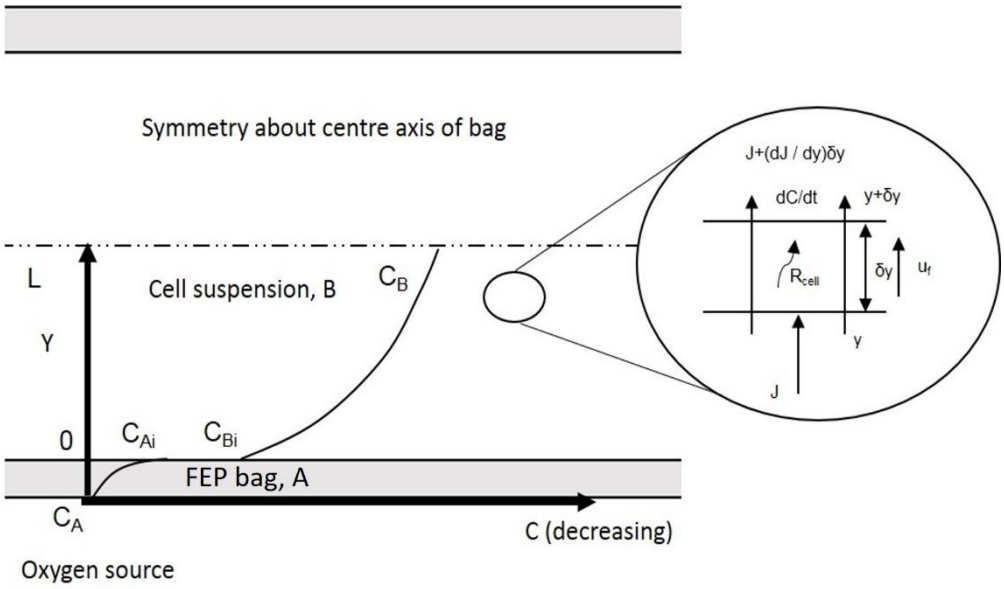

Figure 7-1 Macroscopic diffusion path of oxygen through a FEP bag storage system. Image shows a graphical representation of some of the assumptions that were made. The detailed callout within the figure shows the microscopic element depicting an unsteady state mass transfer with consumption of oxygen by cells (R_{cell}). This element has unit depth.

7.2.2 Modelling of an oxygen transfer system through a gas permeable membrane.

Fick's first law of diffusion is often used to describe steady state diffusion of compounds:

$$J = -D\frac{dC}{dy}$$

Equation 7-1

This law states that diffusion will take place down a concentration gradient and describes accurately a system in steady state. In the case of a hypothermic storage system, the diffusive oxygen flux will fluctuate with time because the concentration of oxygen in the storage

medium will not remain constant due to consumption by the cells. Therefore, it was necessary to construct a non-steady state model for the problem, which can be achieved by considering the mass transfer through finite elements of the medium, shown in Figure 7-1, applied throughout the entire system to be investigated.

Using continuity to balance the number of moles entering and leaving the control volume:

$$\frac{Moles\ in}{unit\ time}\ (at\ y) - \frac{Moles\ out}{unit\ time}\ (\ at\ y + \delta y) - \frac{Moles\ consumed}{unit\ time}\ (throughout\ element\ \delta y) = \ Rate\ of\ change\ of\ concentration \times element\ volume$$

Equation 7-2

Taking Fick's first law to be true at the left boundary, and substituting the fluxes shown in Figure 7-1 into the above equation:

$$\left(\left\{-D\frac{\partial C}{\partial y} + u_f C\right\} - \left\{-D\frac{\partial C}{\partial y} + u_f C + \frac{\partial}{\partial y}\left[-D\frac{\partial C}{\partial y} + u_f C\right]\delta y\right\} - \{R_{cell}\delta y\}\right) A = \frac{\partial C}{\partial t}(\delta y, A)$$

Equation 7-3

Which simplifies to:

$$\frac{\partial C}{\partial t} = D\frac{\partial^2 C}{\partial y^2} - \frac{\partial\left(u_f C\right)}{\partial y} - R_{cell}$$

Equation 7-4

In this case of the movement of dissolved oxygen through a cell suspension during storage all of the system was static, so the term u_f to account for movement in the system will be zero. Considering the Peclet (Pe) number for the balance between advection and diffusion:

$$Pe = \frac{u_f L}{D}$$

Equation 7-5

With u_f equal to zero, $u_f L = 0 < D$, therefore the system is diffusion controlled and the term involving u_f can be neglected, which showed that movement of molecules through the system is controlled by diffusion. This result was similar to Fick's second law, a partial differentiation equation (PDE) for the variation of concentration in both time and space, with an extra term to represent the oxygen consumption by the cells:

$$\frac{\partial C}{\partial t} = D\frac{\partial^2 C}{\partial y^2} - R_{cell}$$

Equation 7-6

This is a second order partial differential equation that needs to be solved to implement the mathematical model of diffusion from the oxygen releasing material to the cell storage medium. Equation 7-6 was solved by finite differences using an Euler approximation of finite elements. This method uses forward- and central-difference approximations for the differentials in the PDE. The time derivative is replaced by a forward difference approximation so that:

$$\frac{\partial C(y,t)}{\partial t} \approx \frac{1}{\Delta t}[C(y,t+\Delta t) - C(y,t)] + O((\Delta t)), \qquad \Delta t \to 0$$

Equation 7-7

The space derivative is replaced by a central difference such that:

$$\frac{\partial C(y,t)}{\partial y} \approx \frac{1}{2(\Delta y)}[C(y+\Delta y,t) + C(y-\Delta y,t)] + O((\Delta y)), \qquad \Delta y \to 0$$

Equation 7-8

and the second derivative is found by considering the above Equation 7-8 at y+/-Δy/2 combined with:

$$\frac{\partial^2 C(y,t)}{\partial y^2} = \frac{\partial}{\partial y}\left(\frac{\partial C(y,t)}{\partial y}\right)$$

Equation 7-9

So that:

$$\frac{\partial^2 C(y,t)}{\partial y^2} \approx \frac{\frac{\partial C\left(y-\frac{\Delta y}{2},t\right)}{\partial y} - \frac{\partial C\left(y+\frac{\Delta y}{2},t\right)}{\partial y}}{\Delta y/2}$$

Equation 7-10

Which leads to the central difference approximation of:

$$\frac{\partial^2 C(y,t)}{\partial y^2} = \frac{1}{(\Delta y)^2}[C(y+\Delta y,t) - 2C(y,t) + C(y-\Delta y,t)] + O((\Delta y)^2), \qquad \Delta y \to 0$$

Equation 7-11

These approximations can then be substituted into the partial differential equation, which, after rearrangement, obtains the Euler method, so that:

$$C(y,t+\Delta t) = C(y,t) + \frac{\Delta t}{(\Delta y)^2}(C(y-\Delta y,t) - 2C(y,t) + C(y+\Delta y,t))$$

Equation 7-12

7.2.3 Boundary conditions.

It was assumed that oxygen concentration remained constant at the outer interface between the FEP bag and atmospheric gas because of the large volume of the supply. There would be a no flux condition at the inner boundary because of the symmetric nature of the problem, and the concentration of dissolved oxygen would be the same both sides of the interface. Therefore, the resulting boundary conditions are:

$$C = C_{atmospheric}\ at\ y = 0$$

Equation 7-13

And:

$$D\frac{\partial C}{\partial y} = 0\ at\ y = L$$

Equation 7-14

There were two materials to this system; an FEP gas permeable bag that contains medium, and a cell-containing phase comprised of the storage medium and the cells for the therapy. There will be mass transfer between the two materials, and it was assumed that the flux leaving one phase will be equal to the flux entering the other. This transfer was implemented in the model by a finite difference scheme similar to the one previously identified by Hickson et al. [315]. This implementation was modified from that previous scheme by the addition of different concentrations of oxygen to each material at either side of the interface, which was introduced by partition coefficient K such that:

$$KC_{Ai} = C_{Bi}$$

Equation 7-15

And:

$$KD_A\frac{\partial C}{\partial y} = D_B\frac{\partial C}{\partial y}$$

Equation 7-16

Taking a central difference at the point on the interface produces:

$$\frac{\partial C_i}{\partial t} = \frac{KD_A\frac{\partial C_a}{\partial y} - D_B\frac{\partial C_B}{\partial y}}{\Delta y}$$

Equation 7-17

Substituting forward differences in domain A, backward differences in domain B, and a forward difference for the temporal differential term into Equation 7-17, and then rearranging the terms, gave the solved boundary equation:

$$C(y, t + \Delta t) = C(y, t) + \frac{\Delta t}{(\Delta y)^2}\left(\frac{D_B}{K}C(y + \Delta y, t) - \left(\frac{D_B}{K} + D_A\right)C(y, t) + D_A C(y - \Delta y, t)\right)$$

Equation 7-18

Temporal boundary conditions assume that the entire system was initially saturated with atmospheric oxygen, so that:

$$C(y, 0) = C_{sat}$$

Equation 7-19

7.2.4 Identifying the form of cellular consumption of oxygen.

The term R_{cell} in Equation 7-4 covers a number of different possibilities for modelling how the cells will consume oxygen. This term could be zero-order with respect to oxygen concentration, which, as a first approximation, was used for developing this model, but it can also take any other suitable form to describe how a cell will behave when the concentration of oxygen changes. Using the zero-order approximation means that the consumption rate identified will not vary with respect to oxygen concentration. Quantitative data in a study by Southard et al. showed how biochemical reactions respond with temperature changes alone and with no other variables altered [158]. A study by Jorjani and Ozturk produced evidence that the high cell densities, comparable to those used in the storage experiments of this thesis, would not affect the specific value of oxygen consumption [70]. Therefore, it was assumed that Michealis-Menten kinetics would not exist under hypothermic conditions. Zero-order kinetics should be a reasonable assumption to determine what amount of oxygen provision would be required. This represents a case of lowest oxygen availability in the preservation medium because there would be no reduction in oxygen consumption rate with decreased concentration in the medium.

7.2.5 Determining the value for zero order of cellular consumption of oxygen l_i.

The metabolism of T cells under normal conditions was identified to be reliant on both glucose and oxygen for glycolysis and oxidative phosphorylation, respectively. The amount of oxygen consumed is known to be 22pmol/min/10^7 cells [280], and this value was used to quantify the consumption at 37ºC. Oxygen consumption has been shown by Frauworth et al. and Geltink et al. to change level when T cells are activated [136,137]. The effect of temperature on the oxygen consumption rate of T cells was included in the models developed for this Chapter from the results produced by a fluorescent oxygen consumption assay.

7.2.6 The reduction of diffusion coefficient with temperature.

Another factor to be included in the model was the rate at which diffusion takes place due to a reduction in temperature. This factor was modelled by an Arrhenius relationship, with coefficients obtained from previously existing data [297].

7.3 Materials and methods.

7.3.1 Measurement of oxygen consumption by fluorescence quenching.

A commercial kit, Mitoxpress xtra, was purchased from Amsbio, and used according to manufacturer instructions. T cells were cultured in the same method as described in section 3.2.1. After 14 days of culture, cells were counted and suspended to a density of 5x10^6 cells/ml in new complete culture medium. 100µL of cell suspension was transferred to a well of a 96 well-plate and sealed with manufacturer supplied mineral oil according to their method to stop transfer of external oxygen. Plates were transferred to an incubator to achieve temperatures of 37ºC, 32ºC, 27ºC, 22ºC, and to a refrigerator for 4ºC. Plates were read for fluorescence at 40, 80, 120, and 160 minutes. Change in fluorescence was interpreted as oxygen consumed by the cells. Complete culture-medium-only negatives that contained no cells were used to evaluate the background fluorescence, which was subsequently removed from samples containing the cells. Individual sample consumption rate was found by averaging the changes over each time point.

7.3.2 Methods for quantifying the success of extended hypothermic preservation of T cells in cell culture formats.

The methods of high-throughput imaging cytometry, metabolic activity through Alamar blue reduction, intracellular ATP concentration, and enzymatic analysis of lactate production were the same as the methods described in previous chapters. Hypothermic preservation was performed in the same way as previously discussed in a multi-well plate to allow oxygen diffusion from the gaseous headspace. Excess quantities of PBS were included around samples and the plate was then sealed with Parafilm to prevent moisture loss.

7.3.3 Long term recovery of T cells after hypothermic preservation.

After completion of hypothermia, which was implemented in the same manner as described in section 3.2, 50µL of preserved cell suspension for each sample to be tested for recovery was transferred to a new 48-well plate and diluted to a total volume of 500µL with RPMI 1640 medium supplemented with 10% (vol.) FBS and 300 IU/ml IL-2. Individual cell culture samples were assayed for membrane integrity and cells counted by the method described in Section 3.2.2. Cell counts were performed after 24 hours and 48 hours and compared to day 0 control values before the application of hypothermia.

7.3.4 Previously used methods

The methods of cell culture, hypothermic preservation, high throughput cell counting and membrane integrity assay, high throughput metabolic activity assays, ATP measurement, and enzymatic measurement of glucose and lactate compounds used methods detailed in Section 3.2 and Section 4.2.

7.4 Results and discussion.

7.4.1 Identification of oxygen consumption by activated T cells with reduced temperature.

Oxygen consumption was measured by an assay that has its fluorescence quenched by the presence of dissolved oxygen. Therefore, it was not sufficient to generate consumption rates

in terms of oxygen concentrations. This assay was sufficient to show how the consumption changed with temperature, and results are presented in Figure 7-2. It was shown that there was a distinct decrease in oxygen consumption when the temperatures were reduced below 37°C. The largest decrease occurred between 37°C and 32°C, but the subsequent reductions did not produce a significant difference amongst each other. An exponential trend line produced the lowest error in least squares regression compared to linear fit, which suggested that there was an Arrhenius type relationship between the data points with a value of error that was only 0.78 across the five data points.

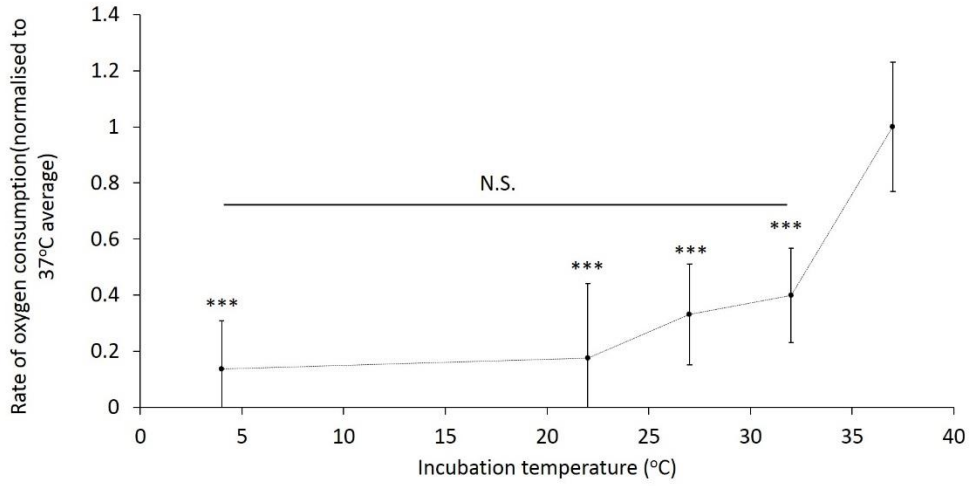

Figure 7-2 Effect of temperature on oxygen consumption rate of activated T cells. Oxygen consumption was measured in a relative sense by examining the quenching of fluorescence of a commercially obtained assay reagent in a sealed well. Results were normalised to the rate of change of fluorescence measured in cells treated at 37°C. N.S. indicates no significant difference, $p > 0.05$. See Section 3.2.10 for the explanation of statistical analysis and consequent labelling. N = 5.

This reduced accuracy of fit may be a result of breakpoints in the oxygen consumption rate. These breakpoints were identified by Southard et al. to exist in oxygen consumption curves with reduced temperature of isolated mitochondria from a range of animals including humans [158]. A further study by Ozturk and Jorjani on oxygen consumption of Chinese hamster ovary cells, baby hamster kidney cells, and hybridoma cells in response to temperature change showed that the data fitted well with an exponential function over the entire temperature range of 37°C to 6°C [70]. That study also agreed with the results presented in Figure 7-2, that oxygen consumption reduced 10-fold over a similar temperature

range. Breakpoints in the response of oxygen consumption may not exist for all cell types, but Figure 7-2 suggested that one may exist for T cells between 37ºC and 32ºC incubation. The results presented in Figure 7-2 agree closely to those of reported in previous studies. Therefore, these results could be used in a computational model of oxygen consumption to examine if there is a requirement for external interventions to provide oxygen during static cold storage.

7.4.2 Validation of the model for predicting oxygen content in a fluorinated ethylene polypropylene (FEP) bag.

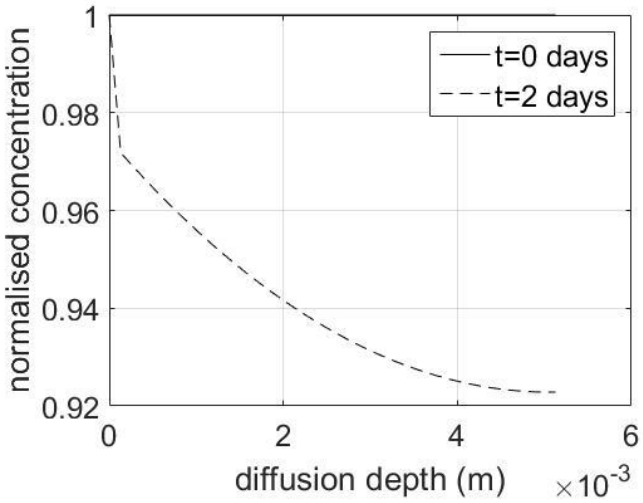

Figure 7-3 Dissolved oxygen concentration profile produced by a validation study. This study held temperature at 37ºC with cell density changing with time according to the rate measured in the documented cell culture study. The area under day 0 and day 2 curves was integrated to find the amount of oxygen that was predicted to be consumed and this was compared to the change in overall oxygen tension measured in the existing cell culture study. The final values that confirm validity are discussed in the relevant text.

The computational model developed for oxygen diffusion through a gas permeable bag containing a cell culture suspension was validated against experimental data obtained from literature. The specific case examined was the use of a fluorinated ethylene polypropylene (FEP) polymer bag by Li et al. that showed a reduction of total oxygen tension from 170mmHg to 158mmHg after 2 days of cell culture [316]. Their results showed that the percentage of oxygen consumed by the cell culture over this time period was 7.06% of the initial concentration. Results in Figure 7-3 showed the normalised oxygen concentration

profile produced by the computational model using the available parameters from the above cell culture study and the T cell oxygen consumption rate obtained from other literature. Numerical integration of the profile to find the total remaining concentration after 2 days compared to day 0 values, the line across y axis at 1 unit in Figure 7-3, showed that the model predicted an oxygen consumption of 6.20% over 2 days of culture. The small difference in total consumed oxygen between the model and the study could be due to the rate at which activated T cells consumed oxygen on a single cell level, which was shown by Frauworth et al. to be dependent on CD28 co-stimulation and presence of IL-2 [136]. The results of the computational model shown in Figure 7-3 detected a small difference to the culture format tested in the other study, which could be explained by slight differences in the measurement of oxygen consumption. Therefore, it can be suggested that the model will suitably predict if the hypothermic preservation of T cells will require added interventions to supply sufficient oxygen.

The validation experiment does show that the FEP bag material provided a noticeable resistance to diffusion of oxygen through the system, as shown by the steep gradient of the leftmost section of the second day concentration curve shown in Figure 7-3. This was a similar result to that found by Avgoustiniatos et al. when FEP bags were examined with computational diffusion and it was shown that a large concentration gradient within the bag material was found at steady state [314]. If improvement to oxygenation is required in a T cell preservation system using a gas permeable bag then alternative container materials may be one direction to investigate.

7.4.3 Temperature and oxygen content.

The main parameter to investigate with the validated model was the influence of temperature on the amount of dissolved oxygen available in the system to the cells during hypothermic preservation. The decrease in oxygen demand of activated T cells with temperature was taken from the data points presented in Figure 7-2. It was shown that as temperature was

decreased, there was a higher concentration of dissolved oxygen maintained after completion of 5 days of preservation, see Figure 7-4. At a high cell density of 20 million live cells per millilitre of medium, a cell density much higher than the cell culture protocol gas permeable bags are intended for, the model predicted that there would be a large decrease in oxygen available to the cells but that this would not be completely depleted to anoxic levels. This suggests that T cells could be cultured to a slightly higher cell density in gas permeable bags than the 1 million cells per millilitre that standard protocols allow for [16,32]. However, at the fixed cell concentration used in the study presented in Figure 7-4, the oxygen tension passed the 5% pO_2 level that would cause changes to the T cell biology [310–313]. With the temperature reduced below 37^{o}C, there was a much higher concentration of oxygen retained in the preservation medium during the 5 days of hypothermia. The oxygen content increased as temperature decreased further. This suggested that the combination of decreased T cell oxygen demand; increased oxygen solubility; and decreased diffusion coefficient, meant that the ability for the static system to maintain oxygen concentration increased with decreased temperature. At the initial cell density conditions, the results shown in Figure 7-4 produced no evidence for the need to oxygenate the cell suspension beyond what can be provided by existing gas permeable cell culture equipment.

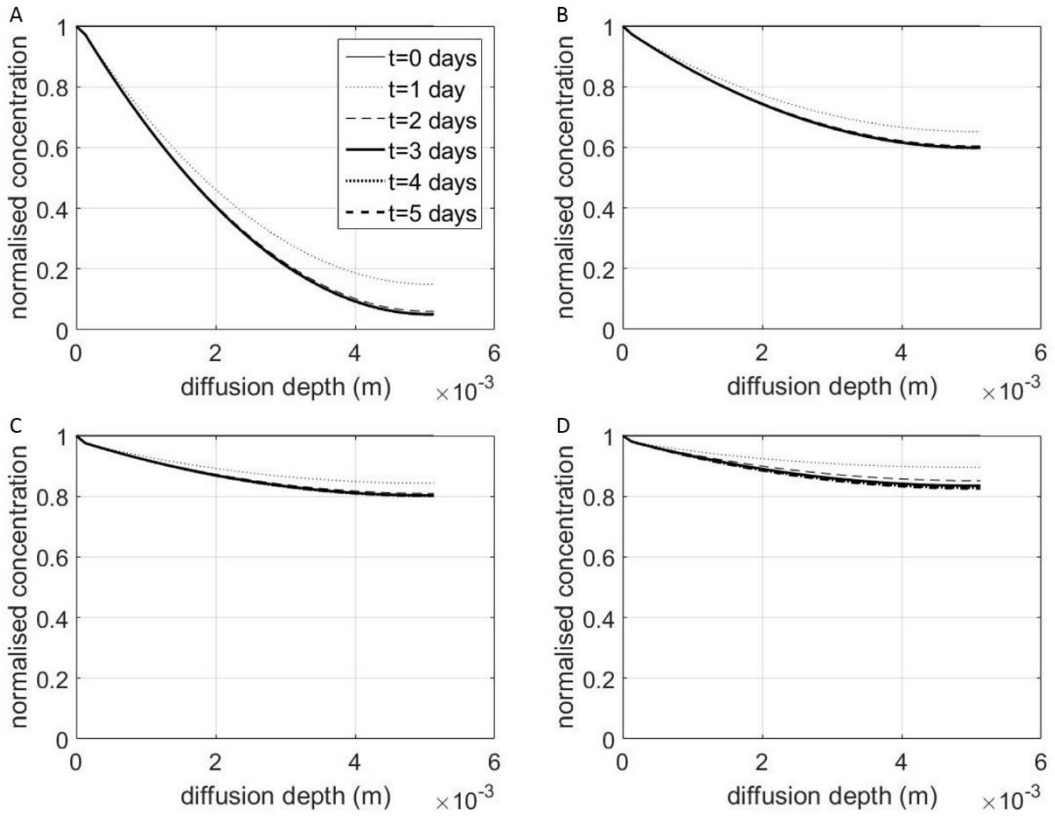

Figure 7-4 The effect of temperature on the dissolved oxygen concentration in the culture bag preservation system. Model parameters that were not temperature dependent were held at fixed values as the temperature was changed. Profiles in each sub-figure show how the concentration gradients evolved with time. The legend shows the selected time points for representative purposes. A) 37ºC, B) 32ºC, D) 22ºC, E) 4ºC. The legend is the same for all subplots.

7.4.4 Cell density and oxygen content.

The value for cell density was initially fixed but this density could change if a different quantity of cells has to be applied to complete the therapeutic dose depending on individual patient conditions. Figure 7-5 presents the results produced by the model with cell density varied between $1x10^6$ to $100x10^6$ live cells per millilitre. It was found that oxygen content of the preservation medium was largely depleted only at the highest cell densities tested, whereas lower cell densities were supported with much smaller changes in the concentration of dissolved oxygen. These results suggested that only dense suspensions of cells, approaching $100x10^6$ cells/ml, may require additional components to maintain the initial oxygen concentration of the preservation medium. Consequently, there was no evidence produced by the model to suggest that additional methods of oxygen delivery were required at the cell density of $10x10^6$ cells/ml to be trialled in the experimental part of this Chapter.

Therefore, standard cell culture methods of oxygenating the medium can be used to investigate the extended duration of preserving T cells with hypothermia, without the effect of changing oxygen tension affecting the results.

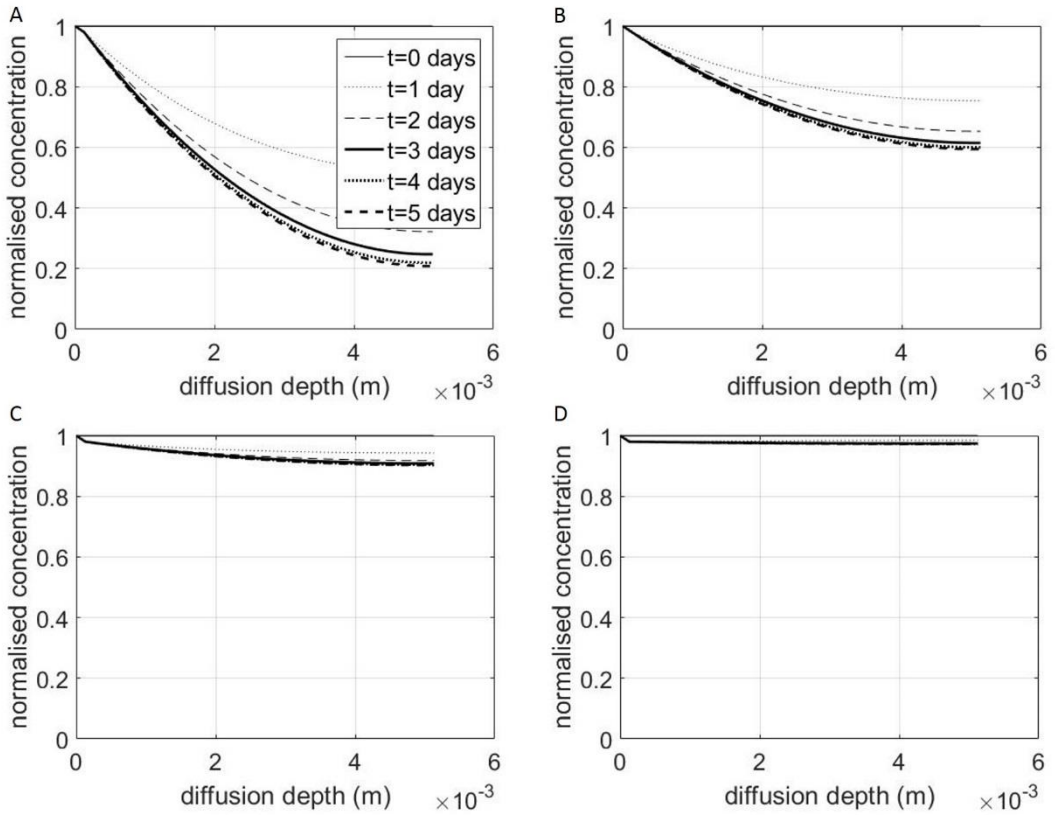

Figure 7-5 The effect of cell density on the normalised concentration of dissolved oxygen present in the preservation medium within the bag system. Model parameters that were not cell density dependent were held at fixed values as temperature was changed. Profiles in each sub-figure show how the concentration gradients evolved with time. The legend shows the selected time points for representative purposes. A) $100x10^6$ cells/ml, B) $50x10^6$ cells/ml, C) $10x10^6$ cells/ml, D) $1x10^6$ cells/ml. The legend is the same for all of the subplots.

7.4.5 Preservation medium thickness and oxygen content.

The cell culture bags examined in this study are a flexible container that can be filled with a different volume of liquid and, when stored horizontally, this decreases the surface area to volume ratio. The change in this ratio will affect the distance at which the cell suspension is located from the gas permeable material and force diffusion of oxygen to occur over a greater distance. This increased distance could lead to the development of reduced oxygen tensions within the cell suspension. Therefore, the effect of medium depth was investigated and the results are shown in Figure 7-6. This investigation found only a small decrease below the

saturated concentration of oxygen at the intended maximum depth, which had a symmetric half depth of $0.5x10^3$ of operation prescribed by the bag manufacturer, see Figure 7-6A. The minimum concentration reached decreased with increased depth of the medium: an increase of 1.3mm of the medium depth decreased the normalised minimum oxygen concentration from 0.85 at 5mm to 0.73 at a thickness 6.3mm, see Figure 7-6A and Figure 7-6B. This large change suggested that the system was quite sensitive to small changes in how the FEP bag is filled.

The 10mm depth of preservation medium presented in Figure 7-6D represents the possibility of one side of a correctly filled bag becoming blocked so that the surface is not exposed to atmospheric gases and diffusion can only take place through the other surface. Limiting oxygen diffusion in this way could expose the cell suspension to reduced oxygen concentration levels. For the 10mm thickness, the minimum normalised concentration level reached 0.58 that of the starting level. This remained above the oxygen concentrations tested in hypoxia studies [310–313]. Therefore, the oxygen depletion from a blocked gas permeable surface should not induce a hypoxic response in the T cells during preservation.

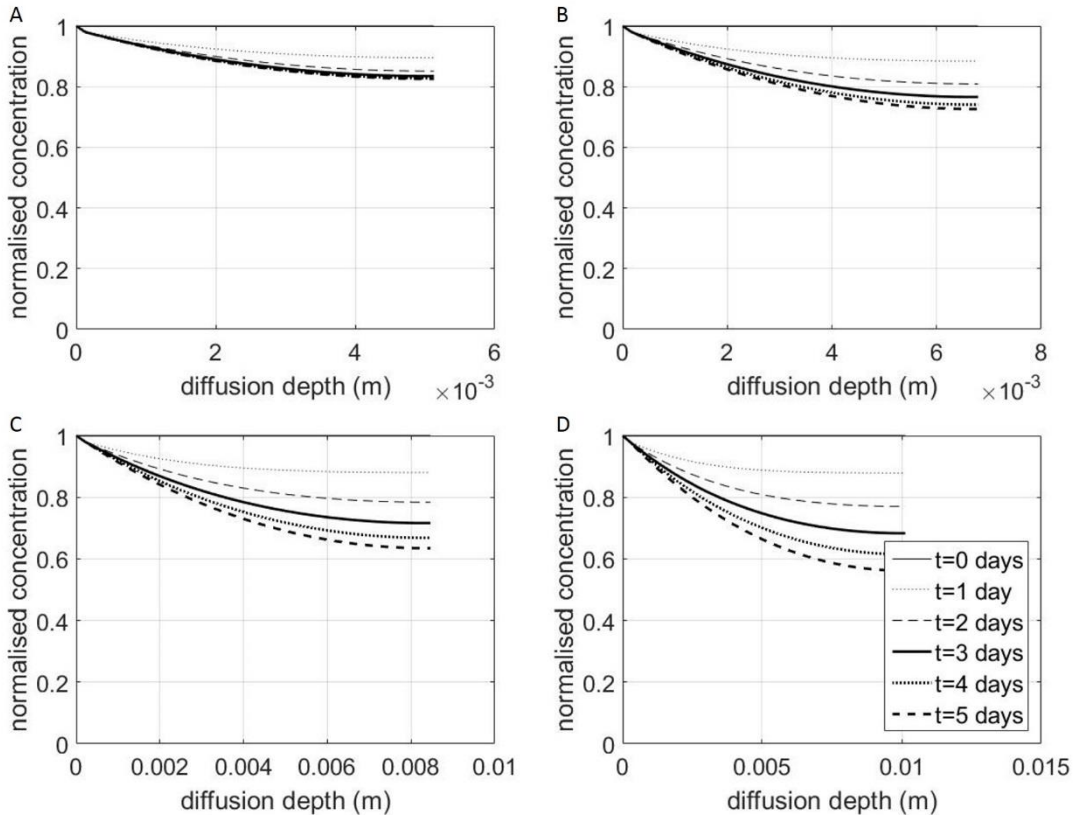

Figure 7-6 The effect of medium thickness, and, therefore, the diffusion depth from the source of oxygen, on the normalised concentration of oxygen present in the preservation medium. Model parameters that were not dependent on medium thickness were held at fixed values as medium thickness was changed. Profiles in each sub-figure show how the concentration gradients evolved with time. The legend shows the selected time points for representative purposes. A) $5x10^{-3}$ m, B) $6.3x10^{-3}$ m, C) $8.1x10^{-3}$ m, D) 0.01 m. The legend is the same for all of the subplots.

7.4.6 Mathematical modelling suggests that oxygenation can be provided by diffusion alone.

It was shown in Sections 7.4.3 to 7.4.5, for the conditions of interest in the experimental study for this Chapter, that the gas permeable bag system would be sufficient to maintain oxygen concentrations in the preservation medium containing T cells. The conditions were; cells at a density of $10x10^6$ live cells/ml; suspended in a gas permeable bag intended for cell culture; and, loaded in a medium volume recommended by the bag manufacturer.

The use of standard materials is not a universal method. Avgoustiniatos et al. showed that a two-dimensional approximation of the islet structure supported inside an FEP bag experienced anoxia, defined as a partial pressure of oxygen at 0.1mm Hg, when computational modelling of preservation conditions for the shipment of the islets was

completed [314]. Islets are particularly sensitive to low oxygen content because they lack enzymes for energy production under anaerobic conditions [317]. Specific results may be dependent on cell type. The requirement for additional oxygenation of islets may also be a consequence of their tissue-like structure compared to isolated, suspended T cells. The islet structure could require greater intervention to maintain oxygen concentrations during preservation, much like it has been shown Kron et al. to be required for organs during hypothermic machine perfusion [87,308]. The investigation for oxygenation during preservation must be completed for each type of biological model.

There may be a need to produce experimental data to support mathematical modelling of oxygenation. A study by Avgoustiniatos et al. that showed negative results for a gas permeable bag used an Arrhenius relationship, with coefficients to adjust the oxygen consumption rate with reducing temperature taking assumed values. They did not collect data for an actual response of islets to reduced temperature. Using this assumed method, they showed that silicone membranes, as opposed to FEP bags, provided better oxygenation of the islets when preserved at 22^{o}C. For the modelling shown in Sections 7.4.3 to 7.4.5, the dependence of oxygen consumption on temperature was measured (see Section 7.4.1) and these results were used, in conjunction with established oxygen consumption rates at 37^{o}C, to determine requirement for external oxygenation of T cells. This approach makes the model and results presented in this Chapter more robust in their use to determine if the FEP bag system would be suitable for storing T cells during hypothermic preservation.

The model found that some deviations from the ideal system could reduce the available oxygen concentration. Firstly, increasing the cell density to increase the dose might require different bag geometries to preserve the cells without inducing low oxygen regions. Cell densities for infusion could be changed when dose escalation trials of the therapy are performed. For example, in an allogeneic CAR-T trial the dose ranged from 0.4x10^{6} to 8.2x10^{6}/kg [36], and Cytokine induced killer T cells have been trialled at quantities of 1x10^{9}

to $1x10^{10}$ cells infused in 300ml of Normosol buffer [62]. These examples suggested that there could be a large concentration difference amongst T cell suspension to be preserved, and that the concentration ranges tested in this computational study were representative of the therapy administration. Sections 7.4.3 to 7.4.5 showed that T cell density during hypothermia does not deplete oxygen concentration until extremely high cell densities of $100x10^6$ cells/ml are required for infusion of the therapy.

Secondly, Sections 7.4.3 to 7.4.5 also showed that there was some flexibility in the volume of medium that an FEP bag could be filled with, and therefore medium depth, to avoid conditions of hypoxia. One result of particular interest from testing of the medium depth was the case where one side of the FEP bag was obstructed during the storage, which produced decreased oxygen concentrations in the cell suspension. This result suggested that a gas permeable bag may have to be packed externally with a material that allows atmospheric gasses to reach all surfaces of the bag during preservation and transportation.

Subject to the constraints discussed above, the overall conclusion from the computational study was that sufficient oxygenation would be available to avoid hypoxic conditions during the extension of $10x10^6$ cells/ml T cell preservation in a standard cell culture format for 5 days. Therefore, an extended duration of preservation using the methods developed in Chapters 3, 4, 5, and 6 can be investigated experimentally without the introduction of oxygen concentration as a variable.

7.4.7 Extended hypothermic preservation of T cells in cell culture formats.

The following study used standard cell culture formats to contain the T cells during hypothermic preservation and provide the oxygenation, which was shown by computational investigation to be sufficient. The effect of extended hypothermia on preservation of CD3/CD28 activated T cells was examined by identifying the cell membrane integrity, see Figure 7-7. Two interventions to cell preservation were tested. Firstly, activated T cells were subjected to a double thymidine block to synchronise the cells to the G1 phase of the cell

cycle, before the application of hypothermia. Secondly, thymidine blocked T cells in combination with 10% (wt./vol.) Ficoll PM70 (ficoll) were added to a modified UW (mUW) preservation medium prior to hypothermia. In conjunction with these interventions, the cell populations were exposed to two temperatures of hypothermia and Figure 7-7 shows results for all four conditions tested. The four conditions all resulted in relatively stable viable cell counts at completion of 5 days of hypothermia. The minimum normalised average came from thymidine blocked with ficoll added cells, which was recorded at 0.82±0.12 after 5 days of hypothermia at 22^{o}C. Comparison between the four conditions by two-way ANOVA found that there was no significant difference in viable cell count between the thymidine treated groups and those with combined with ficoll. However, there was a significant difference between temperatures, where groups at 22^{o}C resulted in significantly lower viable cell count compared to 4^{o}C. Within each group, only thymidine treated cells preserved at 4^{o}C resulted in no significant decrease in viable cell number compared to the value on day 0, see Figure 7-7A. The introduction of ficoll to the medium at 4^{o}C caused significant decrease in viable cell count after 96 hours onwards. Overall, the majority of T cells remained viable with increased preservation time during this study, which suggests that the application of a thymidine block and addition of ficoll to the preservation medium can allow preservation up to 5 days with only small decreases in viable cell number. These small decreases should be acceptable in application of the therapy [69]. A 5-day preservation may also allow more regulatory release tasks to take place during hypothermic preservation of the cells [33].

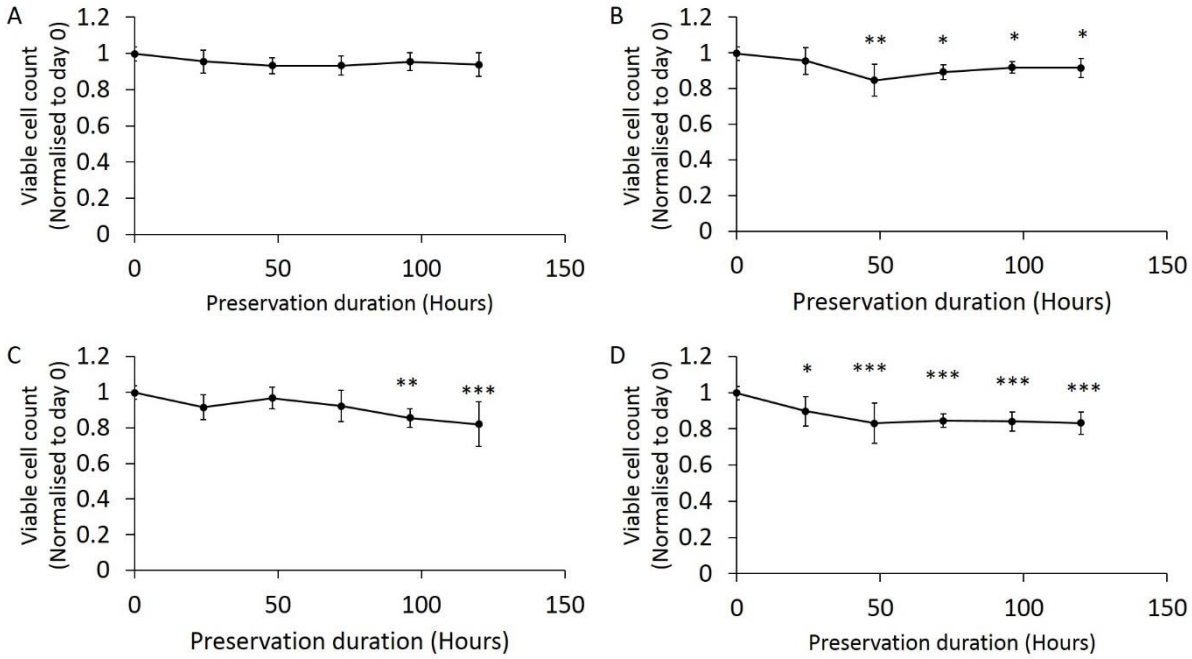

Figure 7-7 The effect of 5 days of hypothermia on the viable cell count of CD3/CD28 activated T cells. Viable cell count was determined by imaging cytometry of total number of cells present, exclusion of Draq 7, comparison to day 0 viable cell count, and the small change in volume of medium present. A) Combination of 4°C and thymidine treatment. B) Combination of 22°C and thymidine treatment. C) Combination of 4°C, thymidine treatment and ficoll supplementation. D) Combination of 22°C, thymidine treatment, and ficoll supplementation. See Section 3.2.10 for the explanation of statistical analysis and consequent labelling. N =6.

Metabolic activity on recovery was measured by the reduction of Alamar blue and the results are shown in Figure 7-8. These results showed that the activity recovered after hypothermia was not as stable compared to the membrane integrity (see Figure 7-7) with a preservation duration up to 150 hours. At 4°C, thymidine paused cells displayed no significant decrease in metabolic activity recovered from day 0 quantities until 4 days of preservation, whereas at 22°C, there was a significant decrease in metabolic activity recovered after day 1 of hypothermia, which decreased to near undetectable levels from day 2. Supplementation of the thymidine treated cells with ficoll in the preservation medium at 4°C displayed no significant difference from day 0 metabolic activity: there was decreased activity at 3 days, but this did not become significant during the 5 days of preservation. At 22°C, ficoll supplementation also arrested the decline in metabolic activity during short-term preservation up to 2 days: after this period there was a rapid decrease in the metabolic activity recovered. Overall, the combination of thymidine block and ficoll at 4°C preservation

temperature maintained a significantly higher metabolic activity after 5 days of preservation, after normalisation for the differences that were observed on day 0.

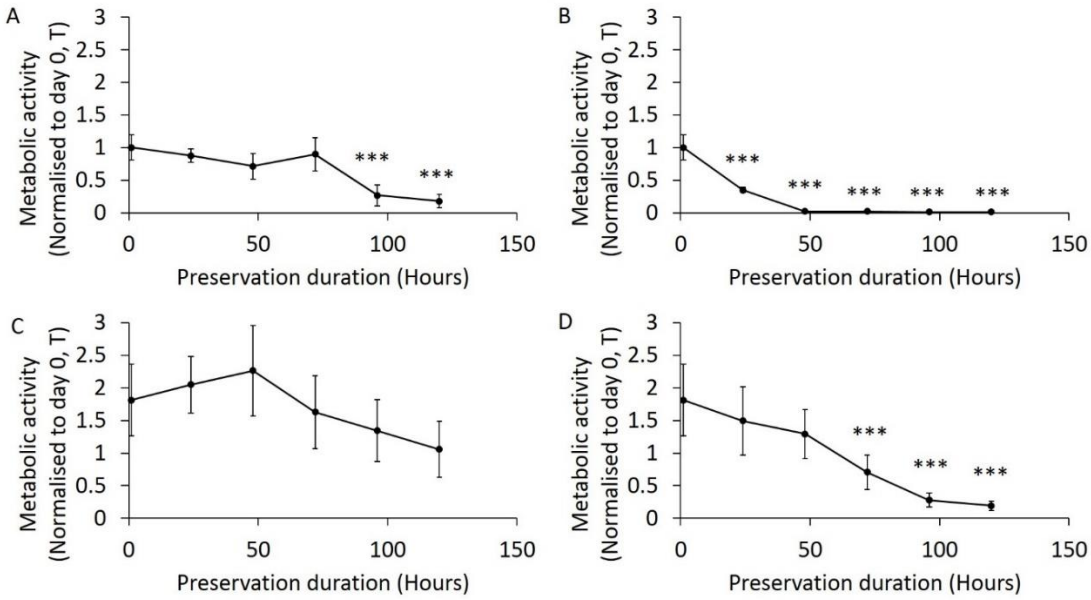

Figure 7-8 The effect of 5 days of hypothermia on the metabolic activity upon recovery to 37°C after preservation of CD3/CD28 activated T cells. Metabolic activity was assayed by the reduction of Alamar blue over a four-hour period at cell culture temperatures. A) Combination of 4°C and thymidine treatment. B) Combination of 22°C and thymidine treatment. C) Combination of 4°C, thymidine treatment, and ficoll supplementation. D) Combination of 22°C, thymidine treatment, and ficoll supplementation. All values were normalised the reduction produced by thymidine treated cells on day 0, with individual background readings subtracted. See Section 3.2.10 for the explanation of statistical analysis and consequent labelling. N = 6.

The addition of ficoll to the thymidine treated cells was shown in Figure 7-7 to minimise the decrease of metabolic activity on recovery after hypothermia with extended 5-day preservation, therefore, cell activity during the hypothermic preservation was investigated. The first indicator of cell activity tested was the concentration of intracellular ATP present at different time points, because a decrease of that parameter of control levels would indicate a difference between metabolic production and biochemical consumption reactions. The results relating to ATP concentration are shown in Figure 7-9.

With thymidine treatment at 4°C, the concentration of ATP remained relatively stable with only a small decrease at day 4 of preservation, but this became a larger decrease at day 5. At 22°C, large, significant decreases in ATP were observed following 1 day of preservation and almost all had gone by day 2.

At 4^{o}C, supplementation of the preservation medium with ficoll had a small negative effect on ATP concentration with a significant decrease occurring 1 day earlier than the thymidine treated group at the same temperature. There was, however, no significant difference detected between the two conditions in two-way ANOVA. Supplementation at 22^{o}C produced a noticeable delay in the decrease of ATP concentration during the preservation period with no significant decrease occurring until 3 days of preservation, compared to the un-supplemented condition, which decreased significantly after 1 day, see Figure 7-9D and Figure 7-9B, respectively. There was no significant difference between the ATP content of T cells preserved with the ficoll additive between 4^{o}C and 22^{o}C, see Figure 7-9D and Figure 7-9C.

At five days, there was no significant difference in the ATP between the four conditions tested in two-way ANOVA, which suggested that all populations had experienced similarly decreased ATP content compared to initial concentrations.

Chapter 5 and Chapter 6 showed that, if the T cells maintained some ATP and metabolic activity on recovery, then these cells displayed the same cytotoxic effect compared to before preservation. The small, non-zero quantities of metabolic activity on recovery shown in Figure 7-8 study suggest that cytotoxic activity may be recovered beyond three days of hypothermic preservation.

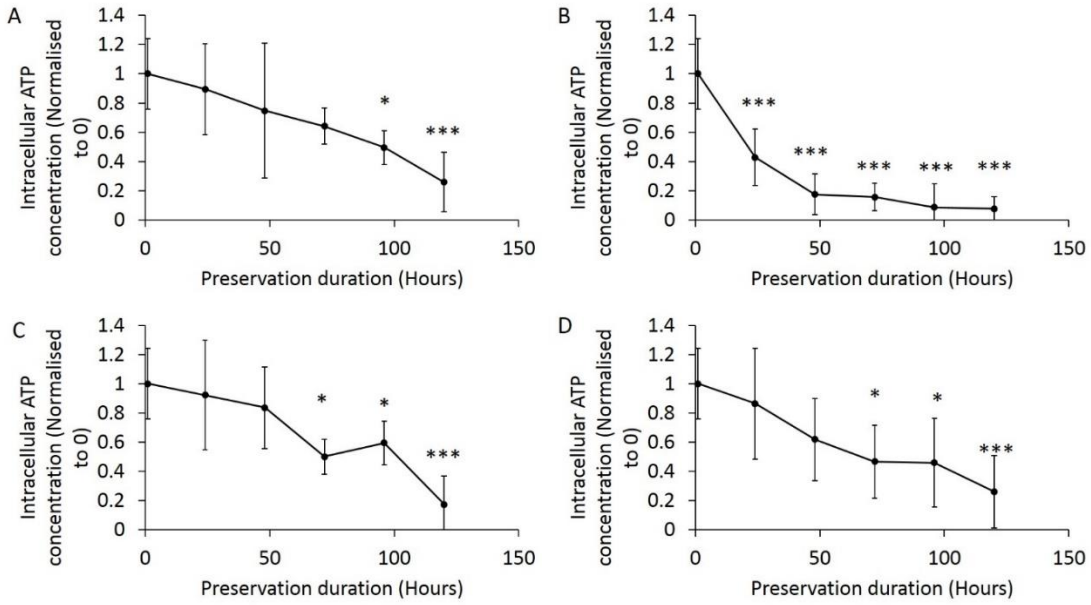

Figure 7-9 The effect of 5 days of hypothermia on the intracellular ATP content of activated T cells with a combination of four different treatments. ATP was measured from independent samples for each time point. A) Combination of 4ºC and thymidine treatment. B) Combination of 22ºC, and thymidine treatment. C) Combination of 4ºC, thymidine treatment, and ficoll supplementation. D) Combination of 22ºC, thymidine treatment, and ficoll supplementation. Intracellular ATP was measured by lysing cells, and examining supernatant with a firefly luciferase assay. A $\log_{10}$ transform of the data was required to validate ANOVA assumptions. See Section 3.2.10 for the explanation of statistical analysis and consequent labelling. N = 6.

The second indicator of metabolic activity during preservation was the accumulation of lactate, which would indicate the continuation of glycolytic metabolism [136,137,146]. The results in Figure 7-10 show the concentration of lactate found in the supernatant of the preservation medium. At 4ºC, with thymidine treatment only, the preserved T cells produced only a small quantity of lactate during the 5 days of hypothermia, see Figure 7-10A. At 22ºC, the thymidine-only treatment produced more lactate compared to 4ºC, with a continually increasing concentration. This rate of lactate production remained fixed with a linear trend fitting the profile with little error, see Figure 7-10B. Supplementation of the preservation medium with ficoll increased the rate of lactate production at both temperatures. The increase in the rate of production was small at 4ºC, see Figure 7-10C. The results showed a significantly increased rate of production at 22ºC, see Figure 7-10D. Both increased temperature and supplementation of the preservation medium with ficoll increased the metabolic activity of the T cells during hypothermia.

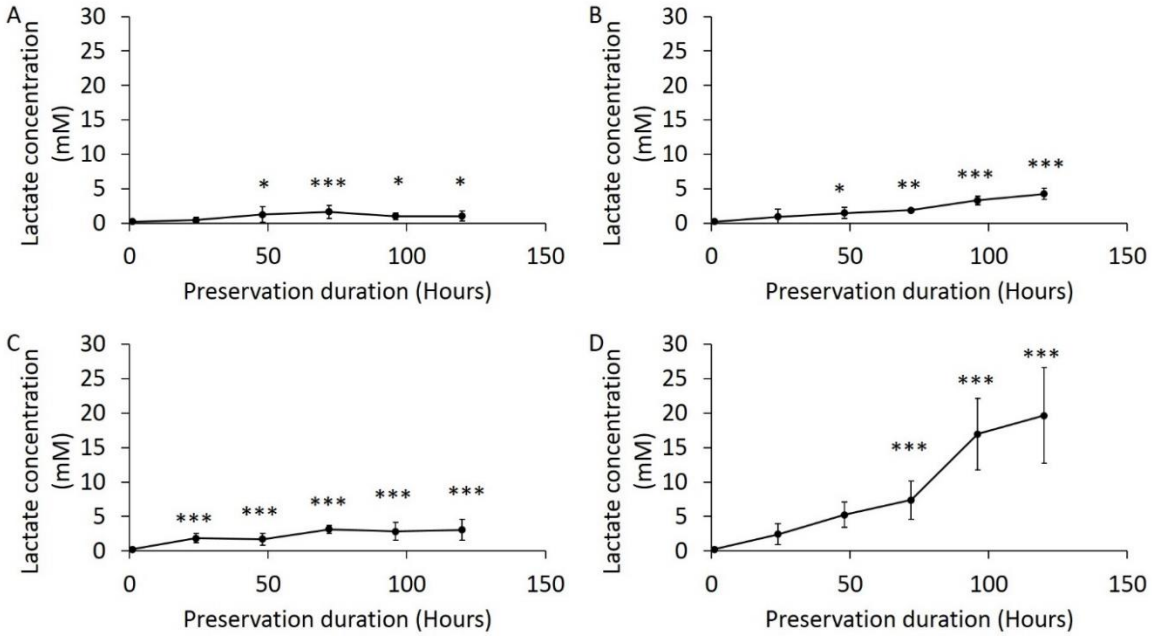

Figure 7-10 The effect of 5 days of hypothermia on the extra cellular concentration of lactate present in the preservation medium. Samples were taken from independent samples for each time point. Lactate concentration was measured by enzymatic assay of medium supernatant. A) Combination of 4°C and thymidine treatment. B) Combination of 22°C and thymidine treatment. C) Combination of 4°C, thymidine treatment, and ficoll supplementation. D) Combination of 22°C, thymidine treatment, and ficoll supplementation. See Section 3.2.10 for the explanation of statistical analysis and consequent labelling. N =6.

As a final study in Chapter 7, the long-term in-vitro recovery of hypothermic T cells was investigated by tracking the viability of the cells on return to normothermic conditions. Firstly, T cells preserved with only thymidine treatment and a temperature of 4°C mainly displayed no significant change to day 0 viability after 24 hours of recovery to normal conditions, see Figure 7-11A. There was a small decrease after cells had been preserved for 120 hours followed by 24 hours of recovery. Increasing the duration of normal conditions to 48 hours showed that the decrease in viability of the recovered cells became greater, with significant difference existing between the two durations of normothermic recovery.

At 22°C, considering thymidine treatment only, there was a much greater decrease in recovered viable cell count after the initial 24 hours of recovery compared to the previous 4°C preservation. This effect can be seen by a greater significant difference occurring after the shortest duration of preservation, see Figure 7-11. Following a further 24 hours of recovery of cells from 22°C, the cell viability decreased further.

At 4°C with ficoll supplementation, the decrease in membrane integrity became significant after 24 hours of preservation followed by 24 hours of normothermic recovery. There was a further significant decrease after 48 hours of recovery. Finally, cells preserved at 22°C with ficoll additive displayed the lowest recovered viabilities after both 24 hours of recovery and 48 hours. Hence, the addition of ficoll was suggested to negatively impact the long-term recovery of the preserved T cells.

The results of Figure 7-11 showed great promise that the long-term recovery would be achievable, even after extended preservation to 4 days, because the T cells remained viable after 24 hours. Maintaining cell viability for this duration could take them beyond the onset of apoptosis that occurred during the exposure to hypothermia. This was true for preservation after 4°C hypothermia; however, cells preserved at 22°C recovered with a decreased viable cell count after 24 hours of recovery. This decreased viable cell count confirmed that apoptosis of T cells was greater after preservation at the warmer temperature compared to 4°C. The results of apoptosis during preservation were presented in previous sections, where it was found that cells preserved at 22°C showed greater phosphatidylserine exposure and nuclear condensation. See Section 4.3.9 for results related to apoptosis. The attribution of cell death in the first 24 hours of recovery to apoptosis that occurred during hypothermia is justified by the knowledge that the process of apoptosis takes 2 to 3 hours to complete [318]. Therefore, apoptosis was not a process that would quickly result in a reduced viable cell count immediately after recovery from hypothermia. There was a lower amount of apoptotic cells in supplementation-free medium at 4°C.

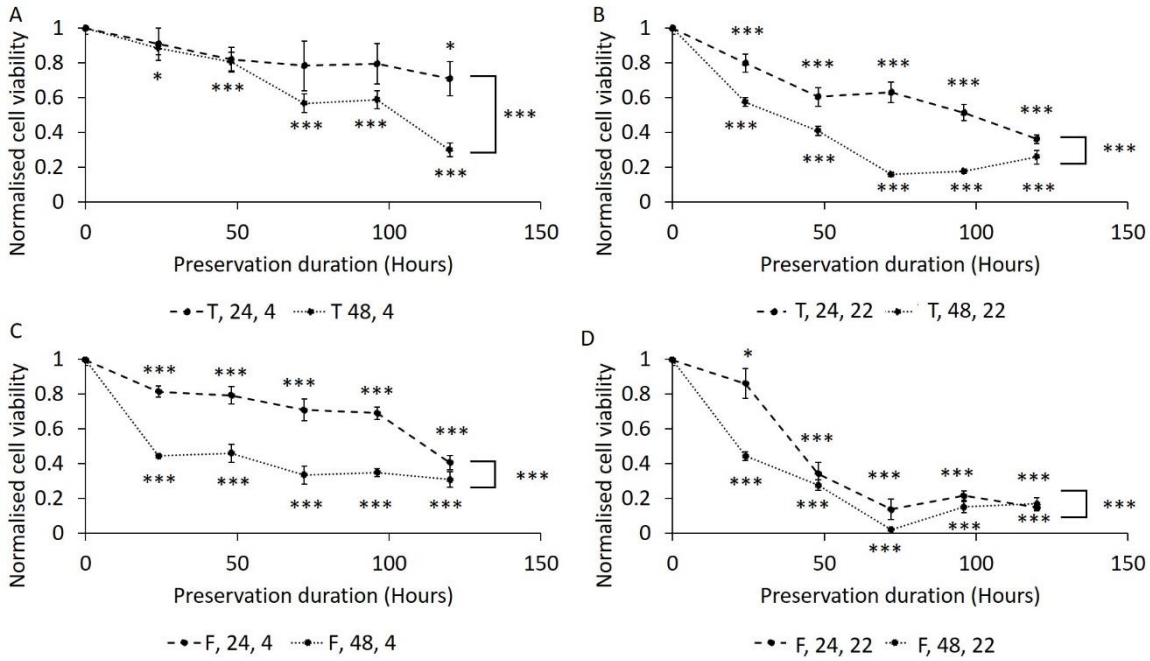

Figure 7-11 The effect of 5 days of hypothermia on the viable T cell number after recovering from preservation and returning to normal culture conditions. Recovery from preservation was performed by diluting cells to concentration of $1x10^6$ live cells/ml in complete cell culture medium and incubating at 37°C in a 5% carbon dioxide incubator. Cultures were tested for viable cell count after 24 hours and 48 hours of recovery culture. Two-way ANOVA with Tukey post hoc tests found that, in all cases, the viable cell count fell significantly after 48 hours of culture. A) Combination of 4°C and thymidine treatment. B) Combination of 22°C and thymidine treatment. C) Combination of 4°C, thymidine treatment, and ficoll supplementation. D) Combination of 22°C, thymidine treatment, and ficoll supplementation. See Section 3.2.10 for the explanation of statistical analysis and consequent labelling. N = 3.

The significant decrease in viable cell count after 48 hours compared to 24 hours of recovery that was observed in all four of the conditions tested may be related to the recovery without suitable antigen stimuli for the T cells. Antigen withdrawal is a well understood mechanism of T cell death. In results of Figure7-11, only IL-2 was supplemented into the medium with the cells suspended at low density for recovery. Such cytokines have been shown by Sadeghi et al. to further reduce the ATP concentration when supplemented into T cell cultures [22]. Necrosis of cells is known to be a consequence of insufficient ATP availability [163]. The recovery with only IL-2 present for generating the results of Figure 7-11 may have further depleted the ATP of preserved cells and caused cell necrosis. T cell death after stimulation by removal from antigens is a well described phenomenon. Hildeman et al. showed that this death took place over a period of days [319], whereas, Mitall et al. showed that cell death due to reactivation by CD3/CD28 took place in less than 16 hours [126]. The cell death after

recovery shown in Figure 7-11 took place between 24 and 48 hours of recovery. This period of days suggests that the cell death observed was partly a result of withdrawal of the T cells from antigens. A more suitable recovery procedure may be required to avoid cell death.

The decrease in viability towards 48 hours of recovery reported in this study could also be related to molecular mechanisms that are activated during hypothermia, but only led to the commencement of apoptosis on recovery to normal conditions. Salahudeen et al. showed there was activation of molecular mechanisms, that were related to apoptosis in human renal cells, after recovery to normal culture conditions from hypothermic storage [320]. They found a maximum in caspase-3 expression at 25 hours of normothermic recovery. This time-scale of cell death was similar to that observed in T cell death after preservation shown in Figure 7-11. Uemura et al. reported that antioxidant medium additives reduced the production of Caspase-3 [321]. However, similar antioxidant compounds were already present in the medium used to obtain the results shown in Figure 7-11. Mathew et al. identified that the pathways that activate apoptosis were present after hypothermic preservation in human renal cells, but those pathways could be blocked by pharmacological compounds that inhibit all caspase activity [289]. That requirement for pharmacological compounds was emphasised again by Ginis et al. in a study that attempted the preservation of human mesenchymal stem cells [322]. Further measures to inhibit apoptosis caused by hypothermia may also improve the recovery of T cells shown in Figure 7-11.

Two mechanisms have been identified that could cause the T cell death after preservation found in Figure 7-11. A greater understanding of T cell response to hypothermia is still required to determine whether the reduced recovery presented was related to T cell biology or the biology of hypothermia. However, this Section of the thesis adds the knowledge that the response to hypothermia in activated T cells was more extreme when the cells were preserved at warmer temperatures and included a compound that stimulated cell activity.

The results of this Section showed that treatments during hypothermia, which appeared to stimulate cell metabolism by the supplementation of ficoll at both temperatures or by thymidine-only treated cells at 22^{o}C preservation, produced significantly lower membrane integrity after hypothermia and further reduced that integrity with recovery at 37^{o}C.

Kurano et al. showed that, in culture conditions, 4.4mM of lactate present in the supernatant inhibited the growth rate of Chinese hamster ovary cells [323]. Cobert et al. showed that the lack of accumulation of lactate in the perfusion medium indicated an avoidance of injury to myocardial tissue in the machine perfusion of hearts for transplant [228]. Lactate could be an important parameter to monitor cell activity during hypothermic preservation. Furthermore, the results of this Section showed that it was only the thymidine-only treated cells at 4^{o}C that avoided a build-up of large quantities of lactate in the preservation medium. This was also the only treatment to avoid both significant decreased viable cell number during the hypothermic preservation and lower decreases on recovery. The treatments that supported cell activity during hypothermia, shown by lactate production, did not translate into metabolic activity on recovery, shown by cellular Alamar blue reduction. Therefore, the accumulation of lactate has an important negative effect during preservation.

The cells that were active during hypothermia also displayed a lower viable cell count after 24 hours and 48 hours of normothermic recovery. This suggested that there was an inverse trend between continued T cell activity during hypothermia and recoverability on return to normal conditions. Long-term recovery of the T cells was improved by methods that paused cells without stimulation, which suggested that preserved cells treated without stimulation during hypothermia may have greater persistence on recovery to normal temperatures. The T cells stimulated during hypothermia may not be as effective as the thymidine paused cells because they are not as persistent on return to normal conditions. Persistence of the T cell after preservation would be an important quality parameter to maintain: Yang et al. have shown that long-term persistence of CAR T cells is an important factor for long term disease

remission in patients [41]. To maintain this important factor during hypothermic preservation, the results of this Section show that treatments, which will combine with hypothermia to limit cell activity, must be adopted. To further improve the persistence of T cells preserved under hypothermia, future research should focus on methods to further reduce cell activity and, thereby, compound the reduction in reaction rate already available by hypothermia.

7.5 Summary.

Limited evidence was available about how the oxygen tension would change during the preservation of T cell suspensions under hypothermic conditions. Therefore, Chapter 7 has mathematically investigated the concentration of oxygen dissolved within the preservation medium to establish if decreased oxygen availability could reach levels that have been shown to induce a hypoxia-related change to T cell biology. It was shown that gas permeable bags would make a suitable container for the cells during hypothermic preservation at cell densities typically required for therapies that use T cell infusions. A more general conclusion is that cell culture equipment can be used for hypothermic preservation without introducing any hypoxia related affects. Therefore, a trial was conducted to extend hypothermic preservation to 5 days. This found that membrane integrity was stable during and after hypothermia in conditions that inhibited cell activity during hypothermia, but these cells lost metabolic activity on short-term recovery. A negative relationship was established between stimulating cells during hypothermia and decreased viable cell count with increased duration of in-vitro cell recovery.

8 Conclusions and Future Work.

8.1 Optimisation of preservation medium by design of experiments.

Some historically beneficial components of preservation media that had a negative effect on the preservation of CD3/CD28 activated T cells were identified by Design of Experiments methods, and were removed to significantly improve the membrane integrity after preservation. These components were found in a range of existing publicised preservation media, which shows that it is vital to examine all components of the preservation process to improve results.

The duration of preservation was not included in the DoE design table, but was included in the study by developing several discrete models at day 0, day 1, and day 3. Although the effects of many components remained stable with time, the negative effect of adenosine became apparent with increased exposure to hypothermia, which suggests that, although rare, the requirement for different components may change with increased time of preservation. The use of multiple DoE models over time, even in fractional-factorial models, allowed some indication of the kinetics of preservation to be examined.

The significant improvement in membrane integrity resulting from DoE optimisation was confirmed with further trials, but only in these OFAT-type trials was the large decrease in recovered metabolic activity truly appreciated, which suggested that existing preservation media alone were insufficient to effectively preserve T cells. This study provided an indication that the inhibition of T cell metabolic activity during hypothermia would benefit the recovery of activity afterwards, as shown by the positive effect on the metabolism of T cells on recovery by a component that was suggested to induce autophagy in human cells by other studies, and by a component that was shown in this study to non-destructively inhibit glucose metabolism. Because significant effects for improvement were being reported, the relative nature of the DoE models made it difficult to understand the large quantity of metabolic activity that was lost, which highlights the need to have a functioning process

before using DoE for optimisation. The need to improve metabolic activity on recovery became an objective for the following studies.

8.2 Effect of antioxidants on T cell preservation.

The addition of metabolism stimulating compounds had little to no effect towards 3 days of hypothermia: the only lasting effect was the inhibition of apoptosis at 4^{o}C provided by ATOC, although these compounds slightly stimulated the metabolism initially, which was in agreement with a number of other studies on different biological models.

Glutathione is the major component in cellular defence against ROS, and this compound produced a significant increase of cell metabolic activity recovered after preservation when included at a concentration of 1mM or above; increasing beyond this concentration towards that of pre-existing preservation media reduced the activity recovered. Formulation of the preservation medium must, therefore, consider the recovery of cells with components in the preservation medium present: even antioxidant concentration and type can have a profound effect on cell recovery after preservation. The supplementation of the modified preservation medium suggested that the support of T cell metabolism in this medium could not be achieved to avoid the loss of function established previously.

Apoptosis of T cells during hypothermia proceeded to a much greater extent at 22^{o}C compared to 4^{o}C. This was not expected, because all existing literature suggested that a product containing live cells would experience more injury with greater reductions in temperature but with a reduced requirement for metabolites. The model of activated T cells used in this study can be suggested to oppose this notion, which suggests that the apoptotic response of different cell lines to temperature may vary.

8.3 Pre-treating and pre-conditioning T cells to improve viability and functionality after hypothermic preservation.

This study confirmed that inhibition of T cell activity before hypothermia is a requirement to achieve functionally recoverable T cells from preservation: cryopreservation pre-

conditioning avoided T cell death during hypothermia, but this did not result in improved activity after recovery from hypothermia. Improvement to activity on recovery was provided by interventions that forcibly inhibited proliferation, through synchronising T cells to a resting G1 phase of the mitotic cycle, which was maintained during hypothermia. The inhibition of mitosis as a pre-treatment, in combination with pre-conditioning, resulted in no difference of therapeutic action between fresh cells and preserved cells after 3 days of 4^{o}C hypothermia. The inhibition improved preservation at 22^{o}C but did not achieve full recovery of therapeutic action of cells preserved at this temperature. Altogether, these results suggested that the activity of T cells during preservation must be reduced before commencing hypothermia to achieve successful preservation.

8.4 Cell sedimentation and its effect on T cell preservation outcome.

This study indicated that the settling of T cells was not negatively affecting their hypothermic preservation at 4^{o}C, but complete suspension by encapsulation did inhibit a decline of ATP during preservation, which indicated that the production and consumption of the nucleoside triphosphate was balanced at this temperature. However, the cells did not recover function with the gelatin, the component that allowed encapsulation, remaining present. The saccharide component, ficoll, which reduced settling velocity but only delayed formation of a sediment, had a large effect on stimulating T cell metabolism during preservation and on recovery, and this component also led to normal therapeutic action on recovery. The stimulation during hypothermia was not related to direct metabolism of the ficoll because the compound was inhibitory on T cell proliferation and metabolism at 37^{o}C. The behaviour of isolated cells with the high molecular weight saccharides requires further research to develop a biological understanding of the mechanism that provides their ability to both stimulate and inhibit cell activity, as reported in this study. The stimulation of T cell activity at 22^{o}C did not facilitate preservation at this temperature.

8.5 Oxygen supply and management during hypothermia for extended preservation.

Computational modelling of oxygen diffusion and consumption found that oxygen would not be depleted during hypothermic preservation that used standard cell culture formats, avoiding the requirement to perform this investigation practically. The results from this modelling permitted a study on extending preservation to 5 days without the effects related to oxygen concentration interfering with the results. This study showed that mitotically inhibited T cells, without additional treatment, lost their stability of recovered metabolic activity after 3 days but retained viable cell numbers throughout 5 days of preservation. However, populations supplemented with a metabolic stimulant maintained stability of recovered metabolic activity but suffered from reduced viable cell number during hypothermia. The inhibited-only method retained higher viable cell numbers after return to normal conditions compared to supplemented method that stimulated activity, which suggest that inhibited activity alone is the most suitable method for recovery of T cells after preservation. However, the decline in metabolic activity after 3 days of preservation with inhibited-only activity suggests that inhibition of mitosis does not block all cell activity. Further investigation into the activity that continues during hypothermia is required to extend preservation beyond 3 days.

8.6 Overall conclusion.

This thesis has provided evidence that Design of Experiments (DoE) methods are capable of identifying suitable components that optimise the hypothermic preservation processes. Unsuitable compounds that would have been included in a generic preservation medium formulation were identified and removed, and this significantly improved the results. However, when testing compositions with limited evidence of the mechanism of action, the sole benefit of a marginal decrease in apoptosis from the study did not outweigh the complicated set-up of multiple DoE standards for testing, although a similar large number of OFAT trials would have been required to establish the marginal effects.

The lack of positive results produced by multiple factor DoE models may have been related to the lack of initial evidence: the deployment of multiple DoE models over multiple time points improved on the knowledge presented in existing literature. The DoE models in this study replicated the short term stimulation provided by the antioxidant compound and metabolite supplementation reported in the in existing literature, but also added the new knowledge that the metabolic activity recovered decreased with extended duration of hypothermia despite the continued presence of the additives. Further studies confirmed that the inhibition of T cell proliferation, either by inhibition of the mitotic cycle or by addition of a long chain saccharide, rather than direct stimulation of cell metabolism, was a requirement to achieve preservation.

Although the use of optimised preservation media provided indistinguishable viable cell numbers between hypothermic preservation at 4^{o}C and 22^{o}C, recovery of T cells from the latter temperature was not achieved by antigen addition; inhibition of proliferation; stimulation of cell activity; or a combination of the latter two. This reasonably exhaustive list suggests that the preservation of T cells under ambient conditions may be difficult to achieve. However, the combination of the studies identified a method to achieve the project goals of preserving cells with high viability and maintenance of therapeutic action with a preservation temperature of 4^{o}C and inhibition of T cell proliferation before commencing hypothermia. This preservation was maintained successfully over 3 days, but measured characteristics declined in quality when moving beyond this duration. Therefore, the answers provided by the results presented in this thesis to the research questions posed at the end of Chapter 2 are that the combination of hypothermia and specialised media are insufficient for activated T cell preservation, and that non-frozen preservation is only achieved with novel interventions that forcibly control cell response before, and during, the application of hypothermia.

A review of related literature suggested that cell damage as a result of the application of hypothermia may increase as temperature is reduced towards 0°C. However, this study suggests that in the case study of activated T cells, more cell damage occurs when the cells were exposed to 22°C compared to 4°C. Therefore, in the study of the hypothermic preservation of this cell type, a contrasting graphical conclusion compared to Figure 2-3 is presented in Figure 8-1.

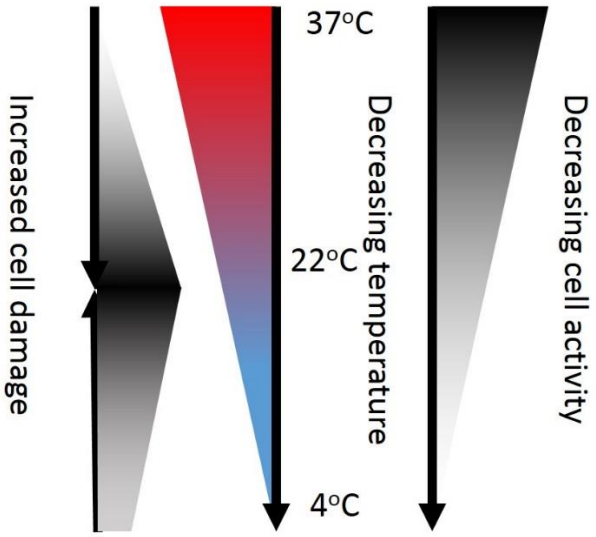

Figure 8-1 Conclusion of cell damage with hypothermia for the specific case of T cells. From the results presented throughout this study, it can be concluded that activated T cells experience more damage at 22°C compared to that experienced at 4°C preservation. However, damage at 4°C was increased over 37°C, which may limit extended application of this low temperature for preservation purposes. Activity during hypothermia decreased continuously as expected with decreasing temperature.

8.7 Future work.

This thesis has identified a method to achieve the preservation of T cells, without the need for cryogenic temperatures, by inhibiting cell proliferation before the onset of hypothermia. However, preservation extended beyond the 3 days fully accomplished in this study may be desirable to allow greater flexibility of tasks to be completed during the preservation. This thesis also sets the direction for inhibiting further T cell activities before attempting hypothermia to achieve longer duration of preservation. The inhibition of both proliferation and metabolic activity before preservation was identified in this thesis, but it was apparent

that other mechanisms were still affecting the cells after extended 5-day preservation due to loss of metabolic activity. These mechanisms should be identified in future work to extend the time period for which activated T cells can be preserved in hypothermic conditions.

Section 2.6.5 discussed the instability in the quantity and population of the RNA sequence occurring between day 3 and 4 of hypothermic preservation, but this was related back to the onset of apoptosis [173], which, because of the difficulty in avoiding a reducing viable cell number on recovery of the T cells after 3 days of hypothermia, the results in this thesis would agree with. Research to gain a better insight into the molecular mechanisms that cause apoptosis from low temperature exposure may be valuable, and is detailed as a requirement in recent forward looking reviews on the established cryopreservation process [14]. It is suggested that RNA silencing (iRNA) techniques may achieve extended preservation by inhibiting caspase activity [324]. This is suitable for organs where machine perfusion can be used to remove the iRNA before implantation [325]. However, for a CGT product in suspension in liquid medium, it may be challenging to remove the additive before infusion without further processing. Further studies are required to investigate the in-vivo effect of preservation media additives, similar to those that investigated ME_2SO infusion with a cell therapy [15]. A biological study into the inhibition of apoptosis-forming pathways may be supplemented by an engineering study to identify methods of removing such a compound before infusion of the CGT product.

Materials to reduce cell sedimentation were investigated in this thesis, and it was found that these have a profound effect on cell metabolism during hypothermic preservation. A further use for these materials may be in avoiding cell damage that could potentially arise from the physical effects of transportation. Stem cells have been shown to suffer cell damage as a result of mechanical loading that could occur during transport [209]. However, the proposal to use gas permeable bags investigated in this thesis would avoid the need for the gas-containing headspace used in that mechanical study, which could have allowed movement

of the bulk liquid and, as a result, the development of shear forces. Further investigations would be beneficial to develop further understanding of the effects that physical loading could produce during preservation.

The studies in this thesis used a limited dataset to establish a 3 day preservation window with no loss of function and the avoidance of wastage, however, larger trials may be required to confirm if this is applicable to a larger population of CGT products and to fully validate that wastage is avoided at this duration. This increased data set could then be used in a cost of goods model to verify that the proposed methods have reduced the cost burden of transportation, suggested to become a challenge as CGT products are scaled-up in production volume [10].

The requirement for forced inhibition of T cell proliferation before hypothermia, which successfully achieved preservation at 4^{o}C and offered slight improvement at 22^{o}C, is similar in nature to the controlled decrease of cell activity in hibernating animals, although there are suggestions that the latter can be achieved without much cooling below body temperatures [163,326]. Should the logistics chain of CGT products require successful preservation at 22^{o}C, or similar temperatures at ambient conditions to reduce energy, it can be suggested that the regulating mechanisms used by hibernating animals be replicated and applied to human cells to further reduce cell activity beyond mitosis inhibition.

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