Novel cryo-protective agents to improve the quality of cryopreserved mammalian cells

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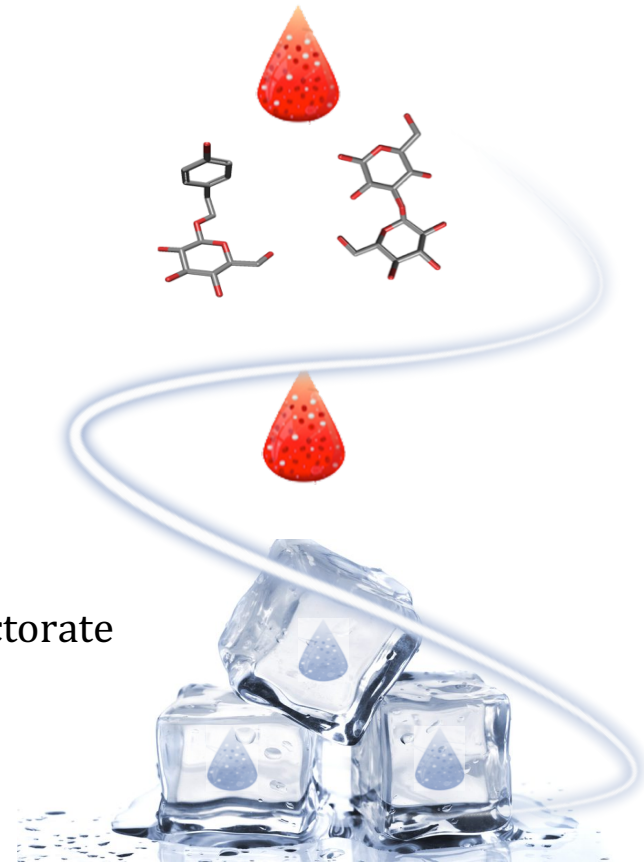

A thesis submitted for the degree of Doctorate of Philosophy- Department of Chemical Engineering and Biotechnology

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To the memory of my father
"Abdullah Bin Mulaafikh" (1939-2015 AC)
Who introduced me to the real world and armed me with knowledge, courage and dignity. A man who had a timeless influence on my life and without his support I would not be able to pursue my education and my dreams would not be real. Hence, I owe him my past, present and future accomplishments.

To my wonderful mother "Dalal Alaamriah" for her unconditional love and prayers

To my beloved husband "AbdulRahman" for being a wonderful partner, motivating and supporting me throughout this journey

To my beautiful little daughter" Heaven", who participated in my thesis writing by pressing the keyboards randomly and excitingly, making me check and correct constantly.

And finally, to my siblings for being always there for me

Abstract

Cryopreservation is a promising approach to long-term biopreservation of living cells, tissues and organs. The use of cryoprotective agents (CPAs) in combination with extremely low temperatures is mandatory for optimum biopreservation. CPAs (e.g., glycerol, trehalose, dimethyl sulphoxide (DMSO)), however, are relatively cytotoxic and compromise biopreserved cell quality. This is usually resultant in oxidative damage, diminishing cell functionality and survival rate. The growing market of cell therapy medicinal products (CTMPs) demands effective cryopreservation with greater safety, of which the currently available CPAs are unable to provide.

The present study was aimed at developing cryomedia formulation to enhance the cryopreservation of nucleated and anucleated mammalian cells. Here, eleven compounds of a polyol nature were selected and examined for their cryoprotective properties. These compounds are derived from plants and honey, thereby ensuring their safety for human consumption. The selection was based on their molecular structure and chemical properties.

Here, the presented study is divided into three main phases: 1) Screening the compounds panel for cryo-additive effects on cells during and post-cryopreservation and optimising the dose response and time course for trehalose and glycerol with and without the novel compounds; 2) Assessing the influence of biophysical criteria on biospecimen cryopreservation (e.g., bio-sampling procedure, cell age, donor age); 3) Establishing the mechanisms of action underpinning the modulatory effect of novel CPAs on biological pathways during cryopreservation.

For the stated purposes, red blood cells (RBCs) obtained from sheep and humans were used to screen the compounds for novel cryo-additive agents. Cryosurvival rate was employed as an indication of the compounds' cryoprotective performance. Cellular biochemical profiles, including lipid and protein oxidative damage as well as key redox enzymatic activities (e.g., lactate dehydrogenase (LDH), glutathione reductase (GR)) were measured. The study revealed that nigerose (Nig) and salidroside (Sal) were significantly effective in protecting cells during the freeze-thaw cycle and recovery phases. Both compounds promoted the activity of GR and reduced oxidative stress mirrored by diminished LDH activity. This was also reflected in the protein and lipid oxidation levels, which was limited to a comparable level with the cells' prior freezing.

Further studies on human leukaemia (HL-60) were carried out to elucidate the molecular and biological pathways associated with cryodamage and the modulatory effects of adding novel CPAs. The proteome profile and the corresponding biological functions were evaluated and

showed that Nig and Sal protected cells against cryodamage. The additive compounds (Nig and Sal) demonstrated a unique and overlapping modulation effect pattern. Nig was found to highly influence proteins engaged with metabolic and energetic pathways, whereas Sal greatly affected nuclear and DNA-binding proteins.

The current study concluded that novel CPAs have high potency in protecting cells and each compound has a unique effect on the cellular proteome. These features can be applied to designing cryomedia formulae with higher protective efficiency for targeted applications in cell-based therapy and biopharmaceutical industries.

Declaration

I, Noha A. S. Al-Otaibi, hereby declare that this dissertation is the result of my own work I carried out in the Department of Chemical Engineering and Biotechnology, University of Cambridge, unless collaboration is specifically stated in the acknowledgement. In this thesis, the undertaken research utilizing human samples was regulated and licensed by the Human Tissue Act (HTA) in England. This thesis, including references, tables and figures altogether does not exceed 65000 words set out by the Engineering Degree Committee at the University of Cambridge.
None of this work has previously been submitted for a degree, diploma or other qualification at any university. Some of the work in this thesis, however, have been presented at the following conferences:

- Alotaibi N *et al* (2016). 9th Saudi Scientific International Conference, Birmingham, UK
- Alotaibi N *et al* (2016). 6th International conference and Expo on Proteomics, Atlanta, USA
- Alotaibi N *et al* (2017). 2nd Annual Biobanking Summit, Berlin, Germany
- Alotaibi N *et al* (2018). Biospecimen Research Symposium: quality matter, Luxembourg

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Publications

The following are list of publications arising from the present work:

Original articles

- Alotaibi NAS, Slater NKH, Rahmoune H (2016) Salidroside as a Novel Protective Agent to Improve Red Blood Cell Cryopreservation. **PLoS ONE** 11(9): e0162748. https://doi.org/10.1371/journal.pone.0162748
- Alotaibi NAS *et al* (2018) Human Leukaemia cells (HL-60) proteomic and biological signatures underpinning cryo-damage are differentially modulated by novel cryo-additives - Submitted to **GigaSceince**.

Book chapters

- Noha A.S. Al-Otaibi, Juliana S Cassoli, Daniel Martins-de-Souza, Nigel KH Slater, Hassan Rahmoune (2018) Molecular Characterization of Human Leukemia 60 (HL-60) Cells as a Model of Acute Myelogenous Leukemia post cryo-preservation. **Methods and Protocols, Methods in Molecular Biology.** (Chapter 30. In Press).
- Noha A.S. Al-Otaibi, Nigel.K.H. Slater, Hassan Rahmoune (2018) Cryopreservation of Red Blood Cells. **Methods and Protocols, Methods in Molecular Biology**. (Chapter 31. In press).

Peer reviewed conference contributions (results not published elsewhere)

- Al-Otaibi N, Slater N, Rahmoune H (2016) Differential effect of glycerol and trehalose on RBCs cryosurvival quality, **J Proteomics Bioinform** 6:9 (Suppl). http://dx.doi.org/10.4172/0974-276X.C1.083
- Al-Otaibi N, Slater N, Rahmoune H (2018) Key factors affecting the quality of human RBCs cryopreservation. In Biospecimen Research Symposium: quality matter, Luxembourg: **ISBER**. Feb 27-28; P21

Patent filed

- Al-Otaibi, N.A, Slater, N.K.H., Rahmoune, H., (2018) High efficiency cryo-additive agent to improve mammalian cells biopreservation. Saudi Arabia, patent application number PCT/SA2018/000006

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List of abbreviation

Aa	Ascorbic acid
ATMP	Advanced therapy medicinal product
ATP	Adenosine triphosphate
CCM	Curcumin
CPAs	Cryopreservation protective agents
CR	Cryosurvival rate
DMSO	Dimethylsulphoxide
DNPH	2,4-dinitrophenylhydrazine
DPBS	Dulbecco's phosphate buffered-saline
DTNB	Dithiobis(2-nitrobenzoic acid)
DTT	Dithiothreitol
Erl	Erlose
FDA	Food and drugs administration (US)
FITC	fluorescein isothiocyanate
G	Glycerol
G6PD	Glucose-6-phosphate dehydrogenase
Gen	Gentiobiose
Glu	Glutathione
GR	Glutathione reductase
GSH	Glutathione in the reduced form
GSK	GelxoSmithKlines
GTMP	Gene therapy medicinal product
h	hour
Hb	Haemoglobin
HL-60	Human leukemia cells
hRBC	Human red blood cell
ipCPA	Impermeable cryoprotective agent
LCMS	Liquid chromatography mass spectrometry
LDH	Lactate dehydrogenase
Mezo	Melezitose
min	minute

mM	Milli-molar
NADH	Nicotinamide adenine dinucleotide
NADPH	Nicotinamide adenine dinucleotide phosphate
Nig	Nigerose
nm	Nano-meter
PA	Protective agent
PBMCs	Peripheral blood mononuclear cells
pCPA	Permeable cryoprotective agent
PK	Pyruvate kinase
PLP	poly (L-lysine isophthalamide)
PS	Phosphatidylserin
Q3G	Quercetin 3-glucoside
Qur	Quercetin
RBCs	Red blood cells
ROS	Radical oxygen species
SAGM	Sodium adenine glucose mannitol
Sal	Salidroside
sCTMP	Somatic cell therapy medicinal product
sec	second
sRBC	Sheep red blood cell
T	Trehalose
TEP	Tissue engineered product
Tur	Turanose
hct	Haematocrit

1 Biobanking and biopreservation of cell therapy medicinal products

1.1 Introduction

The biobanking of biospecimens has become a promising approach across the healthcare services and food industries. The biobanking of cell therapy medicinal products (CTMPs) plays a critical role in healthcare, such as in the case of red blood cell (RBC) transfusion and bone marrow transplantation, which are highly utilised for treating patients suffering from serious illnesses[1,2]. In 2014, the biobanking market was worth approximately 6.04 billion US dollars in the United States[3] with predictions of continuous growth and expansion in Germany and China[3]. The major advancement in various areas, such as cell therapy, stem cell research, cancer therapy, reproductive medicine and precision medicine, is driving the global growth of biobanking and biopreservation.

At present, there is a serious clinical need for optimal preservation and storage of on-demand biospecimens[4] to assure easy access and immediate use of good quality samples. Cryopreservation meets this demand, however, there are a number of problems that the current practice is fraught with, resulting in a low cryosurvival rate[5], loss of functionality[6], cell instability[7,8] and death owing to limited protection against cryo-injury[9]. Additionally, there is a lack of understanding of how freeze-thaw processes along with cryoprotectants (CPAs) (e.g., glycerol and dimethylsulphoxide) affect cell integrity and function. Such a major gap in our understanding of the molecular basis underpinning biopreservation is currently hindering progress in cell-based therapy, hence, the current perception of applied biopreservation approaches' efficiency has changed.

Previously, the entire focus was on the alteration of the chemical and physical properties of biospecimens in response to the low temperature, ignoring their molecular nature during and after biopreservation. Re-focusing the discovery strategy on the molecular alteration of biopreserved cells is essential for identifying the perturbed pathways and developing new CPAs that can specifically target the cryodamaged pathways that will dramatically enhance cryomedia formulation in order to optimize cell biopreservation and recovery post-thaw.

This chapter reviews the current status on biobanking and biopreservation of CTPM.

1.2 Current status of CTMP industries

The market size of advanced therapy medicinal products (ATMPs), including CTMPs and tissue engineering, has grown quite rapidly over the current decade **(Figure 1.1)**. It has developed dramatically since 2012 to reach 30,000 million US dollars in 2018[10]. Somatic cell therapy medicinal products (sCTMP) are the most active ATMPs studied and adapted by clinicians for patient treatments. They constitute half of the available trails **(Figure 1.2)**[11]. It has a great potential to treat serious diseases, such as cancer, cardiovascular and heart failure, Alzheimer's disease, Parkinson's disease, etc.[12,13].

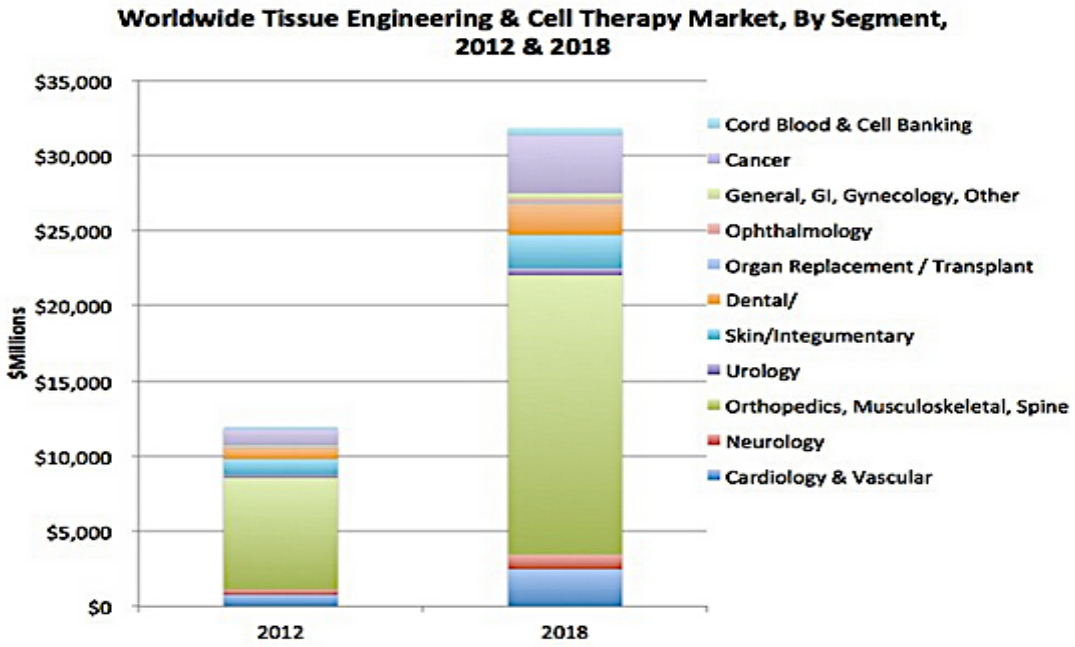


Figure 1. 1 Global market of tissue engineering and cell therapy by clinical application in 2012 & 2018. *(Adapted from MedMarket Diligence, LCC[10])*

There has been major investment in sCTMP by large therapeutic and pharmaceutical companies that attracted to the promising applications of CTMPs. For instance, the pharmaceutical company, Novartis, a leading firm in chimeric antigen receptor in T-cell (CAR-T) molecules, has purchased GlaxoSmithKline's (GSK) cancer portfolio at a cost of 16 billion US dollars and has invested 20 million US dollars in clinical trails[14]. Pfizer and AstraZeneca other large players that have invested in sCTMPs/ATMPs[14]. The huge investments indicate that therapeutic cell industries are entering an exciting and revolutionary era.

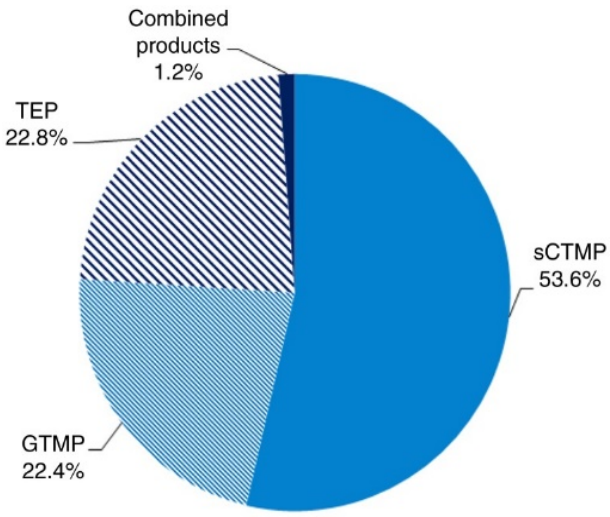


Figure 1. 2 Percentage of advanced therapeutic medicinal products (ATMP). *Somatic cell therapy medicinal products (sCTMP), gene therapy medicinal products (GTMP), tissue-engineered products (TEPs) and combined products (data adapted from Hanna et al.)*[11]

CTMPs, however, has its unique set of challenges. It requires a high degree of technical bioprocesses to meet good manufacturing practices (GMPs) compliance and scalable production. Bioprocessing governs the whole procedure from collecting cells until delivery to the end-patient. It is subdivided into serious steps that must meet the objectives of quality and cost-effectiveness. Failing to meet these objectives will limit patients' access to treatments. In this context, the Provenge company learned a valuable lesson when they adapted the 18-hour non-frozen shelf life of a dendritic cellular immunotherapy product - the short shelf-life involved a highly complex and unfeasible process that eventually was a major obstacle in delivering treatment[15]. From this emanates the need for biopreservation, which is a central procedure in reducing bioprocess costs and facilitating logistics and transportation, therefore, many cell-therapy companies began to develop cell stability through effective biopreservation practices, focusing on cryopreservation[16]. This requires addressing the present challenges of biopreservation and tackling them in order to minimise the risk of wasting resources and ensuring that patients can access high-quality and functional CTMPs.

1.3 Biopreservation challenges

The durability and quality of biopreserved cells are essential to assuring the accessibility of patients to necessary treatments. The most convenient approaches in biopreservation are hypothermia and cryopreservation, which are based on the application of low temperatures to

preserve cells. This permits variant storage duration of CTMPs *ex vivo* that falls between days to decades depending on the selected biopreservation mode.

Most CTMPs are stored under hypothermic conditions (refrigerating), which diminishes cell activity. The fragility and complex nature of stored cells, however, increases their sensitivity to stressors and changes in their environment. As a result, hypothermic conditions have a detrimental effect and compromises cell quality. Conversely, cryopreservation is more powerful. Unlike hypothermic storage, it halts cell activity totally and allows for a prolonged storage duration, minimising the risk of biological contamination and meeting durability conditions. Yet, the toxicity of the applied CPAs and lack of understanding of the biology of cryopreserved cells limits its efficiency and yields low cell quality. This was seen in several examples, such as cryopreserved peripheral blood mononuclear cells (PBMCs), where there was a drastic reduction in the expression of biomarkers versus fresh samples[17], meaning that cells in was unequal to cells out from the cryopreservation. Investigating this variation and causation (e.g., cryomedia, cooling and thawing rate and distinct cell responses), therefore, are essential.

The following chapter provides background of biopreservation principles and discusses the application of CPAs, the freeze and thaw rates, concentrating on cryopreservation and their bioprotection and/or biodestruction considering variant cells' responses. More importantly, it focuses on the molecular biology of cryopreserved cells and CPA's protective effects and their side effects to pave the way for the thesis aims, outline and justification

2 Biopreservation approaches

Cells are usually preserved at different temperatures - either normothermia, hypothermia (4-10°C) or cryopreserved below the freezing point of water. The last two conditions are widely applied to store cells, tissues and organs for therapeutic applications (**Figure 2.1**). Each of these conditions has its pros and cons as explained in the following sections.

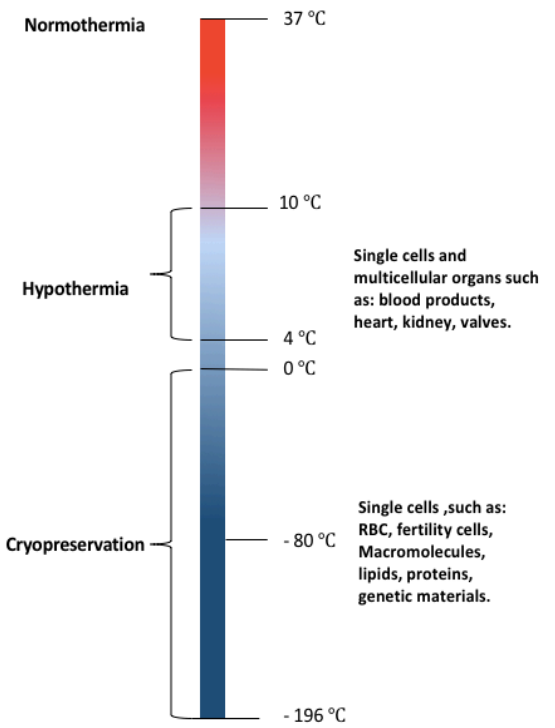


Figure 2.1 Graph illustrating the temperature range of different biopreservation conditions.

2.1 Normothermic conditions

Storing cells at normothermia (or physiological temperature - 37°C) is suitable for cells that will be used immediately or shortly after sampling. This is commonly employed for cell cultures, where they are maintained under controlled conditions (e.g., temperature, pH, CO_2).

In theory, normothermia conditions foster logistics and transportation when maintaining cells in secured containers to avoid leakage. In a recent study, a gel-like capsule media was developed and utilised to stabilize cells during shipment at room temperature, though the gel media can liquefy at 37°C, which may affect cells with gel media compositions, making it unsuitable for warm destinations[18]. Furthermore, it is unknown if the fluctuating temperature under such conditions would influence preserved-cell functionality.

Normothermic conditions, however, are disadvantageous for long-term biopreservation. Cells are subjected to a high risk of contamination if no proper caution is taken during regular maintenance[19]. Plus, the active metabolism and substrate exchange between cells and their environment leads to more ATP production[20], which is concomitant with by-products (e.g., free radical oxygen species (ROS))[21] that impair cell functions. This was seen with RBC oxygen impaired delivery and accelerated process *ex vivo* by ROS[22] and in sperm. The accumulation of ROS during energy production increases lipid peroxidation[21] and affects sperm motility.

Consequently, sperm become exhausted and this eventually leads to low fertility[23]. For these reasons, this approach is limited for active bioprocessing involving cell production, maintenance and utilisation.

2.2 Hypothermic conditions

The use of low temperatures for storing biospecimens has been well-known and established for a century[24, 25]. Unlike normotheria, lowering storage temperatures minimises cellular damage by controlling thermal energy, reducing cellular metabolism and related damaging metabolites[26]. This results in extending the durability of cell storage with a reversible effect[9]. Storage at low temperatures can be divided into two main categories: hypothermia and cryopreservation (**Figure 2.1**).

Hypothermic temperatures are restricted between 10 and 4°C. They have been extensively employed for preserving cells and organs, such as blood products (e.g., RBCs[27] and platelets[28]) as well as the heart, kidney and pancreas[29]. The protocol is easy to apply and permits the extension of the shelf-life of biospecimens for days and up to weeks. Organs like the heart can be stored for 24 hour or less[29], whereas RBCs can be stored for a longer period (up to 42 days)[27,30]. Although hypothermic storage extends the durability of cells, it is still relatively short and cells remain metabolically active, leading to accumulation of metabolites causing irreversible cellular damage[30]. In addition, there is a high risk of microbial contamination, which limits its efficacy.

2.3 Cryopreservation

Cryopreservation is a powerful approach to preserving biospecimens and ensuring long-term biopreservation. Cryopreservation relies on the use of a combination of ultra-low temperatures (-80°C or below) and CPAs to protect cells. It arrests biological activities and maintains cellular structure and function. Such an approach is of great benefit for preserving valuable samples for therapy and research, like in fertility, cancer and life-threatening illnesses treatments as well as genetic and disease screening.

The first application of cryopreservation dates back to 1949, when Polge, Parks and Smith coincidently discovered the use of glycerol as a CPA for cryopreserving spermatozoa[31]. Later, Smith demonstrated that glycerol can be utilised to cryopreserve human RBCs[32], followed by the discovery of dimethylsulphoxide (DMSO) cryopreserving capabilities by Lovelock and Bishop[33]

and its suitability for various cellular biopreservation compared to the former CPA. These sequential findings created the basis of cryopreservation science.

Cryopreservation is a multistep process (**Figure 2.2**). The first step is selecting the appropriate CPA solution and concentration that could be variant depending on cell type and features along with the CPA itself [34]. This also affects the next step, which is the rate of adding CPA to minimise osmotic shock[35] and adjust cells to a new osmotic level if the osmotic gradient is applied. At present, there are few CPAs approved by the Food and Drugs Administration (FDA) for clinical applications, therefore there are restricted choices available clinically. The subsequent step is to change the temperature rate during freezing and thawing, which is another critical point that can affect cells. Finally, there is the removal or dilution of CPA immediately after thawing.

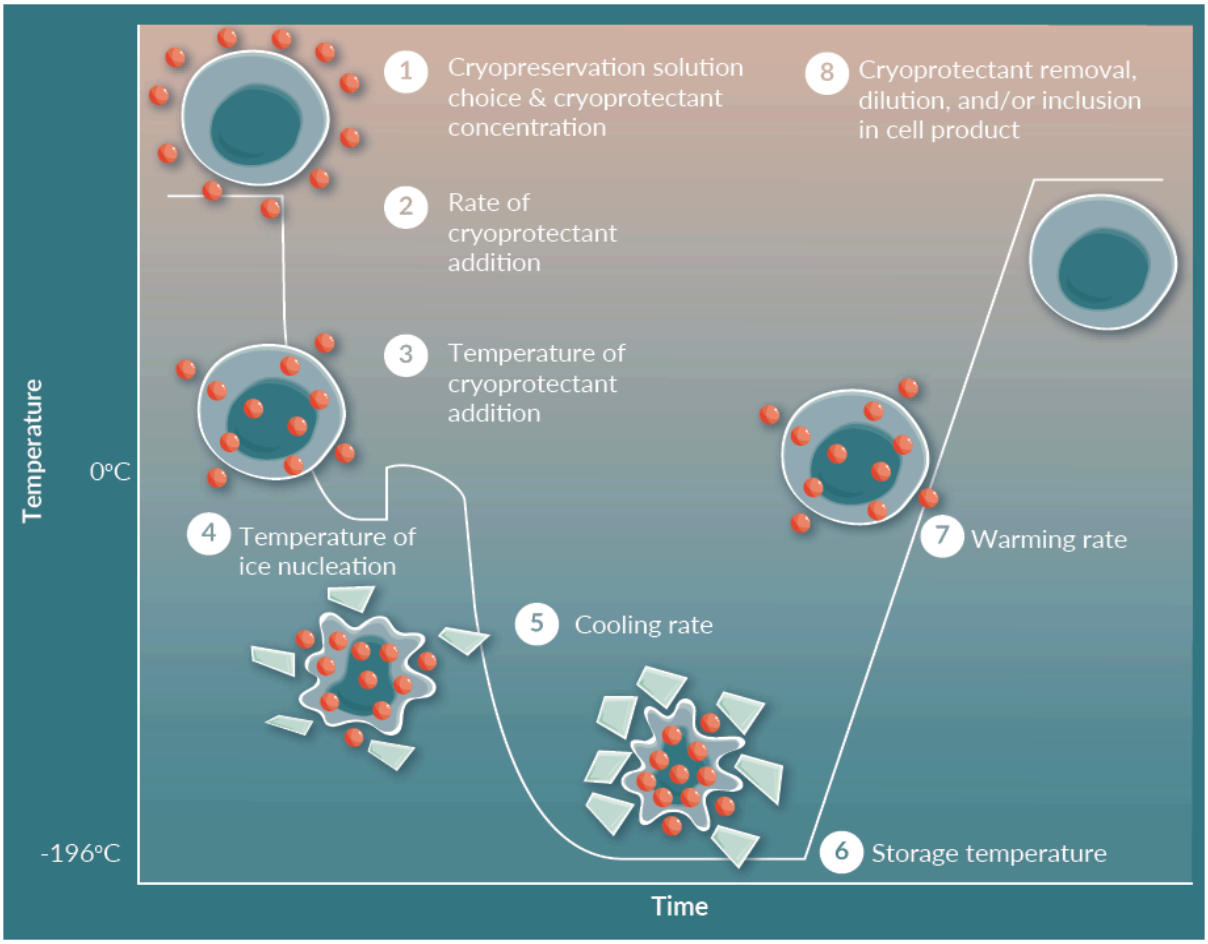


Figure 2.2 Critical steps involved in a cryopreservation procedure. *Depicted is the cryopreservation steps that are subject to risk and optimisation in cryopreservation (adapted from Hawkins et al. [36]).*

2.3.1 Common cryopreservation protocols for mammalian cells

2.3.1.1 Cooling rate

The cooling rate can inflict damage upon cells if the cell character (e.g., size, water and CPA permeability through membrane and cell sensitivity to CPA) as well as CPA concentration are not carefully considered - these factors have a substantial effect on cellular survival rate post-thaw. During slow freezing, cells dehydrate and eventually collapse owing to the formation of extracellular ice crystals concomitant with a gradual increase in osmotic pressure within the cell membrane[37] (**Figure 2.3**). The lengthy exposure to solutes' high concentration is toxic and termed "solution effects"[58,63], which triggers unwanted events (e.g., protein denaturation and loss of membrane integrity)[40,41]. In contrast, fast cooling promotes the formation of intracellular ice crystals[37] based on the incomplete dehydration of the cell[58,60,61,62]. Thus, selecting an optimal cooling rate is important for achieving a sufficient dehydration and freezing without compromising the cells' membrane integrity. Slow or fast cooling, therefore, can be successfully applied, as will be discussed in the following sections, to achieve optimal cooling rates under certain conditions[62,64].

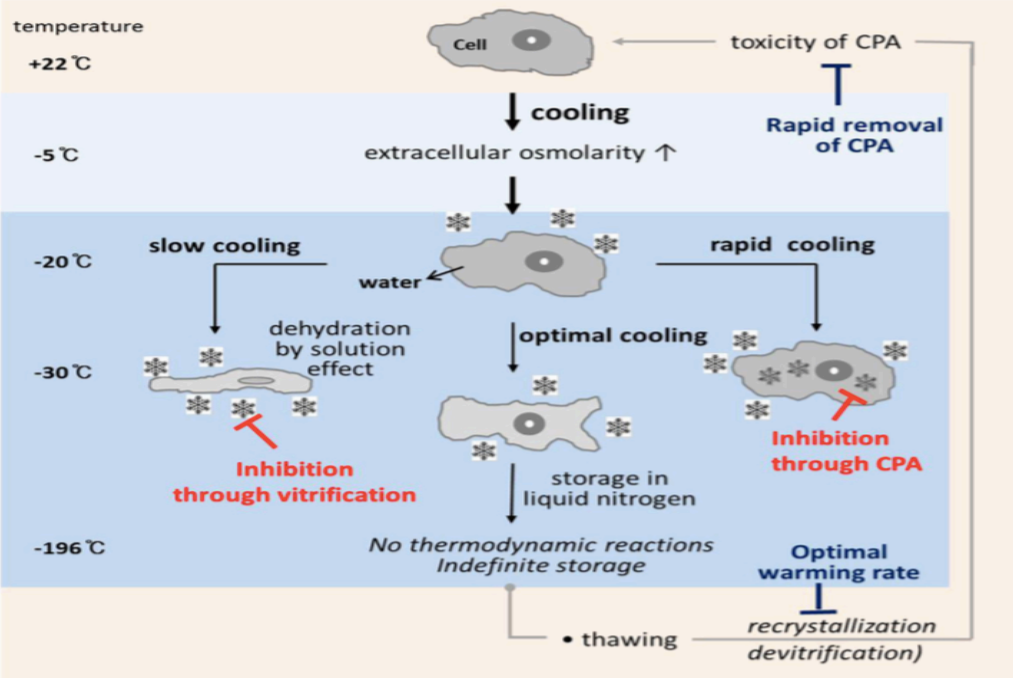


Figure 2.3 Illustration of the events that take place during freeze-thawing of cells. *Formation of ice crystals initiated extracellularly at -5 ℃ during the temperature decrease causes cells to dehydrate. If cells go through slow cooling, they will collapse at -30 ℃. During optimal cooling, cells will shrink with no intracellular ice formation. Rapid cooling will cause ice formation intra- and extracellular (Taken from Jang et al.[46])*

2.3.1.1.1 Slow cooling rate

Slow cooling is a conventional method applied to a wide range of cells. It is based on the assumption that during cooling slowly, cells are dehydrated sufficiently and intracellular ice crystal formation is prevented when reaching sub-zero temperatures[38]. Initially, cells equilibrate within CPA media, which is commonly prepared at a relatively low concentation[47], for a short time (e.g., approximately 10 min) and then slow cooling follows. The temperature is reduced by 1-10 °C/min. The cooling rate is variant depending on cell type[48]. For instance, the optimum cooling rate for pig hepatocytes is -1°C/min[49] whereas for mammalian ovarian tissue, it ranges between -0.3°C/min and -0.5°C/min[50]. The slow cooling rate controls the movement rate of water from the cells to the surroundings and controls the osmolality of the external fluid to achieve solute equilibrium and prevent osmolar stress. As a result, ice nucleation occurs in the surrounding environment while cells are gradually dehydrated, leading to intracellular freezing prohibition[51]. Usually, the temperature is constantly decreased until it reaches -20°C or lower, then cells are cooled faster by being plunged into liquid nitrogen at -196°C, where intracellular vitrification takes place[52].

2.3.1.1.2 Vitrification

Vitrification is another freezing method that transforms cells from a liquid state to a glassy state with no ice-crystal formation. It depends on the use of a high concentration of CPA and ultra-rapid cooling. In practice, incubating cells in CPA media substitutes intracellular water followed by direct immersion in liquid nitrogen at -196°C[53]. These steps prevent the formation of intracellular ice crystals and diminish lethal mechanical injury[54]. There are several naturally non-toxic CPAs that can be appropriate for vitrification, such as sugars and polyols[55,56].

Vitrification is time-saving, easy to carry out, inexpensive and requires no special equipment[57]. It has been applied successfully on RBCs[8], oocytes and embryos[58,59]. It is debatable whether vitrification is as efficient as or better than slow freezing. A number of studies have suggested that it is more efficient in preserving morphologically normal cells compared to slow freezing[60,61]. Others have reported, however, there are no major differences between the two approaches with regards to cell survival morphology and their apoptotic status[62,63]. Certainly, one of the downsides of this approach is the application of a high concentration of CPAs, which is cytotoxic and leads to osmotic injury[64].

2.3.1.2 Thawing rate

In the thawing step, it is advisable to avoid the gradual increase in temperature as this will cause lethal damage to cells resultant from ice recrystallization[65] (**Figure 2.3**). Instead, a sufficiently rapid warming is recommended either by using a water bath or devices that have a setup temperature between 37-40 °C. Warming should not exceed 120 sec to 3 min in order to minimise unnecessarily lengthy exposure of cells to CPAs[66] (**Figure 2.3**).

Fast-thawing has demonstrated better results compared to slow-thawing rates in fetal rat islet function[67]. Islets thawed rapidly featured a better response to stimulants compared to samples that were thawed slowly[67]. In another study by Pugliesi *et al.*[68], thawing equine spermatozoa fast efficiently maintained the viability and fertilization capability of the cells.

2.3.1.3 Additional and removal procedures of CPAs

Adding and removing CPAs exposed cells to a drastic damage, resultant from osmotic stress and/or chemical toxicity if performed inappropriately, resulting in cellular stress tolerance reduction and low viability. Selecting CPA concentration is important for deciding upon the addition protocol, which could be a single step if the CPA concentration is relatively low (roughly 10%), otherwise it can performed gradually[47]. Adding CPA will induce a cellular shrink-swell response[47] whereby cells will shrink and dehydrate as an initial response to the osmosis shock, and they will then swell as CPAs penetrate into cells through the membrane and replace the lost water[69]. The penetrating CPAs reach an equilibrium state depending on their extracellular concentration, cellular or tissue type and their density or thickness. Reaching equilibrium takes several minutes, as in the case of a protocol that utilises glycerol, or it could extend to hours as with a trehalose-loading protocol using synthetic polymers, such as PP-50, an amphipathic polymer consisting of poly (L-lysine isophthalamide) grafted with L-phenylalanine . The addition step is reversed when removing CPAs, which is usually started by gradually diluting CPA solution and decreasing osmotic stress that will improve cellular revival[70].

2.3.2 Cryomedia compositions and cryoprotection mechanisms

As discussed earlier, cryopreservation can be accomplished by applying two different cooling rates that require either freezing or vitrification solutions. These solutions contain carrier media to provide cells with support at low temperatures[47] in the presence of CPAs. They may also feature salts, pH buffers, osmotic agents, nutrients or apoptosis inhibitors. The solutions are usually prepared at or close to isotonic concentrations, and are occasionally referred to as base perfusate[47].

2.3.2.1 Cryoprotective agents and their protective mechanisms

CPAs are chemical substances that protect cells during cryopreservation. In total, there are approximately 56 compounds that possess cryoprotective abilities when applied to different cellular systems during cryopreservation[71,72]. Notably, there has been an enormous amount of research undertaken to understand freezing damage mechanisms and the protective role of CPAs[57]. The main cell injury that occurs during cryopreservation is attributed to imbalanced osmolarity and some associated biomolecules instability. When temperature reaches the freezing point of water, free water molecules begin to aggregate and begin forming ice crystals surrounding cells, thus forcing water immigration from intra- to extracellular, thereby leading to cell shrink. This can be manifested to affect other water bound with biomolecules such as proteins and lipids, causing deformation and dysfunctionality. CPAs structurally possess functional groups (such as hydroxyl and carbonyl groups) that have the affinity to form hydrogen bonds with water. Consequently, they will eliminate cell dehydration and mitigate the damage resultant from increased hyperosmosis[74,75,76]. Additionally, they can substitute water bound to biomolecules and enhance their stability[77,78]. The interaction between CPAs and the phospholipid bilayer and its stability was confirmed by Crowe *et al* [79]. The stabilising effect of CPAs has also been observed on other cellular compartments such as meiotic spindle proteins[80].

However, the protection mechanisms of CPAs are not fully understood, owing to the complexity of cryopreservation process and the variety of cells. The behaviour of water during cooling and warming remains a major factor influencing cell viability during cryopreservation[81]. Thus, understanding the physical and chemical properties of water and CPAs as well as their interrelationship and interaction with cellular biomolecules alongside throughout the cryopreservation process remains essential to comprehending the role of CPAs. The permeability of CPAs through the cell membranes is a key trait to identify their protection mechanism. Therefore, CPAs can be classified based on their permeability into two main classes: (1) permeable and (2) impermeable.

2.3.2.1.1 Permeable CPAs (pCPAs)

pCPAs are generally small, non-ionic molecules that are highly soluble in water, even at low temperatures. They can pass through cellular membranes and equilibrate within the cytoplasm in exchange for intracellular water during dehydration without over dehydrating the cell. They become solid at a lower temperature than water freezing, and subsequently suppress ice formation[81] and mitigate cellular physical damage that could occur in cellular compartments and membranes. Moreover, pCPAs reduce salt-induced stress by dissolving solute and reducing

concentrations in the remaining water fraction intracellularly until the cell is cooled to a sufficiently low temperature[81]. pCPA permeability is controlled by their viscosity and the membrane properties of the cell itself [82,83]. The latter mentioned is variable between different cell types as well as the varying ages of cells[84,85]. However, the most commonly employed pCPAs, including the applied ones in the present study, are introduced in the following.

Dimethylsulphoxide (DMSO)

DMSO is an amphipathic molecule that can dissolve in both water and organic solvents. It can be used as solvent for a large variety of applications[86]. It gained prominence in cryopreservation specifically as it was among the first discovered CPAs[87]. Many successful cryopreserving protocols were developed using DMSO to cryopreserve oocytes[88,89], embryos[90,91] and ovarian tissues[92]. Furthermore, it was reported to have an additional benefit of removing and scavenging ROS [87]. Yet, there is a restriction in using DMSO based on a number of toxicity concerns[93,94] that will be discussed in a later section (2.3.3).

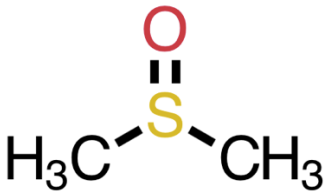


Figure 2.4 DMSO's chemical structure.

Alcohol-based CPAs

There are a number of alcoholic compounds and derivatives that have been investigated as CPAs consisting of polyols, such as methanol[95], propylene glycol[31], ethylene glycol[96], butylene[97] and glycerol[98], the most widely used triol CPAs.

Glycerol was the first discovered pCPA and the most heavily investigated for preserving mammalian spermatozoa[43], including human sperm[42]. It is a triol sugar that has high solubility in water and permeability into cells. It was suggested that glycerol has colligative properties that decrease salt concentrations during freezing[44]. Various studies have suggested that water-pumping protein, aquaporin, plays a role in glycerol permeability[45,46].

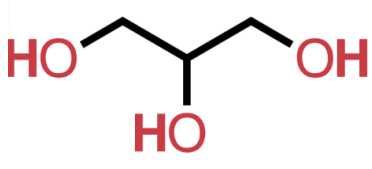


Figure 2.5 Glycerol's chemical structure.

2.3.2.1.2 Other pCPAs

There are other polyols, like adonitol, methanol, ethylene glycol, amides and imides (e.g., formamide and acetamide), that serve to protect cells but have not been widely employed owing to their limited and low efficiency[81].

2.3.2.1.3 Impermeable CPAs (ipCPAs)

ipCPAs are large molecules usually comprised of long chains of polymers that are unable to permeate through cellular membranes. They are water soluble and thought to increase the osmolarity around cells, which results in cellular dehydration and reduces ice crystal formation intracellularly[22]. The combination of high concentrations of ipCPAs and fast cooling promotes vitrification and stabilizing cellular proteins and membranes[102]. Their protective mechanism is based on preventing ice formation extracellularly as well as intracellular through dehydration[103]. There are several classes of ipCPAs, such as certain forms of sugars, macromolecules and polymers. Sugars are classified based on their chemical structure into: mono-, di- and polysaccharides (glucose, trehalose and raffinose, respectively). A number of these sugars are permeable (e.g., glucose) and others are impermeable (e.g., trehalose). Sugars have garnered unique interest over the last decades. They have been found to protect protein activity[104,105] and reduce protein chemicals[106,107] along with possessing thermal denaturing heat capacity[108,109], which leads to protein stabilization. In particular, trehalose has been identified as a universal protein stabilizer and been involved in many freezing and desiccation studies[110,111,112].

Trehalose

Trehalose is a non-reducing disaccharide consisting of two glucose units binding via an α,α-1,1-glycosidic bond (α-D-glucopyranosyl(1→1)-α-D-glucopyranoside). The sugar is found abundantly in nature, mainly in cells and organisms able to resist freezing, such as bacteria, insects, invertebrates and plants[113]. Thus, it has stimulated much research into understanding its function and role in protecting biospecimens under stressful conditions[114].

Trehalose has unique properties - for instance, it has a 2.5-fold greater hydration range compared to other sugars[115], and this is potentially based on trehalose's strong affinity for creating hydrogen bonds between hydroxyl groups of trehalose and water molecules; as a consequence, it increases protein stabilization[116].

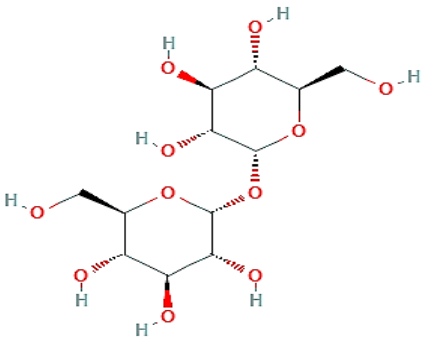


Figure 2.6 Trehalose's chemical structure. It consists of two glucose units bound via an α-D-1, 1- α-D glycosidic bond.

Trehalose has been used as an additive for oocyte[117], embryo[118], sperm[119] and RBC vitrification protocols[120]. It is considered a safe and non-toxic CPA in comparison to previously mentioned CPAs (e.g., glycerol and DMSO)[121]. Its success as a CPA, however, is conditional to its presence in- and outside of the cell membrane at a sufficient enough concentration[120] to improve cellular tolerance against freezing and dehydration**.** Naturally, it is not permeable across mammalian's cellular membranes, therefore it requires permeablisers[122,110] (**Table 2.1**).

There is a clear dispute on the optimum quantity of intracellular trehalose to accomplish successful tolerance during freezing. This was seen for the same cell types, for example, in a study by Shirakashi *et al.*[123] that suggested cryopreserving RBCs loaded with 63.7 mM trehalose can lead to an approximately 70.9% RBC cryosurvival rate, while another study by Lynch *et al.*[120] indicated that 200 mM and above is required to achieve a 74±4% cryosurvival rate. In another study by Holovati *et al.*, the amount of trehalose loaded into RBCs was as low as 15 mM and the resultant cryosurvival rate was 66.5%[122]. The significant differences between loaded trehalose concentrations and the similarity in the ultimate results remain unexplained. Moreover, the use of trehalose as a cryoprotectant to replace toxic CPAs for mammalian cell biopreservation was limited to the number of cells, mainly RBCs[120]. This is ascribed to the effectiveness of trehalose-loading methods, which influence the sufficient concentrations for cryopreservation.

Trehalose-loading techniques

Interestingly, trehalose is not synthesized by mammalian cells nor permeates through their membranes[49,123] despite the presence of trehalose enzymes in mammlian intestinal villae membrane[125]. Thus, many ongoing studies have developed different methods to permeate

trehalose into the mammalian cells[126,122,124] (**see Table 2.1**). It has been established that the presence of trehalose intra- and extracellularly enhances cellular tolerance to freezing and desiccation[127,128].

Several specific techniques have been developed to permeabilise trehalose through cellular membranes under certain conditions. These approaches introduced trehalose into cells and demonstrated a variable level of effectiveness in cryopreservation (**Table 2.1**).

TREHALOSE-LOADING TECHNIQUES	CELL TYPE	INTRACELLULAR TREHALOSE CONCENTRATION	CONDITIONS	EFFICACY
CARRIER				
LIPOSOMES[123]	RBC	~15 mM	Incubating time: 4-6 h	66±5% recovery rate post-freeze-thaw
MEMBRANE PERMEABILISER				
BACTERIAL PROTEIN ALPHA-HEMOLYSIN[124]	3T3 fibroblasts and human kertinocytes	200 mM		Survival rate > 80%
PH-RESPONSIVE BIOPOLYMERS [104]	RBC	251±6 mM	Incubating time: 6-9 h	Cryosurvival rate between 59-74±4%
FLUID-PHASE ENDOCYTOSIS[125]	Human mesenchymal stem cells	20-30 mM	Incubating time: 24 h *Requires cellular functional microtubules	Not determined
ELECTROPORATION[126]	RBC	63.7 mM		70.9%
MICROINJUCTION[127,128]	Embryonic mouse cells	100-150 mM		Normal development and implantation
ATP-DEPENDENT P2Z RECEPTOR[129]	Hematopieotic stem and progenitor cells	Not determined	Required 200 mM trehalose solution, ionized ATP and incubation time: 75 min	Colonies formed post-thaw: $19x10^2±5x10^2$
CELLULAR ENGINEERING				
EXPRESSION OF TREHALOSE INTRACELLULARLY[130]	Mouse LMTK cell lines and human embryonic kidney epithelial lines (298 cells)	80-100 mM	Reach maximum accumulation at incubation time: 30 h	Improved osmotolerance but not desiccation
EXPRESSION OF TREHALOSE TRANSPORTER[131,132]	Chinese hamster ovary (CHO) cells, *Xenopus laevis* oocytes	23-100 mM	Incubation time: 6 h minimum	Partially improved cellular desiccation tolerance
ENGINEERING PERMEABLE TREHALOSE (TREHALOSE HEXAACETATE) [133]	Rat hepatocytes	300 mM	Incubation time: 9 h	Slow albumin secretion post-loading; No cell lose

Table 2.1. Summary of developed techniques to permeate trehalose into mammalian cells

pH-responsive biopolymer development

There are various membrane-disrupter peptides and polymers that have been widely applied to drug delivery, such as natural and synthetic peptides[140]. These peptides are effective in disrupting the cellular phospholipid bilayer membrane to permeate substances into cells. However, owing

to safety concerns regarding their application and cost-effectiveness, researchers are seeking an alternative[141].

In recent years, a set of amphipathic pH-responsive polymers were developed in the Chemical Engineering and Biotechnology Department at the University of Cambridge to overcome the aforementioned concerns. These polymers can permeabilise cellular membranes through weak attachment with the membrane, creating pores that facilitate the passage of external substances into cells. Further development of the polymers led to synthesizing a range of biodegradable polymers that were composed of the same backbone, poly (L-lysine isophthalamide) (PLP). Grafting PLP with hydrophobic amino acids, such as valine, phenylalanine and leucine, increases the disruption of the cell membrane. These types of polymers were used in previous studies to permeate drugs and cryoprotectants[142,143]. The application of pH-responsive biopolymers was successful in loading trehalose into RBCs[110], however it did not demonstrate the same loading efficacy when applied to nucleated cells. This is attributed to the escape of the biopolymer itself inside the cells through endosomal pathways[144], which affects attachment stability with the cell membrane and consequently the uptaken quantity of trehalose.

- ***PP-50 polymer structure and permeabilisation mechanisms***

PP-50 is composed of repeat units of a PLP backbone that are grafted by L-phenaylalanine at a degree of grafting of 46.2% (Mn=23 kDa) **(FIGURE 2.7).** PP-50 has a weak carboxyl acid and hydrophobic side chain. At low pH (lower than the polymers pKa) the hydrophobocity of the polymers increased, thus resulting in polymer aggregation. These hydrophobic polymers interact with the hydrophobic core of the membrane, creating pores that facilitate membrane permeabilisation, mimicking viral peptides in membrane permeabilisation activity[145].

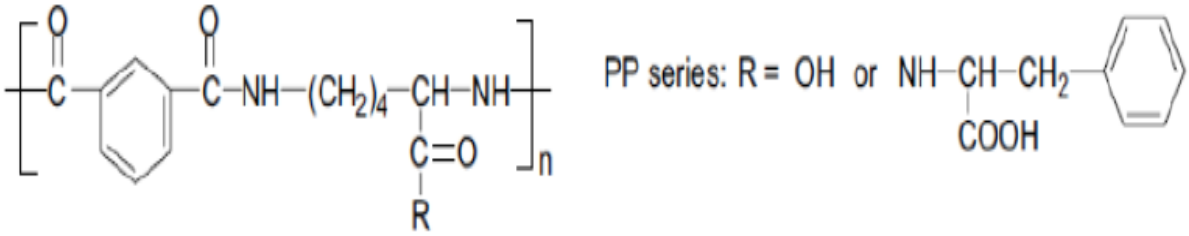


Figure 2.7 pH-responsive PP-50 structure. *PP-50 composed of repeat units of poly (L-lysine iso-phathalamide) (PLP) grafted with L-phenylalanine (PP), R represents the grafted group. (taken from Lynch[146])*

Studying the mediation mechanisms demonstrated that PP-50 localisation is restricted to the cell membrane[146]. An additional study of diffusant PP-50 volume and membrane permeability indicated that PP-50 permeability is resultant from non-Stokesian diffusion; consequently,

hydrophilic molecules are placed between hydrophobic phospholipid hydrocarbons and across the cell membrane. Further examination via atomic force microscopy showed PP-50 on the membrane surface, which was confirmed by the impurity of the membrane. The original status of the membrane can be regained following permeabilisation by a simple washing step using phosphate buffer saline, which is crucial for clinical applications[146].

2.3.3 Cryoprotectant toxicity and detrimental effects

It is clear from the previous discussion that CPAs are a wide range of molecules that are differ in their molecular weight and their permeability into cells. Introducing CPAs in high concentration (molars) is accompanied with non-specific adverse effects such as osmotic stress and cell dehydration[147] that also could induce the oxidative stress[148]. This can cause severe cell damage; for instance, increasing the concentration of DMSO, glycerol and 1,2-propanediol, is linked with the production of non-enzymatic formaldehyde[149], a cytotoxic compound that contributes to cell death[150,151]. The long exposure duration of cells to high concentration of CPA also harm cells development, as reported when exposing bovine blastocytes to a high concentration of ethylene glycol over 10 min[152]. Likewise, introducing a high concentration of propanediol to mouse zygotes was found to have a similar damaging effect on cell development to that observed in bovine blastocytes[153]. These types of reported damages are considered non-specific since it is not limited to specific CPA identity. However, the molecular interaction of CPAs is more closely linked to the permeable CPAs, as they are able to interact with the cells compartments and biomolecules[148].

Moreover, the CPA toxicity effect can be either reversible (e.g., osmotic shock and cellular shrinkage[154,155]) or irreversible. Notably, cryopreservation protocols involving short exposure times to CPAs can reverse the induced damages. Nevertheless, irreversible damage is common in cells lacking self-renewal or repair mechanisms, such as RBCs[156] and embryonic stem cells[157,158]. Furthermore, it is important to distinguish between the effect of CPAs and applied conditions (e.g., cooling and thawing rates) by testing CPAs under various cooling and thawing conditions[81,159].

2.3.3.1 Oxidative damage

Oxidative stress occurs during cryopreservation, mainly when adding CPAs to cells[160]. The increased oxidative stress results in more ROS production[161], which leads to a disequilibrium between the generated ROS and the cellular antioxidant capacity within the redox pathway. A decrease in cellular reduced glutathione (GSH) content was made observed during the freezing

step of sperm[162], indicating that oxidative damage occurs during the initial steps of cryopreservation. Consequently, increased ROS production results in lipid peroxidation[163], DNA instability[164], protein oxidation [165], overall dysfunctional cells and low survival rates[164,162,166] (**Figure 2.8**).

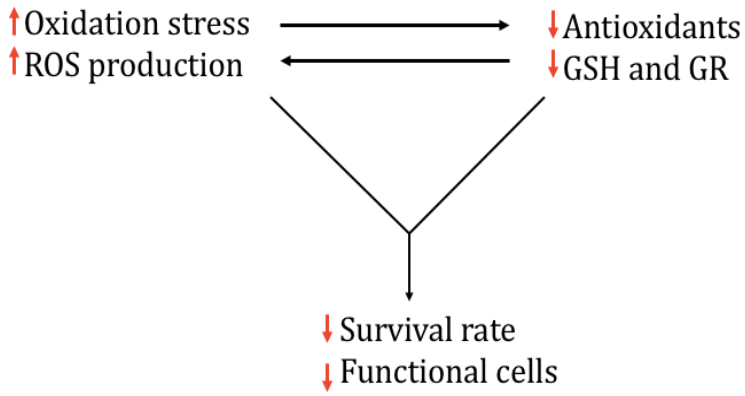


Figure 2.8 The relationship between cellular oxidative stress and antioxidative systems during cryopreservation and their influence on cellular survival and functional rate.

Oxidative stress is non-specific effect, and it has also been observed when applying glycerol[167], DMSO[168] and trehalose[169] to cells. Antioxidant treatments, such as those involving vitamin E analogue, troxol[170], attenuates the detrimental effect of ROS. However, other antioxidants, such as vitamin C and E, were reported to have either a moderate or nearly no antioxidant effects during cryopreservation[171].

2.3.3.2 Osmotic stress during adding and removing CPA

Although applying CPA during cryopreservation mitigates the effect of severe temperature alteration, the high concentration of CPA accumulated intracellularly causes an osmotic imbalance when diluting cells post-thaw in cell culture isotonic solution, which has a detrimental effect on cells. In cryopreserved human mesenchymal stem cells (hMSC), it has a significant effect on cellular viability, filamentous actin distribution, intracellular pH, and mitochondria aggregation[172]. It also has also been found to cause abnormal spindles and morphology in human oocytes, which can potentially influence their viability post-cryopreservation[173]. Similarly, CPA causes a serious alteration in mammalian sperm viability, physiological properties, protein phosphorylation patterns[174], and can lethally damage enzymatic activity and DNA[175]. However, osmotic stress factors and associated cell shock cannot be decoupled since they interact with each other , though the resultant effects can be reversed or limited to a certain extent by minimising exposure time, accelerating freezing and thawing speeds, and gradually diluting CPAs in cells[176,177, 90], which can increase post-thaw cell viability.

2.3.4 Other biochemical effects

Cells naturally have a dynamic and complex system involving active biomolecules that respond distinctly to all forms of environmental stressors, including CPA media and alterations to temperature. The cells' response to stressors involve complex biomolecular events influencing their fate. Measuring the survival rate of thawed cells is a classical parameter that is not precise when determining the efficacy of cryopreservation. This is because during the recovery period a decrease in cellular viability occurs in different cell types[7,178,179]. This is attributed to the activation of apoptosis in cryopreserved cells post-thaw[180,181]. Xu *et al.*[179] reported that exposing cells to DMSO and freezing conditions activate apoptosis through extrinsic and intrinsic pathways, including caspase-8, caspase-9 and p53. Although some CPAs has different mechanisms, yet they lead to the same lethal results. Propylene glycol (ProH), for instance, reduced cells viability via increasing intracellular calcium to a cytotoxic level[182,183,184].

Furthermore, the cryopreservation affects cells' biomarkers[17,185]. it alters the proteome profile of cells, which in some cases can bring about changes in cellular metabolism, function and structure[186,187]. In previous work, there is often no clear demarcation between the effect of CPAs and the cryopreservation protocol itself. Yet, these studies did overlap in the applied CPAs[188,179,185].

2.3.5 Quality assessment methods of cryopreserved cells

The assessment of biopreserved cells or tissues is essential to determining the quality and reliability of the biopreservation protocol and solution. An accurate measurement of viability and functionality are paramount to quality assessments. In the past, classical parameters, such as survival rate or motility, were the only quality measurements[189,190,191]. With the evolution in technologies and developed assays, scientists can obtain more information surrounding the level of stress that heralds cellular death cascades and dynamic changes that impact cryopreserved cells' function and morphology.

Nowadays, there are a wide range of viability assays available, however, selecting the appropriate assay mainly depends on cell types to avoid inaccurate measurement. For instance, the measurement of LDH leakage in media can be used for membrane integrity assessment because of its reliability and easy performance. It is an applicable measurement in single cells as well as tissues and organs[192,193]. Conversely, using fluorescent probes for viability measurement is suitable for many cells excluding hepatocytes, because of their detoxification activity with respect to probes that influencing the accuracy of the measurment[159].

The emergence of developed technologies, such as genomics, transcriptomics, proteomics and metabolomics (collectively termed OMICs) has provided a comprehensive profile of biopreserved cells, including their stressed and compromised biological pathways, which may help designing protocols or solutions in order to modulate the damaged pathways. So far, the majority of OMICs applications in cryopreservation are limited to reproduction medicine and plants[194, 195]. Such as in human sperm characterisation post-thaw[196] and the mechanistic effect of sucrose and cold pre-treatment on potato cryopreservation[197]. The deep analysis OMICs provide stresses the importance of adopting such analytical approach in researches aiming at advancing biopreservation and biobanking for better CTMPs outcome[159].

2.3 Thesis aims, outline and justification

There is high interest and need in identifying effective protective compounds that can offer cells high protection against induced and accumulated oxidative stress during biopreservation. This requires a better understanding of the molecular biology underlying the biopreservation as well as knowledge of the mechanisms of action of the protective agents. The present thesis, therefore, aimed at enhancing mammalian cells cryopreservation through screening novel protective compounds of natural origin that could potentially promote cells survivability, extend their stability and improve their quality for clinical and pharmaceutical applications. Furthermore, this thesis aimed at elucidating the mechanisms of cryo-damages and modulatory effect of the potential protective agents by assessing cryosurvival rate, biomolecular profile (e.g. enzymatic activities, oxidative damages, and proteome changes) and proteome profile prior to and post cryopreservation.

To achieve these objectives, chemicals obtained from natural sources and possess polyols were screened and tested for their cryopreservation capacity using sheep RBCs (sRBCs) as described in **Chapter 4**. All utilised materials and experimental approaches were used frequently in all chapters are described in **Chapter 3.** The proteomic investigating methods are described in the corresponding experimental chapter. The screened compounds were assessed as an additive to the conventionally-used cryomedia glycerol, trehalose and DMSO. The differences in cryomedia used in the study were considered and a comparison between the glycerol and trehalose protocols was carried out in **Chapter 4** with and without the tested compounds. The additive compounds which improved the cryosurvival rate of sRBCs and enhanced their stability post-cryopresrevation in refrigerating media were further assessed for their effects on cellular biomolecular profile as described in **Chapter 4**. This was followed by attempts to translate the findings on human RBCs (hRBCs) biopreservation as described in **Chapter 5**. There were several confounding factors affected the biopreservation, such as the age of the donated blood and biosampling procedures. Consequently, a nucleated peripheral blood cell model, human leukaemia (HL-60) cell, was used for further validation of the effects of the protective compounds (**Chapter 6**). Analysing the redox enzymatic activity changes and the oxidative damages of proteins and lipids in the cryopreserved cells was a way forward to assess the changes occurred during biopreservation. Based on the experimental outcome, biopreserved cells were further screened in a non-targeted way at the proteome level, using liquid chromatography tandem mass spectrometry (LCMS), for proteins profiling to identify effects on individual proteins as well as on protein networks. The obtained data from both approaches were integrated for more complete

assessment of the biopreservation damages and the mechanisms of action of protective agents. This allowed the establishment of CPAs unique effects on anucleated and nucleated cells, which was another important translational goal of this thesis. The experimental workflow is illustrated in **Figure 2.8.**

The long-term outcomes of this thesis can improve the biopreservation of important mammalian cells, including CTMP industries for pharmaceutical and healthcare applications. These are discussed in **Chapter 7**.

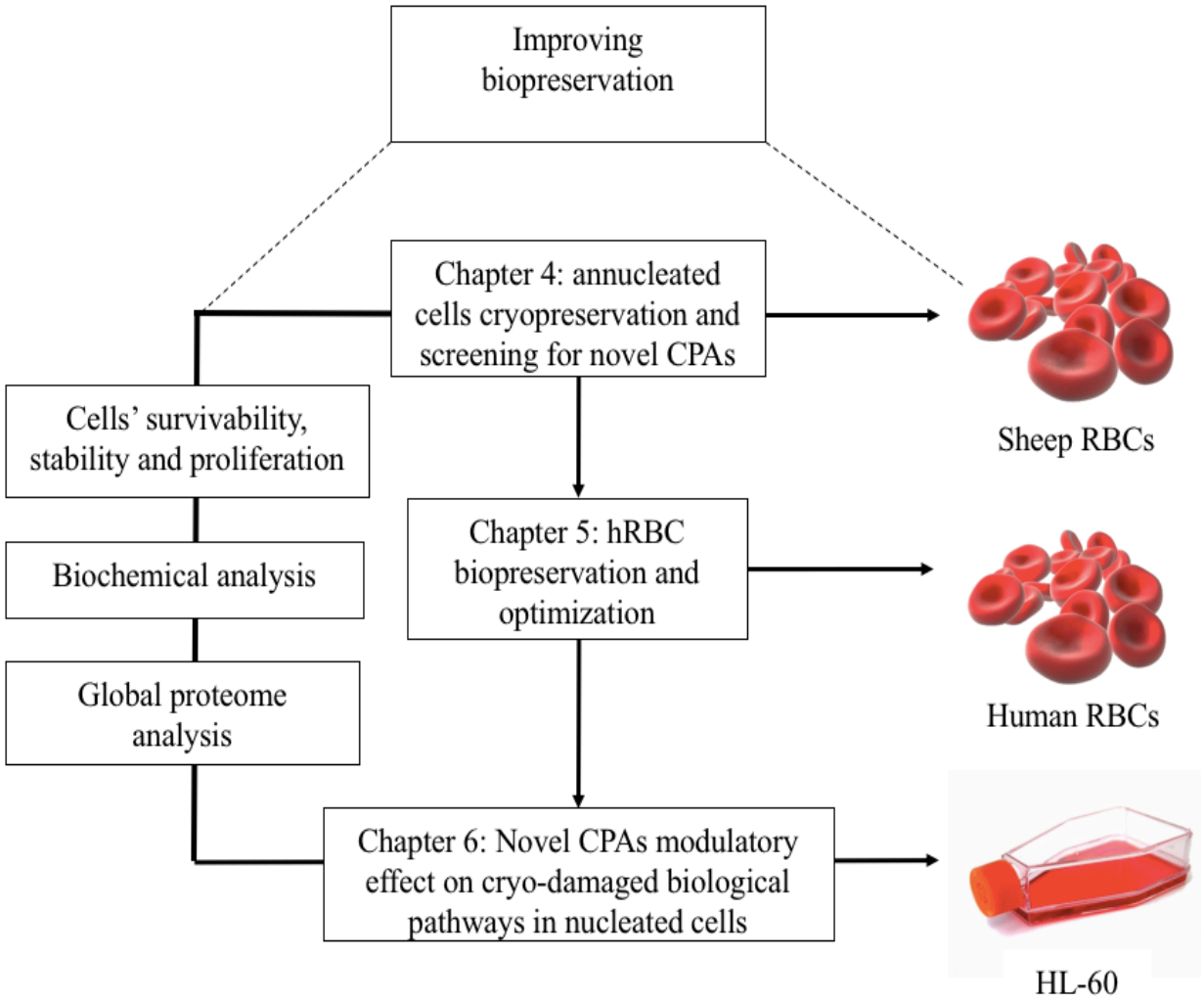


Figure 2. 1 Experimental chapters and workflow

3 General materials and methods

3.1 Introduction

This chapter describes the general research methods and chemicals used in investigating the functionality of biopreserved cells. The quality of cryopreserved cells was assessed via examining their biochemical and proteomic profiles prior to and post cryopreservation in standard or formulated cryomedia. Precisely, this was achieved by comparing the results with and without adding the novel tested compounds at three critical time points: 1) prior to freezing; 2) post thawing; and 3) during recovery. All methods target analysis of key macromolecules and molecular pathways as a means of assessing cell survival through freeze-thaw cycles and ability to function. Therefore, the selected functional analysis focused on enzyme activities and macromolecules (e.g. proteins and lipids) oxidation damage.

In addition, proteomic profiling was used to assess the above parameters in a non-targeted way. Proteomic is a broad-spectrum approach that enables analysis of hundreds or thousands of proteins simultaneously to provide a deeper insight into the dynamic responses of cells to environmental changes and drugs exposure. The principle of this approach is described in the current chapter, whereas the instrumental tools and analytical approaches are detailed in the **Chapters 5 and 6**.

3.2 Cell survival rate and stability

One of the fundamental parameters in assessing the quality of biopreserved cells is the counting of cells survival post thawing in a given period of time. In this study, survival rate of cells and their stability were assessed by two different methods, subject to cell type used in the experiment:

1. Cell count via haemocytometer and trypan blue staining
2. Haemolysis via Drabkin's reagent

3.2.1 Cell count via haemocytometer

It is a standard procedure of cell counting used to determine cell viability. For this, 10 µL of cell suspension was mixed with equal volume of 0.4% trypan blue (Sigma-Aldrich). The mixture was

left for 5 min to allow sufficient permeation and then loaded into the V channel of the haemocytometer device (Neubauer, Spenser Bright Line™; Sigma Aldrich).

The device consisted of a thick slide with counting chamber and cover glass. After adding the sample, the slide was placed on light microscope platform under the objective. The microscope was set on 10x magnification and the counting chamber was visualized. The chamber is divided into 9 squares (1 mm ^{2} each). Each square consists of gridded areas with subdivisions consisting of small squares of either 1/16 mm^2 or 1/25 mm^2 (**Figure 3.1)**. The coverslip over the chamber gives a depth space of 0.1 mm^2, which accommodates a volume of 0.9 mm^3.

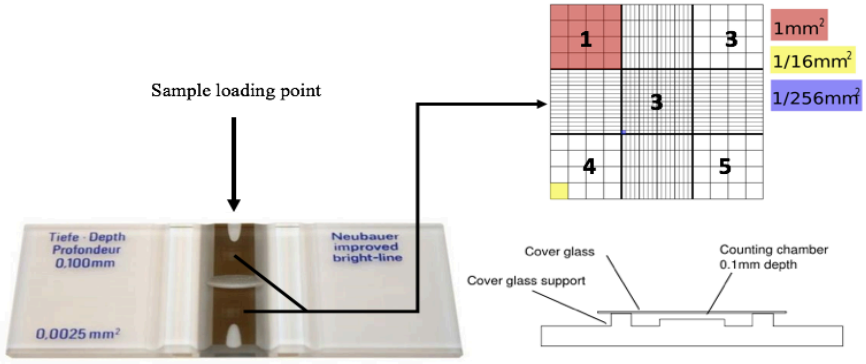


Figure 3. 1 Haemocytometer. *Left) Structure of the instrument and the loading points. Top right) Schematic diagram of the grid areas of each chamber (counting squares are labelled from 1 to 5). Bottom right) Depth of haemocytometer with cover glass on surface.*

The cell number was counted in one of these squares to calculate the total number of cells and density culture. The cells density was calculated using the following equation:

$$Cell\ number\ per\ mL = \left(\frac{number\ of\ counted\ cells}{number\ of\ counted\ squares}\right) \times dilution\ factor \times 10^4 \quad \textbf{(Eq 3.1)}$$

In order to calculate cell viability, the common analysis procedure involved excluding the cells stained by trypan blue. Trypan blue is a negatively charged dye that can penetrate cell membranes in dead or ruptured cells and bind to positively charged proteins in the cytosol. Thus, stained cells were considered dead whereas the bright unstained cells were considered to be living. The following equation (**Eq 3.2**) was used to calculate the viability of counted cells:

$$Cell\ viability\ [\%] = \frac{number\ of\ unstained\ counted\ cells}{Total\ number\ of\ counted\ cells\ (stained+unstained)} \times 100 \qquad \textbf{(Eq 3.2)}$$

3.2.2 Haemolysis Drabkin's reagent

In RBCs, haemoglobin (Hb) is the major component of the cells. It forms about 97% of the total protein of the cell. This protein acts as a carrier of gases such as O_2 and CO_2 between the lungs and the rest of the body. Hb consists of 4 globular protein subunits, each containing a non-protein heme group with a central molecule of iron in the ferrous state. Each heme group can combine with 1 molecule of O_2 or CO_2 gas. Hb carries oxygen from high to low O_2 pressure tissues, where it releases the O_2 and returns CO_2 from the tissues to the lungs.

When RBCs die, or if they are ruptured or damaged, the Hb is released freely in the media. Free Hb can be measured by Drabkin's reagent, a colorimetric method based on oxidation of Hb and its derivatives, excluding sulphemoglobin, to methemiglobin in the presence of alkaline potassium ferricyanide. The methemoglobin reacts with potassium cyanide to form a stable compound called cyanmethemoglobin, which can be measured at 540 nm in a spectrophotometer.

For total Hb measurement, 10 µL of total RBC lysate was suspended in 200 µL Drabkin's reagent while free Hb was measured in supernatants post centrifugation at 750 x g for 3 min. The supernatants were collected and mixed with Drabkin's reagent and the mixtures were incubated 20 min at room temperature. The absorbance of each sample was measured at 540 nm with a SpectrostarNano plate reader (BMG Labtech; Aylesbury, UK). The percentage of haemolysis was calculated as following:

$$RBC\ lysis\ [\%] = \frac{Supernatant\ OD\ 540\ nm}{Total\ Hb\ OD\ 540\ nm} \times 100 \qquad (\boldsymbol{Eq\ 3.3})$$

3.3 Biochemical assays

3.3.1 Enzymatic analytical assays

3.3.1.1 Assays background and performance

Enzymatic assays kits for glutathione reductase, glucose-6-phosphate dehydrogenase and lactate dehydrogenase were purchased from Abcam (Cambridge, UK). In each case, the analysis is based on colorimetric measurement of the products resulting from the enzymatic reactions. The measurements were performed using a Spectrostar Nano plate reader at specific times. Enzymatic assays and standard curve preparation for each assay were performed as specified by the supplier, unless stated otherwise.

3.3.1.2 Glutathione reductase activity

Glutathione reductase (GR) reduces oxidized glutathione (GSSG) to reduced glutathione (GSH) utilizing NADPH generated from the pentose phosphate pathway. GSH plays an important role in the redox cycle to protect cells against oxidative stress by keeping a high ratio of GSH/GSSG.

The GR assay is based on the reaction between GSSG and GR enzyme in the samples, which produces GSH. Next, the GSH reacts with 5,5'-dithiobis (2-nitrobenzoic acid) (DTNB) to produce the chromophore 2-nitro-5-thiobenzoate (TNB), which is measured spectrophotometrically at λ_{max} =405 nm.

Initially, 100 µL cell lysate was pre-treated with 5 µL 3% H_2O_2 and incubated at 25°C for 5 min to destroy the GSH. Then 5 µL catalase was mixed with the mixture and incubated for 5 min at 25°C. After that 5 µL was taken from each sample and placed in 96 well plate and the volume was accomplished to 50 µL with assay buffer. A positive control was also used in all assays to assure the accuracy of the used reagents. For this, 10 µL of the positive control was placed into well and adjusted to 50 µL with assay buffer as with the test sample. A reaction mix was prepared to improve well-to-well reproducibility, consisting of 40 µL GR assay buffer, 2 µL DTNB solution, 2 µL NADPH-GNERAT™ solution and 6 µL GSSG solution. Next, 50 µL of this mix was added to each test well. The first and second readouts were measured after 5 and 10 min intervals using the Spectrostar Nano plate reader. In parallel, a TNB standard curve was prepared and tested as described by the supplier. The TNB amount generated from the standard curve (ΔA_{405}= ΔB) was applied in the following equation to measure the GR activity per mL:

$$GR\ activity = \left(\frac{\Delta B}{\Delta T x V C x V}\right) x\ sample\ dilution = \frac{mU}{ml} \qquad \textbf{(Eq 3.4)}$$

Where ΔB is the TNB amount from the TNB standard curve (in nmol), ΔT is the time difference between the first and the second readout in min, VC is the volume change factor during sample pre-treatment and V is the pre-treated sample volume applied into the reaction well (mL). The unit is defined as the amount of enzyme required to produce 1 µmol of TNB per min at 25°C. The oxidation of 1 mole of NADPH to $NADP^+$ will generate 2 mol TNB. Therefore, 1 TNB unit equals 0.5 NADP units.

3.3.1.3 Lactate dehydrogenase activity

Lactate dehydrogenase (LDH) is used as biomarker to assess the presence of damage and cytotoxicity of cells and tissues[198]. The LDH assay is based on the reduction of NAD^+ to NADH by LDH activity. NADH then interacts with a specific probe to produce a colour which can be read at λ_{max} = 450 nm. The assay was performed as detailed by the manufacturer. The quantity of NADH was detected spectrophotometrically at 450 nm by mixing NADH detection buffer with the cell supernatant and lysate. The first readout was taken immediately, and samples were incubated in the dark at 37°C with a final reading taken at 30 min. The activity of LDH was calculated using the production of NADH from the standard curve (ΔA_{450}=B) and applied into the following equation:

$$LDH\ activity = \left(\frac{B}{\Delta T x\ V}\right) x\ D = \ mU/mL \qquad \textbf{(Eq 3.5)}$$

Where B is the generated NADH from the standard curve, ΔT is the reaction time and D is the sample dilution factor in the assay, v is the volume.

3.3.1.4 Glucose 6 phosphate dehydrogenase activity

Glucose 6 phosphate dehydrogenase (G6PDH) is a cytosolic enzyme in the pentose phosphate pathway, a pathway that provides cells with energy by reducing the co-enzyme nicotinamide adenine dinucleotide phosphate ($NADP^+$) to NADPH. The NADPH maintains the level of reduced glutathione in the cells, which helps to protect cells against oxidative damage. The experimental test is based on the conversion of $NADP^+$ to NADPH and measures the absorbance of the generated NADPH at 450 nm wavelength.

RBCs cell lysates equivalent to 10^7 cells were diluted using the G6PDH assay buffer to 5 times. Then 50 µL was taken and placed in 96 micro-well plates and combined with reaction mix composed of 46 µL assay buffer, 2 µL G6PDH substrate and 2 µL G6PDH developer. A positive control was run in parallel with the test as detailed by the supplier. A standard curve was assayed at the same time. The measurement was carried out at 450 nm in the first 5 min to obtain the first readout, and the reaction was left at 37°C for 30 min away from light and the measurement repeated for the final readout. The generated NADH was calculated from the standard curve by subtracting the background from the last obtained read (B in nmol=ΔA_{450nm}=A_2-A_1). The following equation was applied to obtain G6PDH activity:

$$G6PDH\ activity = \left(\frac{B}{(\Delta T) xV}\right) x\ sample\ dilution = \ mU/ml \qquad \textbf{(Eq 3.6)}$$

Where B is the generated NADH in nmol, ΔT is the difference in time between the first read (A1) and the last one (A2) in min, V is the volume of used sample in the reaction in mL.

3.3.2 Oxidation assays

3.3.2.1 Background

Reactive oxygen species (ROS) are normally produced in cells through the aerobic pathways. Over-production or exposure to ROS can cause damage to cellular macromolecules (e.g. lipids, proteins and DNA), causing abnormal or dysfunctional cells. Here, protein and lipid oxidation were examined before and after biopreservation to assess the quality of the biopreservation protocols.

3.3.2.2 Protein oxidation

For proteins, the most reliable and commonly used biomarker for oxidation damage is the measurement of the protein carbonyl content. Protein carbonylation takes place when protein molecules are attacked by ROS, which alters the amine group side chains (e.g. lysine, histidine, arginine and proline) into carbonyl groups. Carbonyl content can be detected and quantified via the reaction between 2,4-dinitrophenylhydrazine (DNPH) and carbonyl group on the protein, forming a Schiff base to generate the corresponding hydrazone (**Figure 3.2**). Hydrazone is a compound that can be analysed spectrophotometrically at an absorbance between 360-385nm. The readout can be standardized to yield protein concentration.

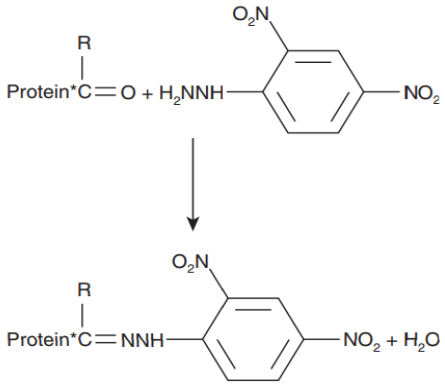


Figure 3. 2 Schematic diagram showing biochemical reaction between DNPH and protein carbonyl group

Cell lysates corresponding to 10^7 RBCs and 10^6 HL-60 cells were used to assay protein carbonyl groups prior to freezing and post thawing. The assay was run using a carbonylation assay kit purchased from Cayman Chemical Company (Ann Arbor, MI, USA). The preparation and assay set up was done following the manufacturer's instructions. Briefly, 200 µL of cell lysates were transferred into two 2 mL eppendorf tubes and labelled as sample and control tubes. Next, 800

µL of DNPH solution was added to the sample tube and similar volume of 2.5 M HCl was added to the control tube. The samples were incubated in dark with regular mixing by vortexing every 15 min for 1 hour. After that, 1 mL of 20% TCA was added to each tube followed by vortexing. Next, the samples were placed on ice for 5 min, then proteins were pelleted by centrifuging samples at 13,000 x g for 10 min. Then pellets were washed following the manufacturer's instructions and then suspended in 1 mL of ethanol/ethyl acetate mixture provided in the kit and washed twice more as above. Finally, the pellets were re-suspended in 500 µL of guanidine hydrochloride. Any remaining debris was removed by centrifugation as above and the supernatants (220 µL) were placed into a 96 well plate and carbonyl groups measured at 375 nm in a spectrophotometer.

3.3.2.3 Lipid oxidation

Lipids can also be disrupted by ROS and electron scavenging imbalance causing cells damage. The damage occurs in a series of reactions, resulting in a reactive aldehyde such as malondialdehyde (MDA) and 4-hydroxynonenal (4-HNE). These products can be measured to assess the oxidative damage. The assay is based on the formation of a malondialdehyde-thiobarbituric acid (MDA-TBA) adduct in acidic conditions at 95°C for 1 h. For this, 10^7 RBCs/mL and 10^6 HL-60 cells/mL were lysed separately in 303 µL of lysis buffer and butylated hydroxytoluene (BHT) prepared as detailed by the manufacturer and centrifuged at 13,000 x g for 10 min at 4°C to remove insoluble material. The supernatant was transferred to a fresh tube and used to assay lipid peroxidation from cells prior to and post cryopreservation. Cells were harvested and prepared for the assay following the manufacturer's protocol. The absorbance of the samples was measured at λ=532 nm using the Spectrostar Nano plate reader following the manufacturer's protocol and the MDA concentration was expressed in nmol.

3.4 Proteomic analysis

3.4.1 Protein extraction

Cells were pelleted by centrifugation at 100 x g for 5 min then supernatant was discarded. Cells pellet was suspended in PBS buffer then centrifuged again, this step was repeated three times. Finally, cells pellet was lysed in 350 µl RIPA buffer and 2.85 µl protease inhibitors and kept on ice for 30 min.

3.4.2 Protein concentration measurement

Protein concentrations were measured using the Bio-Rad reagent (Bio-Rad Laboratories Ltd, UK), which also known as Bradford assay. The reagent binds to protein carboxyl and amino group

in acidic aqueous forming a noncovalent complex with the protein that generates a blue colour. The intensity of the generated colour represents the amount of complex protein-dye formed, which can be estimated by using an absorbance reader. Unknown protein concentrations of samples were determined by measuring against a standard curve of known bovine serum albumin (BSA) concentrations. In each case, the Bio-Rad reagent was added to samples in a 1:30 ratio to give a final volume of 200 µL. Samples were loaded into the plate reader and the absorbance was measured at 595 nm. The protein concentration of each sample was measured in triplicate to improve the accuracy.

3.4.3 Proteins in-solution digestion and purification

3.4.3.1 Reagents

Tris stock (0.4 M, pH 7.8) was prepared by dissolving 12.1 g of Tris base in 200 mL of MilliQ-water and the pH was adjusted to 7.8 with 6 M HCl. Then, MilliQ-water was added to final volume of 250 mL and the solution was stored at 4˚C.

6 M Urea in Tris buffer (pH 7.8) was prepared by placing 2 g of urea in a 15 mL falcon tube and then adding 1.25 mL of 0.4 M Tris stock. The total volume was adjusted to 5 mL by adding MilliQ-water. For peptide purification, Waters C18 Sep-Pack cartridges were used. Sep-Pak Plus™, and equilibrating and eluting solutions were prepared on the same day. To prepare the equilibrating and elution solutions, HPLC grade acentonitrile (CH_3CN) and formic acid (FA) were purchased from Sigma-Aldrich. Equilibrating solution A is consisted of 98% MilliQ-water, 2% CH_3CN and 0.1% FA and eluting solution (B) consisted of 65% CH_3CN, 35% MilliQ-water and 0.1% FA.

3.4.3.2 Procedure

Cells lysates were incubated with cold acetone at -20˚C overnight and then centrifuged at 10,000 x g for 5 min. The supernatant was removed and tube left open on bench for 20-40 min to completely dry. Then, 100 µL of 6 M urea buffer was added to the pellet and the sample was vortexed and sonicated for 2 min. This was followed by adding 5 µL of the dithiothreitol (DTT) reducing agent and vortexing. Then, the sample mixture was incubated for 1 hour at room temperature. After that, 20 µL of iodoacetamide alkylating reagent was added to sample mixture followed by vortexing and incubation for 30 min at room temperature. At the end of the incubation, the urea concentration was reduced by adding 775 µL of MilliQ water and vortexed to mix. Finally, trypsin was added in a 1:50 ratio (trypsin:protein) and the contents were mixed carefully and left overnight at 37˚C to carry out a full digestion. The reaction was stopped by

adjusting the pH of the solution to slightly acidic (pH<6) as determined by using a pH paper detector.

Digested proteins were purified through a SEP-PAK C18 purification column to remove any trace salt following the manufacturer's protocol. Briefly, the column was equilibrated by flushing with 5 mL solution B and then with 10 mL solution A (both solutions prepared earlier). Then, the digested peptides were added gently onto the column and this was washed with 10 mL solution A. After that, peptides were eluted in 65% (v/v) ACN and 0.1% (v/v) FA.

3.4.5 Reversed-phase liquid chromatography tandem mass spectrometry

3.4.5.1 Principle

The principle of LC-MS/MS is based on separating analytes according to their molecular weight and the ion charge ratio (m/z). First, digested peptides travel through liquid chromatography and separated based on their molecular weight, then exposed to ion source electrospray ionisation (ESI) to volatize and ionize the peptides for MS analysis[199]. ESI coupled to MS is used to analyse complex samples. Then, eluted and ionised peptides exposed to heated drying gas at atmospheric pressure. This evaporates the solvent and increase the charge in the peptides. The exceeded like charges on the peptides lead to cohesive forces and ionised peptides desorbed into the gas phase. These peptides pass through a capillary sampling orifice into the mass analyser (**Figure 3.3**).

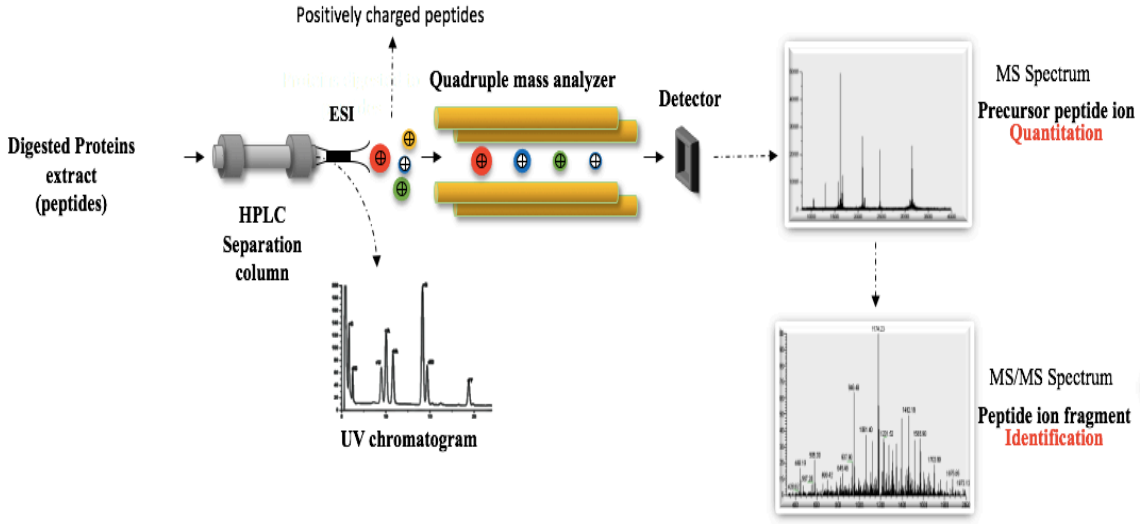


Figure 3. 3 LCMS schematic diagram. Depicted is the LCMC instruments: High pressure liquid chromatography (HPLC), electrospray ionisation (ESI), quadruple mass analyser, detector, mass spectrum (MS) for peptides quantification and MS for peptide ion identification.

The specific LC, ESI and MS instruments used in the study and their setup are discussed in sub-section of the corresponding chapters 4 and 5.

3.5 Statistical analysis

All experiments were performed in triplicates unless otherwise stated. Results were presented as mean ± standard deviation (SD). The equation for the standard deviation of the mean calculation is shown below:

$$SD = \sqrt{(1/N)\sum_{i=1}^{N}(xi - \mu)^2} \qquad \textbf{(Eq 3.7)}$$

Where x represents each value in the population, μ is the mean value of the population, Σ is the summation and N is the number values in the population.

Significant differences between two independent groups were determined using Student's t-test for paired and unpaired observations. *P* values < 0.05 were considered significant. For multiple independent groups, statistical significance was analysed by one-way and two-way ANOVA, multiple comparison was done using Turkey's range test.
Pearson's correlation coefficient (r) was used to determine the degree of correlation between the study variables. The equation for r is as following:

$$r = \frac{(n(\Sigma xy) - (\Sigma x)(\Sigma y))}{\sqrt{[n\sum x^2 - (\Sigma x)^2][n\Sigma y^2 - (\Sigma y)^2]}} \qquad \textbf{(Eq 3.8)}$$

The calculation and data analysis were performed on excel and GraphPad Prism software (GraphPad Software, UK).

4 Identifying novel cryo-protective agents to improve biopreservation

4.1 Introduction

In this chapter, details are provided concerning screening experiments investigating the protective properties of a selected panel of compounds during and after cryopreservation. The experiments involved testing a range of concentrations of each compound in order to determine dose response effects. The efficacy of the compounds was evaluated by measuring the cryosurvival rate, as a first classical parameter. Compounds that improved cryosurvival rates were further evaluated for mechanism of action by examining their effects on cell functionality, such as enhancing cellular activities and protecting macromolecules against oxidative damages.

Finally, this chapter reports on the differences in efficiency between glycerol and trehalose as conventional cryomedia for the cryopreservation of sRBCs in the presence and absence of the selected compounds. Moreover, the effects of the trehalose loading protocol using the synthetic biopolymer PP-50 have not been well-studied in RBCs. Here, an attempt is reported to optimise the trehalose loading protocol by examining a range of trehalose concentrations and different incubation times and assessing the changes that occur in the sRBC functional profile under these conditions.

4.2 Experimental design

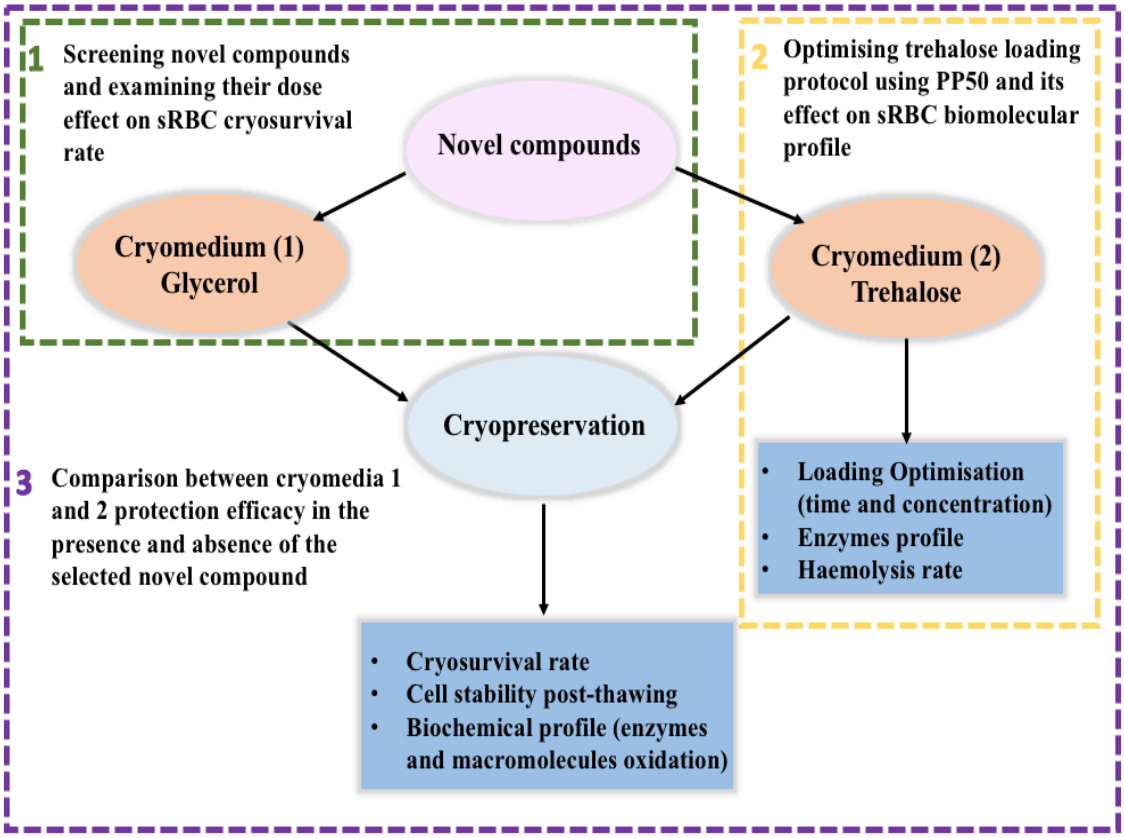


Figure 4. 1 Schematic diagram illustrating the experimental workflow. *The study here was divided into three main experiments. (1) Screening a selected panel of compounds for potential protective properties incorporated into glycerol cryomedium. (2) Optimising the trehalose loading protocol using the synthetic biopolymer PP-50 by examining two conditions, incubation times and trehalose concentrations, and assessing the cells' statuses under these conditions by measuring the haemolysis rate and the enzymatic profile. (3) Comparing the efficacy of glycerol and trehalose cryomedia for the cryosurvival rates and functional profiles of sheep red blood cells (sRBCs) in the presence and absence of the novel additive compound*.

The experimental study in the present chapter is divided into three parts (**Figure 4.1**). The first part involved screening a number of selected compounds that potentially possess protective properties based on their chemical structures and features. The selected compounds were added to PBS for equilibration before freezing, as well as to 20% glycerol solution (cryomedia 1). The compounds were incorporated into the solutions in a series of concentrations: 0, 50, 100, 200, 300, 400 and 500 µM. Initially, sRBCs were incubated for 2 h in PBS with and without the additive compounds for equilibration, and then the PBS was removed by centrifugation as described later (**Section 4.2.1.3**). sRBC pellets were re-suspended in 20% glycerol with and without the additive compounds, then immersed in liquid nitrogen and stored for 24 hours. Next, frozen sRBCs were

thawed in a 37°C water bath and the protective efficacy of the compounds was assessed by measuring the haemolysis rate using Drabkin's reagent.

The second experiment attempted to optimise the trehalose loading protocol using the synthetic biopolymer PP-50. It also investigated the status of key intracellular enzymes, mainly G6PD, LDH and GR during trehalose loading (as described in **Chapter 3**).

The last experiment analysed the additive effect of the best compound candidate in 10% glycerol versus 300 mM trehalose solutions (**Figure 4.2**). The cryosurvival rates of sRBCs were tested after thawing and cells were stored in a saline, adenine, glucose and mannitol (SAGM) solution with and without a protective compound under cold storage conditions (4°C) for 10 days. The stability of the cells was measured every two days during cold storage by measuring the haemolysis rate. The biomolecular profiles of surviving cells were assayed prior to freezing, post thawing and 10 days later after hypothermic storage. The assays included measurements of GR and LDH activities, and the levels of protein and lipid oxidation damage. Enzymatic functional assays and statistical analysis in this chapter were performed as described previously in **Chapter 3**.

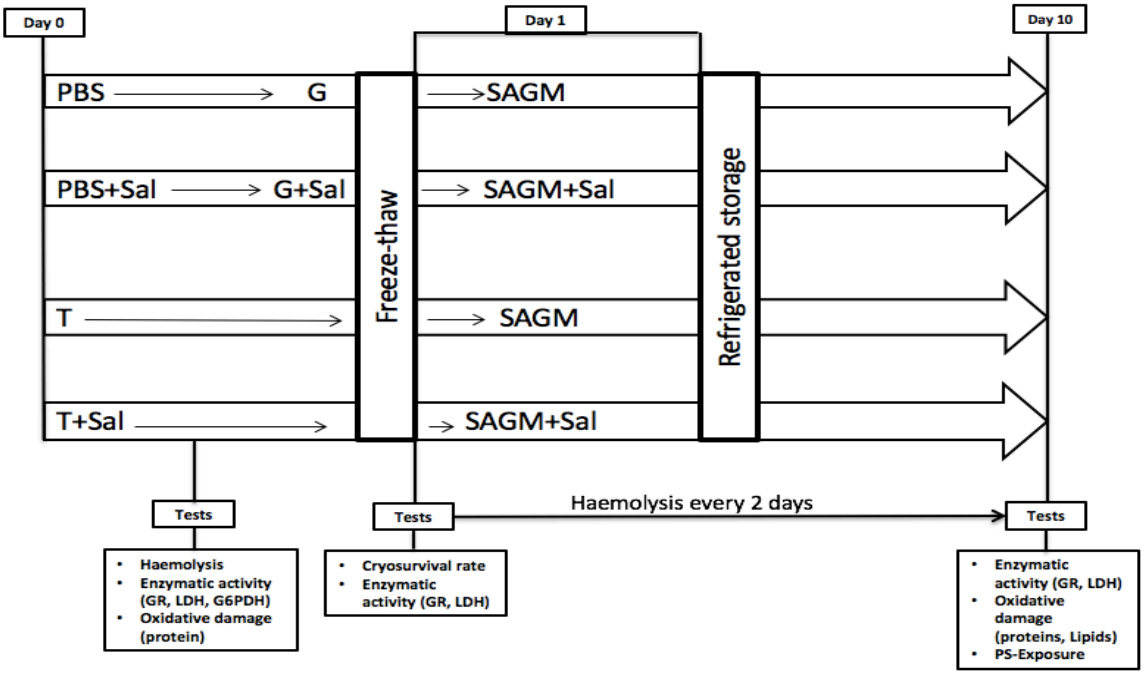


Figure 4. 2 Diagram illustrating the experimental workflow to determine the effects of conventional cryomedia glycerol (G) and trehalose (T) with and without the additive compound salidroside (Sal). *Sheep red blood cell (sRBC) samples were collected and evaluated at three time-points: before cryopreservation, post-thaw and during hypothermic storage at 4°C. The evaluation measured haemolysis rate, enzymatic activities, oxidative damages and phosphatidylcholine (PS) exposure, as shown in the diagram. sRBC stability post-thaw was measured every two days during the cold storage.*

4.2.1 Experimental materials and assays

4.2.1.1 Materials and reagents

Defibrinated sheep red blood cells (sRBCs) were purchased from TSC Bioscience Ltd (Buckingham, UK). Dihydrate trehalose, curcumin, glutathione, ascorbic acid, erlose, turanose, nigerose, salidroside, melezitose, gentibiose, quercetin, quercetin-3-D-glucoside, sterilised filtered Dulbecco's phosphate buffer saline (DPBS), sodium chloride, glucose, adenine, mannitol, Drabkin's reagent and glycerol were purchased from Sigma-Aldrich (Dorset, UK). The synthetic membrane permeabiliser PP-50 was synthesised in-house[143]. The FITC Annexin V apoptosis detection kit was purchased from BD Pharmingen (Oxford, UK).

3.3.1.2 Preparation of solutions

Trehalose (300 mM) was prepared in DPBS and the polymer PP-50 was added to a final concentration of 100 μg/mL for trehalose loading. Glycerol at a concentration of 10% was also prepared in DPBS. This concentration was shown to possess similar cryo-protective effects to those exhibited by 20% glycerol prepared in DPBS (Supplementary data S4.1).

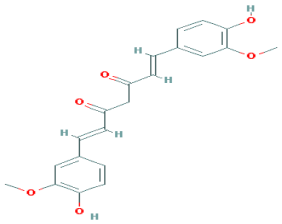
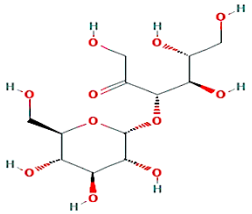
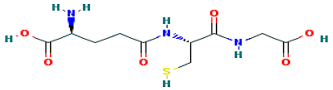
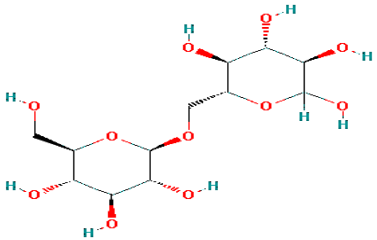
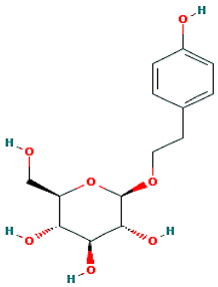
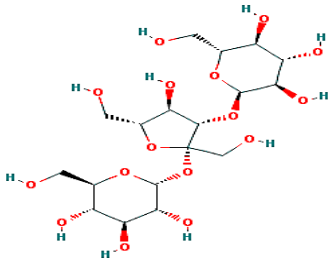
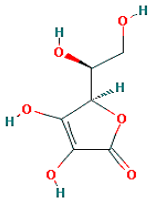
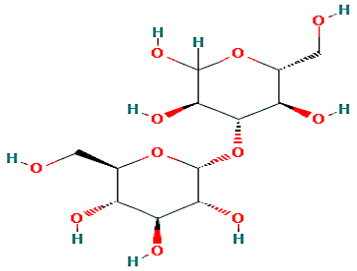
Stock solutions of the selected compounds were prepared at 100 mM by adding the appropriate amount to PBS, with the exception of curcumin, which has poor solubility in water but is more soluble in the presence of glycerol; therefore, curcumin was used only in the freezing medium. Serial final concentrations of 50, 100, 200, 300, 400 and 500 μM were then prepared in either DPBS or the cryomedia, glycerol and trehalose. SAGM was prepared by adding 8.77 g sodium chloride, 9.0 g glucose, 0.169 g adenine and 5.25 g mannitol to 1000 mL sterilised water. Salidroside was added as required to the solution to reach a final concentration of 200 μM.

3.3.1.3 Preparation of sRBCs

Defibrinated sRBCs were pelleted by centrifugation at 10,000 x g for 3 min. Supernatants were removed and the pellets were washed three times with PBS by centrifugation, as mentioned above. For testing the effects of compounds, sRBC pellets were suspended in 1 mL PBS with and without the selected compounds, and then incubated for 2 h.

3.3.1.4 Compounds selected for their potential cryo-protective properties

Compounds utilised in the current study are shown in **Table 4.1**. The selection of these compounds was based on three main criteria. Structurally, compounds had to be classified as phenolic polyols possessing multiple hydroxyl functional groups for organic reactions. They also had to be derived from natural sources, and considered safe for human and animal consumption. Finally, they had to possess antioxidative properties or resemble antioxidants in their structure. These compounds were dissolved in the appropriate solvent, which in this case was DPBS buffer, cryomedium (trehalose or glycerol) and SAGM solution. The prepared solutions were either used immediately or stored at -20ºC until use.

Component name	Source	Abbreviation	Chemical structure	Component name	Source	Abbreviation	Chemical structure
Curcumin[200,201]	Derived from plants	CCM		**Turanose**[202,203]	Honey	Tur	
Glutathione[204]	Derived from plants	Glu		**Gentiobiose** [205]	Honey	Gen	
Salidroside[206,207]	Derived from plants (Rahdiola rosea)	Sal		**Melezitose**[202]	Honey	Mezo	
Ascorbic acid[208]	Derived from plants	Aa		**Nigerose**[202,203]	Honey	Nig	

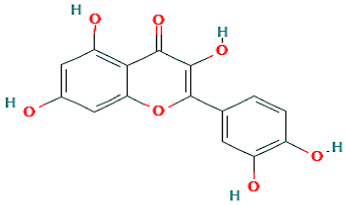
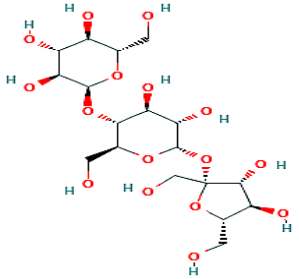
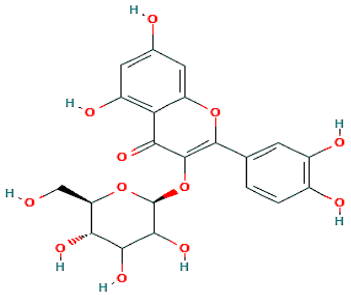
Quercetin[209]	Derived from plants	Qur		**Erlose**[202,205]	Honey	Erl	
Quercetin 3-glucoside[210]	Derived from plants	Q3G					

Table 4. 1 Chemical structures and sources of the selected compounds for potential cryoprotective properties screening assay using RBC biopreservation.

4.2.2 Cryopreservation and cryosurvival measurement

For cryopreservation in trehalose, sRBC pellets were suspended in 1 mL 300 mM trehalose with 100 μg/mL PP-50 to yield a 15% haematocrit (hct), as described previously[143]. The compounds were added to the incubation solution in a series of concentrations, as described previously, and haemolysis was measured after incubation to check the effect of the used compounds. For the glycerol freezing solution, sRBC pellets were suspended in glycerol cryomedium at 15% hct with and without tested compounds, and then left to equilibrate for no longer than 10 min. Cell suspensions were transferred into 2 mL cryogenic tubes, and then submerged in liquid nitrogen and left for 24 h to 1 week. Afterward, the sRBC samples were thawed in a 37°C water bath and haemolysis was measured (as described in **Chapter 3**) for survival assessments.

The sRBC pellets were washed as described before, and then suspended in SAGM with and without the test compound and left to recover for 10 days. During the recovery period, sRBC suspensions were stored at 4°C and haemolysis was measured every two days to assess cells' stability post freeze–thaw and during cold storage.

4.2.3 Phosphatidylserine translocation

The exposure of phosphatidylserine (PS) was tracked by flow cytometry using fluorescence-activated cell sorting. External PS was tagged with Annexin V flagged with the fluorescent molecule fluorescein isothiocyanate (FITC). Annexin V is a calcium-dependent phospholipid-binding protein that has a high binding affinity to PS and is used to identify apoptotic cells by binding to exposed PS. PS in normal intact cells is located in the interphase of the cell membrane. The translocation of PS from the interphase to the external surface of the membrane is a biomarker of cellular apoptosis, and cells stained with FITC are therefore considered to be apoptotic.

The assay was performed following the manufacturer's instructions (BD Pharmingen; Oxford, UK). Briefly, surviving cells obtained from trehalose+/-Sal cryopreservation were collected on Day 10 post refrigeration in SAGM+/-Sal and washed twice in cold PBS. Next, $1x10^6$ cells/mL were suspended in 1X binding buffer and 100 μL of the suspension were incubated with 5 μL of the added Annexin V solution. The mixture was gently vortexed and then incubated in the dark at room temperature for 15 min. After this, 400 μL of 1X binding buffer were added to each tested sample and then samples were vortexed. Two sets of controls were run in parallel with the tested

samples: a biological control (fresh and untreated RBCs) and a technical control (unstained RBCs). The analysis was performed within 1 h using a CyAN™ ADP analyser with excitation at 488 nm, using the Summit™ software for data acquisition and analysis. Each dot represents a single cell and dot density is expressed by contour. The analysed data were selected manually after excluding clumps of cells and debris.

4.3 Results

4.3.1 Primary screening of novel compounds

The effect that adding the selected compounds had on survival rate of sRBCs post freeze–thaw was estimated by measuring the haemolysis rate (**Figure 4.3**). The cryosurvival rates of sRBCs cryopreserved in cryomedia containing 50 – 400 μM CCM or Sal (**Figure 4.3A**) were significantly higher than those in glycerol alone, with cryosurvival rates of 87 ± 3.73% and 92 ±1.04% in CCM and Sal, respectively, in comparison to 72 ± 1.05% in glycerol. In contrast, adding Q3G demonstrated protective effects in a dose-dependent manner. In this case, the cryosurvival rate increased with increasing Q3G concentrations, to reach a maximum level of 81.5 ± 4.5% at 500 μM. The opposite effect was seen with increasing ascorbic acid concentrations, as the cryosurvival rates of sRBCs decreased from 67 ± 4.9% at 100 μM to 31.02 ± 3.5% at 500 μM. The best cryosurvival rates obtained from the ascorbic acid and quercetin treatments occurred when sRBCs were frozen in cryomedia containing 50 and 100 μM of both, however, cryosurvival rates under these conditions remained significantly lower than those observed in the control. A similar outcome was observed in glutathione (Glu) treatments, where the cryosurvival rate was 53.88 ± 1.6% at 50 μM and decreased to 23.2 ± 2.3% at 100 μM.

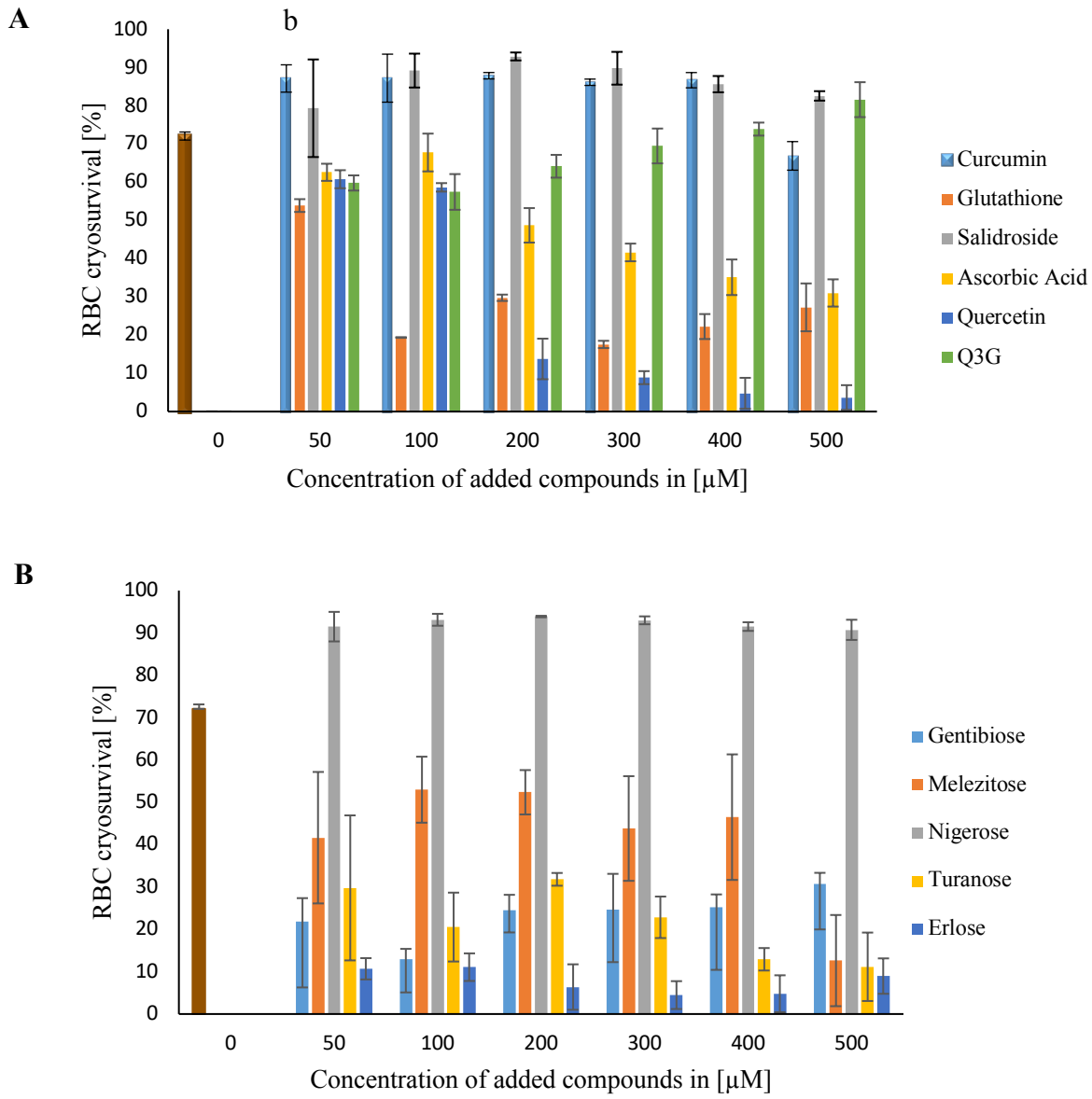


Figure 4. 3 Effects of various concentrations of additive agents on cryosurvival rates of sheep red blood cells (sRBCs). *sRBCs were suspended and frozen in 20% glycerol with and without additive agents for 24 hours. (A) Shows the effect of additive agents derived from plants and (B) shows the effect of additive agents derived from honey. An additive concentration of zero corresponds to sRBCs frozen in 20% glycerol only. All compounds were added separately to 20% glycerol solution for final concentrations of 50, 100, 200, 300, 400 and 500 µM. After thawing, cryosurvival rate was measured using a haemolysis assay. Results were analysed using one-way ANOVA, followed by Turkey's test for multiple comparisons. Data were derived from triplicate samples in each test. Data are expressed as means ± SD. Letters a and b denote significant differences between variables ($p < 0.05$).*

The additive Nig significantly improved the survival rate of sRBCs post-thawing, even when applied at low concentrations (**Figure 4.3B**). For instance, at 50 µM the cryosurvival rate was 91.48 ± 3.48%, nearly 21.64% higher than the estimated cell survival in glycerol alone. Applying Nig at higher concentrations (100 – 500 µM), however, showed similar effects to those observed at 50 µM. Erlose, turanose, gentibiose and melezitose had effects opposite to those of Nig, lowering the cryosurvival rates of sRBCs in all applied concentrations, in comparison to the control. Interestingly, melezitose exhibited the same effects on sRBC cryosurvival rates when applied at 50 – 400 µM concentrations (cryosurvival rate of 47.45 ± 11.14%), but when increasing the concentration to 500 µM, the recovery rate decreased to 12.6 ± 11%.

4.3.2 Optimising trehalose loading and cryopreservation protocol

4.3.2.1 Effects of trehalose concentration and incubation time effects on sRBCs prior to cryopreservation

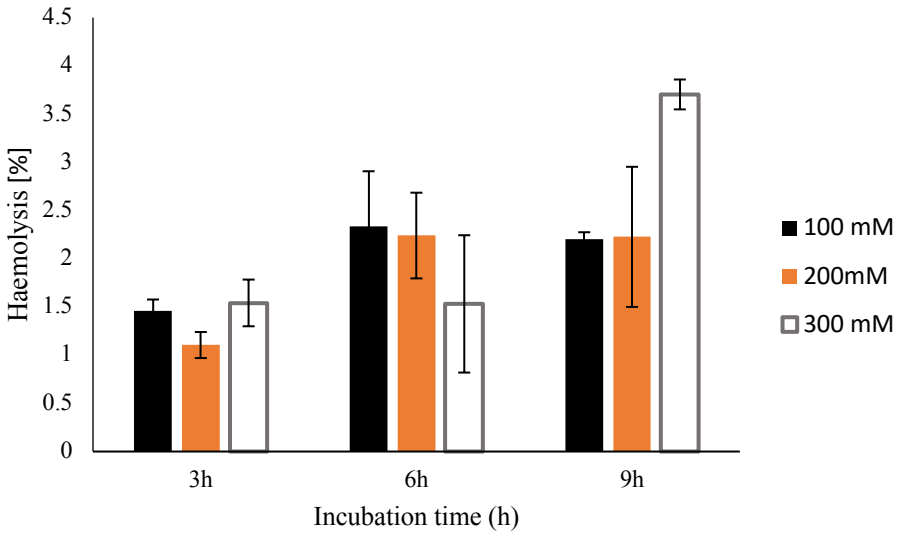


Figure 4. 4 Haemolysis induced in sheep red blood cells (sRBCs) after incubation in different trehalose concentrations for different intervals of time. *The haemolysis of sRBCs incubated in 100, 200 and 300 mM trehalose with 100 µg/mL PP-50 was measured using Drabkin's reagent every 3 hours for 0 – 9 h intervals. Data are derived from triplicates and expressed as mean ± SD; bars and (*) indicate significant variables ($p < 0.01$).*

Haemolysis was induced in all sRBCs by the incubation time and trehalose concentrations tested (**Figure 4.4**). Incubating sRBCs in 300 mM trehalose for 9 h was found to trigger the highest level

of haemolysis (3.7 ± 0.15%), in comparison to the other concentrations, which yielded approximately 2.5% or less. Overall, haemolysis rates of sRBCs resultant from the incubation with a range of different trehalose solutions were low.

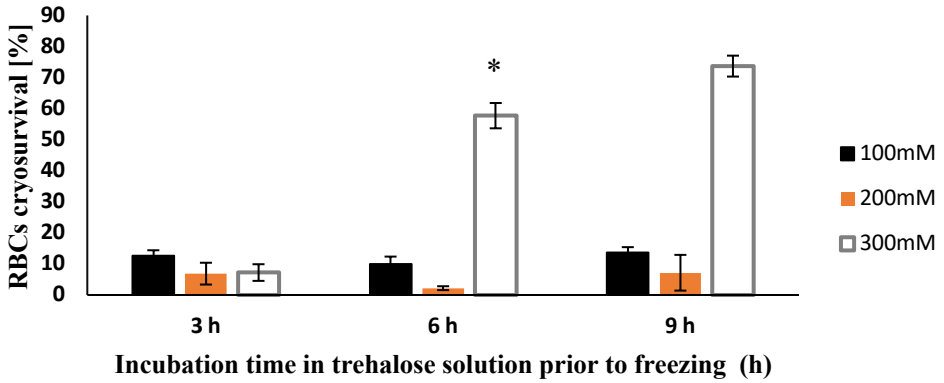


Figure 4. 5 Effects of different trehalose concentrations and incubation times on cryosurvival rates of sheep red blood cells (sRBCs). *sRBCs were incubated in 100, 200 and 300 mM trehalose solution with 100 µg/mL PP-50 for different time intervals (3, 6 and 9 h) prior to cryopreservation, then frozen for 24 h in liquid nitrogen, followed by thawing at 37°C. Cryosurvival rates were assayed using Drabkin's reagent. Data were derived from triplicates, expressed as mean ± SD; bars and * indicate significant variables ($p < 0.01$).*

Trehalose concentration and loading time are major factors involved in successful cryopreservation. The highest cryosurvival rate, 73 ± 3.3%, was obtained when sRBCs were incubated in 300 mM trehalose for 9 h, in comparison to rates of 13.64 ± 1.75% and 7.1 ± 5.75% for sRBCs incubated for the same time period in 100 mM and 200 mM trehalose, respectively (**Figure 4.5**). A shorter incubation time in 300 mM trehalose solution resulted in a lower cryosurvival rate (57.3 ± 4%). Similarly, incubating sRBCs in a lower concentration of trehalose for a shorter period of time was not beneficial for sRBC cryopreservation.

4.3.2.2 Enzymatic activities in sRBCs during trehalose loading

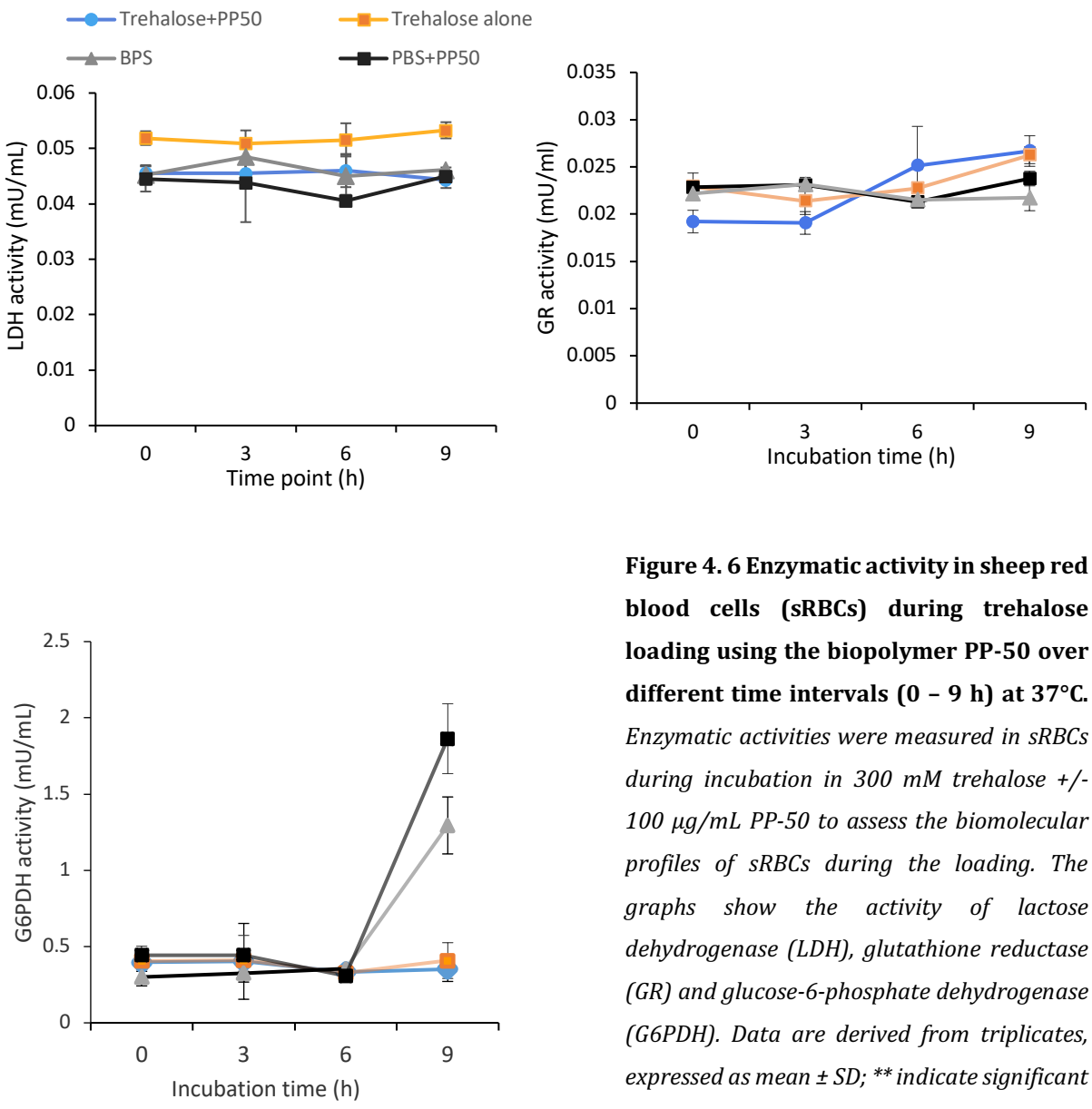


Figure 4. 6 Enzymatic activity in sheep red blood cells (sRBCs) during trehalose loading using the biopolymer PP-50 over different time intervals (0 – 9 h) at 37°C. *Enzymatic activities were measured in sRBCs during incubation in 300 mM trehalose +/- 100 µg/mL PP-50 to assess the biomolecular profiles of sRBCs during the loading. The graphs show the activity of lactose dehydrogenase (LDH), glutathione reductase (GR) and glucose-6-phosphate dehydrogenase (G6PDH). Data are derived from triplicates, expressed as mean ± SD; ** indicate significant differences (p < 0.01).*

Enzymatic activities were measured in sRBCs incubated with 300 mM trehalose +/- PP-50 and PBS +/- PP-50 for different time intervals. LDH activity was steady for all conditions, exhibiting no significant variation, with the exception of when sRBCs were incubated in trehalose alone, in which case the LDH activity was slightly higher (by 0.01 ± 0.0012 mU/mL) than under the other conditions (**Figure 4.6**).

For GR activity, slight differences were observed among different conditions (**Figure 4.6**). GR activity in sRBCs incubated in trehalose + PP-50 increased insignificantly over the incubation

period from 0 and 9 hours, from 0.03 ± 0.002 mU/mL to 0.04 ± 0.0027 mU/mL. sRBCs incubated in trehalose solution alone demonstrated a similar increasing pattern of GR activity over time, from 0.021 ± 0.002 to 0.026 ± 0.0015 mU/mL. Overall, GR activity in sRBCs incubated in the trehalose solution was slightly higher than that in sRBCs incubated in the PBS buffer. The use of PP-50 appeared to have no effect on GR activity. Interestingly, G6PDH activity in sRBCs incubated in PBS buffer with PP-50 showed an increase in activity over time, from 0.44 ± 0.08 mU/mL at 3 h to 1.86 ± 0.004 mU/mL at 9 h. A similar result was observed when sRBCs were incubated in PBS alone, where G6PD activity increased from 0.3 ± 0.008 mU/mL to 1.29 ± 0.004 mU/mL. This was different for sRBCs incubated in trehalose with or without PP-50, in which case the activity of the enzyme remained at the same level from the starting point up to 9 h, where it was estimated to be 0.37 ± 0.033 mU/mL.

4.3.3 The efficiency of conventional cryomedia in the presence and absence of a novel cryoprotective agent (Sal)

The screening of the compounds' additive effects on cryosurvival rates revealed that Cur, Nig and Sal were the most effective compounds for enhancing cell recovery rates post-thaw. Further validations for the additive compounds effects were therefore performed. In the current pilot study, 200 µM of Sal was examined in glycerol and trehalose for its effect on cells' biomolecular profiles and stability post freeze–thaw.

4.3.3.1 Effects of Sal on sRBCs after incubation and freeze–thaw in glycerol +/- Sal versus trehalose +/- Sal

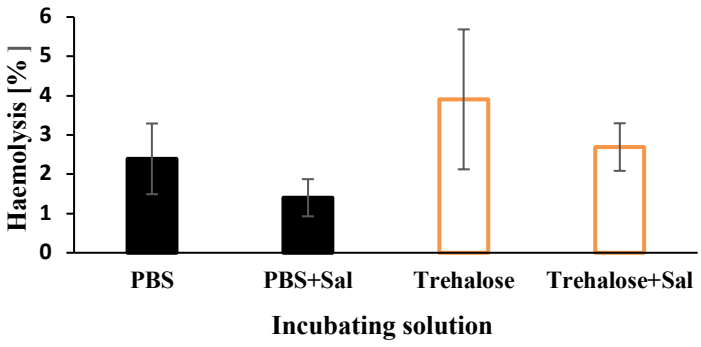


Figure 4. 7 Haemolysis induced in sheep red blood cells (sRBCs) post incubation in PBS and trehalose with and without salidroside (Sal). *Haemolysis of sRBCs was measured after 9 h of incubation in PBS or trehalose/PP-50 with and without Sal at 37°C. Data are derived from triplicates, expressed as mean ± SD ($p > 0.05$).*

Before freezing sRBCs, the effect of Sal on the rate of haemolysis during trehalose loading at 37°C was investigated using Drabkin's reagent. The results showed that during the pre-freezing incubation conditions, trehalose triggered haemolysis at a rate of 3.9 ± 1.8% (**Figure 4.7**). Adding Sal reduced haemolysis to 2.7 ± 0.6%. A similar trend was seen in sRBCs incubated in PBS versus PBS containing 200 μM Sal; the rate of haemolysis was reduced from 2.4 ± 0.9% in PBS alone to 1.4 ± 0.4% in PBS with Sal. Overall, haemolysis rates were considered insignificant in all conditions, as they were below 4%.

4.3.3.2 Effect of Sal on cryosurvival of sRBCs post thawing

The effect of Sal on sRBC cryosurvival was assessed 24 h after thawing by measuring the haemolysed versus surviving cells using Drabkin's reagent (**Figure 4.8**). sRBCs frozen in 10% glycerol with Sal showed a survival rate of 61.1 ± 4.8%, which was approximately 1.6-fold greater than that observed for cells frozen in glycerol alone (37.9 ± 4.6%). Cryosurvivability of sRBCs frozen in trehalose with Sal (61.2 ± 1.4%) was significantly greater than that of cells frozen in trehalose alone (54.8 ± 1.7%). Overall, Sal enhanced the cryosurvival rates of sRBCs after freeze–thaw by similar manner in both glycerol and trehalose.

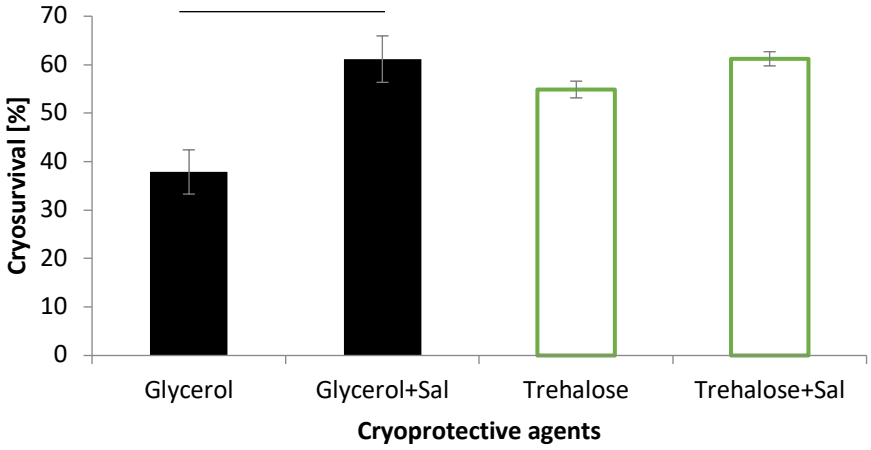


Figure 4. 8 Effect of salidroside (Sal) on cryosurvival rates of sheep red blood cells (sRBCs) in glycerol and trehalose cryomedia. *sRBCs were frozen in either glycerol or trehalose with or without Sal. After thawing, survival rates of sRBCs were measured using Drabkin's reagent. Data are expressed as mean ± SD; (*) and bar indicate a significant difference ($p < 0.05$).*

4.3.3.3 Effect of Sal on sRBC survival after thawing in cold storage for 10 days

During the first two days post-thawing, no haemolysis was observed in sRBCs resultant from trehalose/Sal post-thaw and storage in SAGM/Sal (**Figure 4.9).** By Day 4, the rate of haemolysis under these conditions gradually increased to reach 12.1 ± 2.5%. The haemolysis rate continued to increase at a low rate, finally reaching a level of approximately 16.7 ± 1.3% by Day 10. sRBCs that had been frozen in trehalose alone and stored in SAGM without Sal exhibited a significantly higher haemolysis rate (29 ± 8.4%) compared to the effects observed in the presence of Sal between Days 4 and 10 ($p < 0.05$). sRBCs frozen in glycerol alone exhibited a higher haemolysis rate (60.4 ± 5.0%) during storage in SAGM media than that observed under the trehalose conditions described above. Introducing Sal to the glycerol and SAGM afterward resulted in a lower haemolysis rate than that of cells frozen and stored without Sal. Haemolysis rates under both conditions [glycerol (SAGM) and glycerol+Sal (SAGM+Sal)] were considerably higher in comparison to trehalose alone with and without Sal. The haemolysis rate in glycerol+/-Sal eventually reached 60.4 ± 5.0% on Day 10.

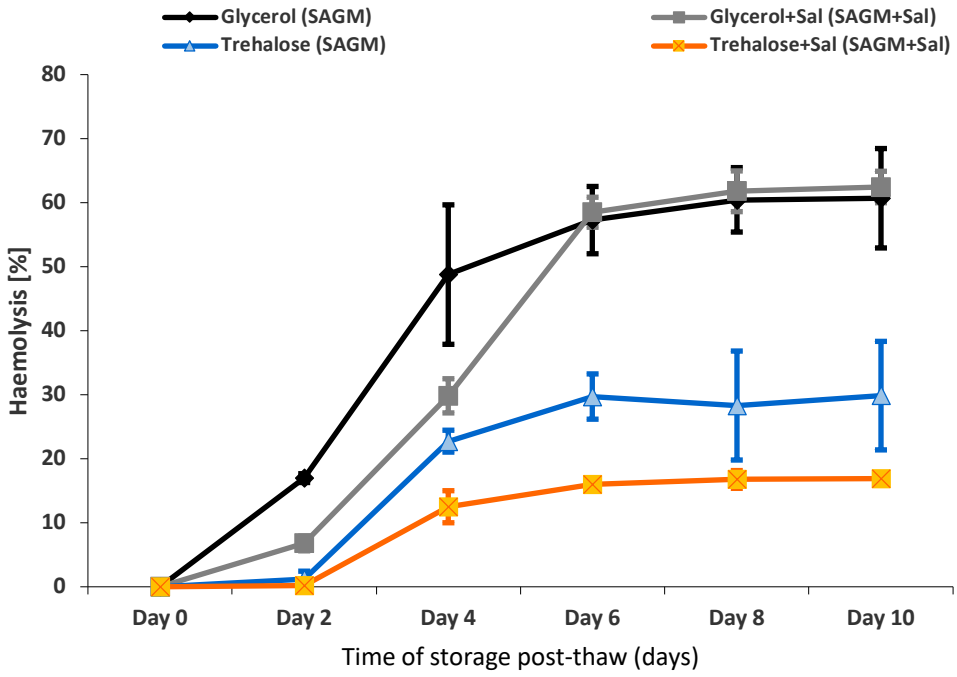


Figure 4. 9 Post-thaw haemolysis rates of sheep red blood cells (sRBCs) stored and refrigerated in either saline, adenine, glucose and mannitol (SAGM) solution alone or in the presence of salidroside (SAGM+Sal). *Survival rates of sRBCs cryopreserved in glycerol or trehalose solutions with and without Sal were assessed every two days for up to 10 days post-thawing by measuring sRBC haemolysis using Drabkin's reagent. Data are obtained from triplicates and expressed as mean ± SD; $p < 0.05$ is considered significant*.

4.3.3.4 Effect of Sal on enzymatic activity in sRBCs after one day and ten days in cold storage

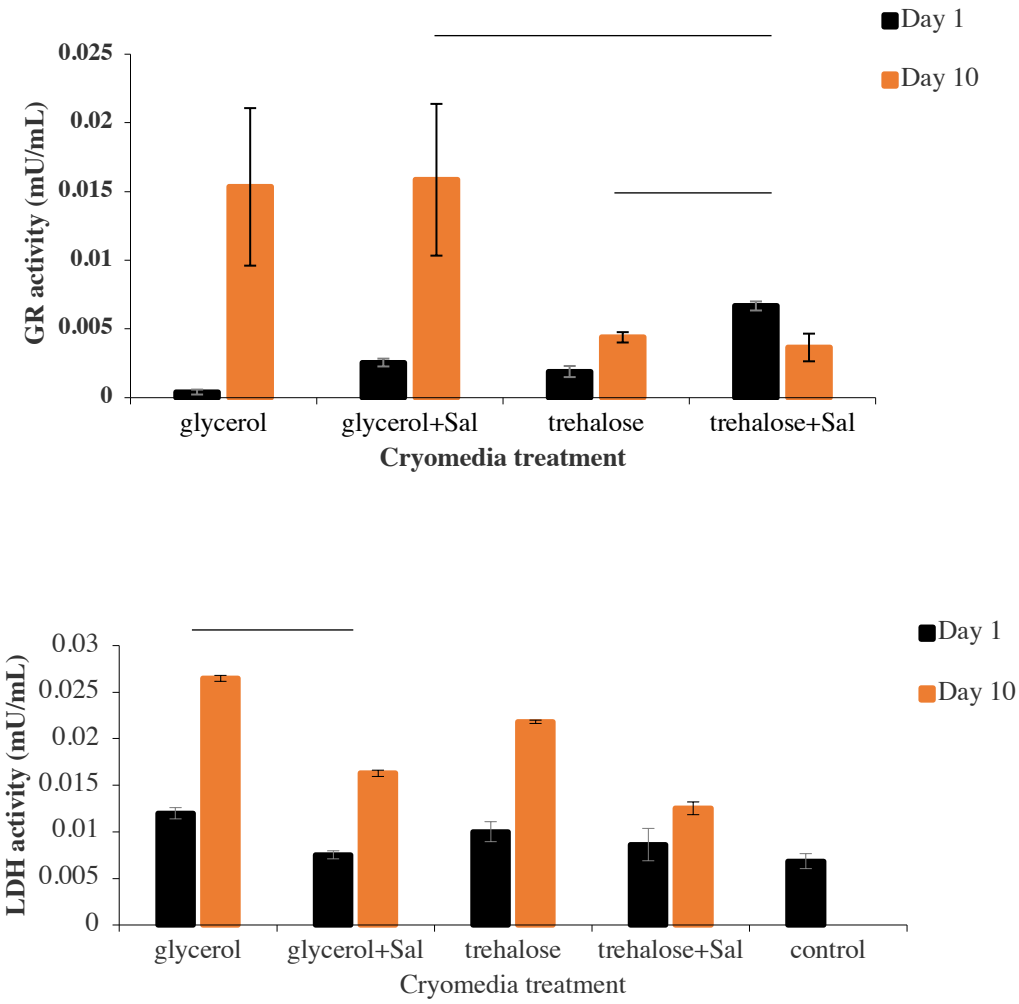


Figure 4. 10 Intracellular enzymatic activity in sheep red blood cells (sRBCs) measured after thawing at Day 1 and Day 10 of cold storage in saline, adenine, glucose and mannitol media with and without salidroside (Sal). The top graph shows the intracellular activity of glutathione reductase (GR) and the bottom graph shows that of lactose dehydrogenase (LDH). Data are obtained from triplicates and expressed as mean ± SD; bars and * indicate significant differences ($p < 0.05$).

Incorporating Sal into the cryomedia and the refrigerating media post-thaw led to an increase in GR enzymatic activity in sRBCs on Day 1, both in trehalose (0.0066 ± 0.003 vs 0.0004 ± 0.00017 mU/mL) and in glycerol (0.0025 ± 0.00028 vs 0.0019 ± 0.0004 mU/mL; **Figure 4.10**).

On Day 10, GR activity increased considerably with and without Sal in glycerol-frozen sRBCs, reaching 0.015 ± 0.005 mU/mL, approximately 37 times greater than the activity observed on Day 1. In contrast, in the trehalose-frozen cells, GR activity in the absence of Sal showed a smaller increase (10-fold) from Day 1 to Day 10 (increase of 0.004 ± 0.003 mU/mL). In the presence of Sal, the activity decreased over the 10 days to reach 0.006 ± 0.0003 mU/mL.

Intracellular LDH activity in sRBCs was also measured on Day 1 and Day 10 after thawing. In general, sRBCs stored with Sal showed lower LDH activity than LDH in sRBCs stored without Sal. On Day 1, the activities of LDH in sRBCs without Sal were 0.01 ± 0.0006 mU/mL and 0.01 ± 0.007 mU/mL under the glycerol and trehalose freezing conditions, respectively. LDH activity increased by Day 10 to reach 0.026 ± 0.0003 mU/mL and 0.0218 ± 0.0002 mU/mL in the same samples, respectively. The presence of Sal resulted in a lower level of intracellular LDH activity on Day 1, under both the glycerol (0.007 ± 0.0004 mU/mL) and trehalose (0.008 ± 0.002 mU/mL) freezing conditions. On Day 10, LDH activities of the stored sRBCs in the presence of Sal were 0.016 ± 0.0003 mU/mL and 0.012 ± 0.0006 mu/mL in glycerol and trehalose, respectively (**Figure 4.10**).

4.3.3.5 Oxidative damage to sRBCs post-storage

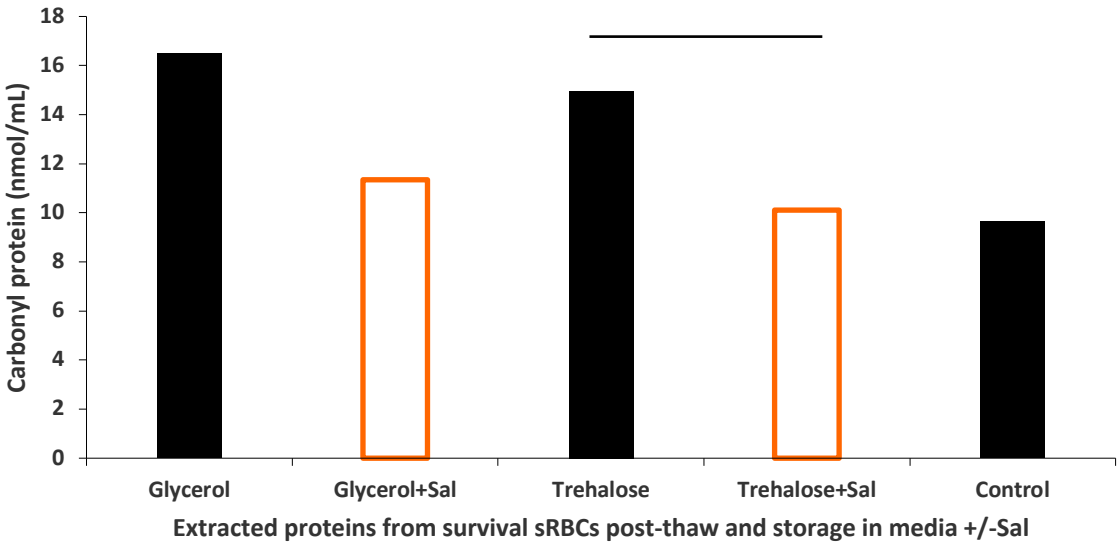


Figure 4. 11 Effect of salidroside (Sal) on protein carbonylation in sheep red blood cells (sRBCs) when stored in saline, adenine, glucose and mannitol (SAGM) media post thaw. *The protein carbonylation level was determined in sRBCs cryopreserved, thawed and stored in SAGM+/-Sal. The control represents the protein carbonylation level of sRBCs not subjected to freeze–thaw. Data are derived from pooled triplicate samples and represented as the mean ± SD; * and bars indicate significant differences (p-value < 0.05).*

The potential protective effect of Sal against protein oxidation in sRBCs post cryopreservation and recovery was assessed. Carbonylated proteins in sRBCs post-thaw were compared to the control, which reflected carbonylation of proteins in fresh sRBCs not subjected to freeze–thaw (**Figure 4.11**). Carbonylated protein levels in sRBCs stored in SAGM immediately post thaw were 16.49 nmol/mL and 14.99 nmol/mL under the glycerol and trehalose freezing conditions, respectively. sRBCs cryopreserved and stored in media with Sal showed significantly lower carbonylated protein levels of 10.1 nmol/mL and 9.63 nmol/mL for the glycerol and trehalose freezing conditions, respectively ($p < 0.05$).

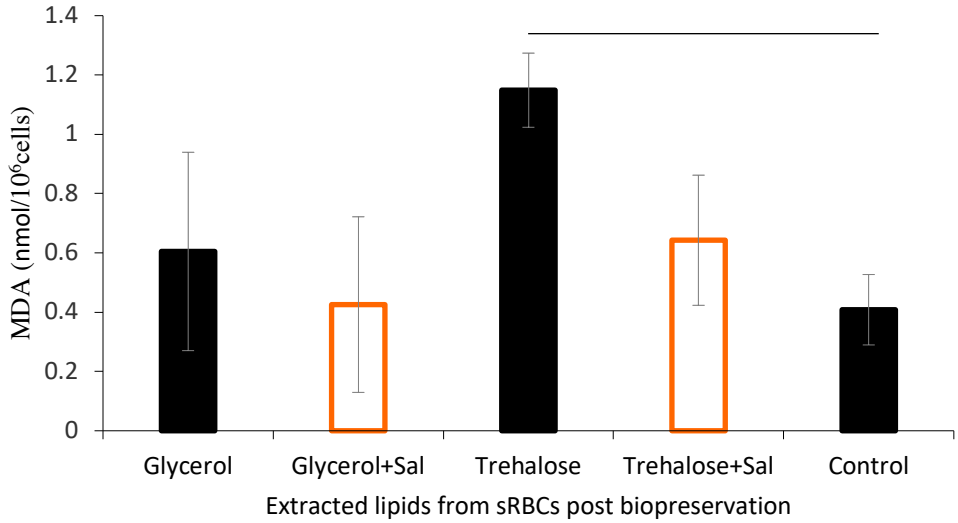


Figure 4. 12 Effect of salidroside (Sal) on lipid peroxidation in sheep red blood cells (sRBCs) in saline, adenine, glucose and mannitol (SAGM) media post-thaw. *Lipid peroxidation, based on malondialdehyde (MDA) levels, was measured in sRBCs stored in SAGM+/- Sal post-thaw. The control represents extracted lipids from fresh sRBCs not subjected to freeze–thaw. Data are derived from triplicates and expressed as mean ± SD; * and bars indicate significant differences (p < 0.05).*

The oxidative damage suffered by sRBC lipids in relation to the presence of Sal was determined by measuring malondialdehyde (MDA) levels **(Figure 4.12**). The results demonstrated that glycerol had no effect on lipid peroxidation levels in sRBCs after thawing and refrigeration (0.4 ± 0.29 vs 0.6 ± 0.22 nmol/10^6 cells under control and glycerol freezing conditions, respectively). In contrast, trehalose induced significant lipid peroxidation, reaching 1.14 ± 0.125 nmol/10^6 cells. Interestingly, adding Sal reduced MDA in trehalose-loaded sRBCs to 0.64 ± 0.2 nmol/10^6 cells, which was similar to that in the control.

4.3.3.6 Influence of trehalose +/- Sal on apoptosis of sRBCs

The externalisation of PS, the apoptotic biomarker, was determined in trehalose-loaded sRBCs after freeze–thaw and storage in SAGM in the presence and absence of Sal (**Figure 4.13**). Data were acquired at Day 0 for control, and then post thawing and refrigeration on Day 10. There was less than 1% PS exposure in sRBCs under all conditions (control, trehalose [T] and trehalose + Sal [T+Sal]), indicating that none of the tested conditions promoted the exposure of PS. Although the control exhibited slightly higher PS exposure than did T and T+Sal, the difference was considered insignificant.

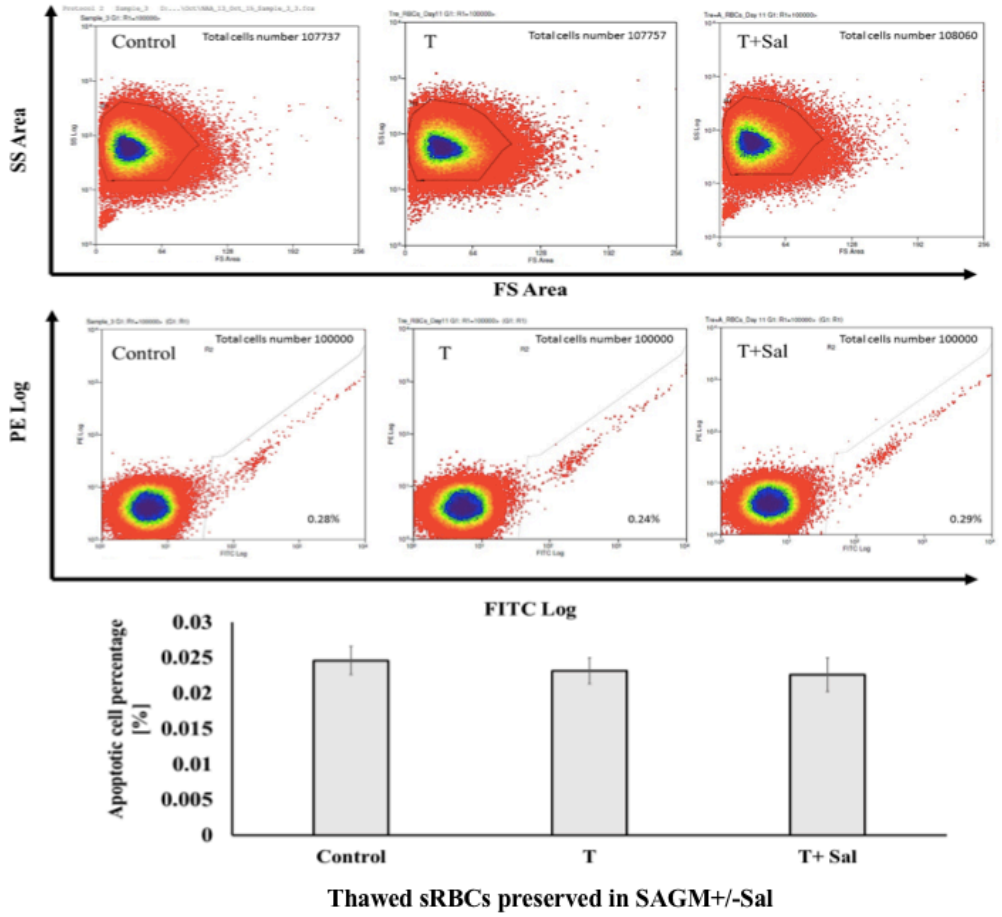


Figure 4. 13 Effect of salidroside (Sal) on apoptosis of sheep red blood cells (sRBCs) post thawing and storage in saline, adenine, glucose and mannitol (SAGM) media. *sRBCs cryopreserved using trehalose (T) +/- Sal and maintained in SAGM+/-Sal were tested for early apoptosis on Day 10 post thawing using the phosphatidylserine (PS) biomarker. Externalisation of PS in sRBCs after storage at 4°C for 10 days was determined using the fluorescent conjugate annexin-V. Analysis was performed via flow cytometry. The control represents fresh sRBCs without any further treatment. The top panel shows the sorting of cells by population size and the bottom panel shows cells sorted according to their emission of FITC. Data are obtained from triplicates and expressed as mean ± SD; p >0.01 is considered significant.*

4.4 Discussion

This study aimed to improve the protective efficacy of cryomedia by introducing an effective protective compound to enhance cryosurvival rates and limit oxidative damage to cells. For this purpose, several compounds derived from natural sources were screened in sRBCs. The analysis took into account the effects of any previous applications of these compounds, if they existed, on cell vitality, as well as the particular cellular models used (e.g. nucleated and anucleated). The dose effectivenesses of the additive compounds were examined using a range of concentrations (50 – 500 μM). The best cryosurvival rates after freeze–thaw, compared to those of cells in glycerol alone, were obtained by adding CCM, Nig or Sal to the cryomedia. In the experiment, these three compounds enhanced cryosurvival rates of sRBCs in a dose-independent manner. CCM is one of the most-studied plant derivatives in medicine, as an antioxidant and anti-inflammatory agent[211]. Additionally, it has been utilised as a health-beneficial food spice for a long time with no side effects reported[200]. The protective effect of CCM on sRBCs in the present study corresponds with previous studies of sperm cryopreservation that utilised CCM at the same doses[212,213]. Altogether, these findings indicate that this compound is likely to be effective and safe in the biopreservation of a variety of cell types[201]. The antioxidative mechanism of CCM was proposed previously by two research groups. The first group suggested that CCM donates a hydrogen atom in one of its phenol rings under physiological conditions[214,215], whereas the second group suggested that CCM donates a hydrogen atom in a central methylenic group under acidic conditions[216].

The protection provided by Nig and Sal to sRBCs during cryopreservation is comparable to that found for CCM. The addition of either of these compounds increased sRBC survival by 20% in comparison to conventional cryomedia. The existence of both compounds, Nig and Sal, in human diets without recorded side effects suggests that they are safe for consumption. Sal is the active compound in *Rhodiola rosea*, a Chinese traditional herb that has been reported to relieve fatigue and exhaustion resulting from prolonged stress, as well as having anti-apoptotic[217] and antioxidative properties[206]. In contrast, little is known about Nig. It is found in honey[203] and a few studies have reported its application as a substrate for some enzymes only[218]. Considering the present findings, however, it should be noted that CCM, Nig and Sal share a common structural feature, as they all contain multiple hydroxyl (-OH) functional groups (CCM, Nig and Sal contain 3, 8 and 5 -OH groups, respectively). This suggests that they may act as antioxidants by donating their H-atom to neutralise free radical species. This possibility requires further investigation,

however, using isotopically-labelled compounds to trace atom transfers in reaction products will most likely be useful.

Contrary to expectations, sRBC cryosurvival rates were unexpectedly low when antioxidants such as ascorbic acid, glutathione and quercetin were applied. Interestingly, previous studies have shown that these molecules act in a dose-dependent manner[219]. For example, the effective concentration of glutathione in a previous investigation was greater than the concentrations applied in the present study. In addition, glutathione is an unstable compound[220,221]; therefore, it is less favourable for cryopreservation applications because all cellular molecules are subject to destabilisation by extreme low temperatures.

Furthermore, there are discrepancies in the protective effects of quercetin. Here, quercetin did not demonstrate any protective effect on sRBCs, whereas previous studies have reported that it effectively protects sperm during cryopreservation[209]. This could be due to the antioxidant and pro-oxidant functional properties of the compound[222,223], which influence cellular glutathione content[219]. Quercetin has also been found to decrease intracellular lipid peroxide levels when incorporated into the medium at high concentrations (mM)[219]. Additionally, it has been found to protect RBCs in a hypotonic environment, but it has also been observed to promote haemolysis rates during temperature changes[224]. These complicated and interacting effects limit quercetin's protective applications. Nonetheless, applying quercetin could be beneficial for mitigating adverse effects that occur when bringing medium to body fluid isotonicity level under controlled temperatures.

The application of trehalose as a CPA requires the use of a loading approach into the cells, as trehalose can enter cells naturally, but only in very small amounts[225], which are not sufficient for cryopreservation. Lynch et al.[110] established a unique loading protocol using the synthetic biopolymer PP-50. Despite the successful applications of this protocol, it remains limited to RBC cryopreservation only. In addition, this protocol requires a lengthy incubation time to load trehalose into RBCs, a condition that has not been investigated adequately at the biomolecular levels of incubated cells. In this study, we attempted to reduce the trehalose concentration and loading time, but reducing these two parameters was ineffective. This could be due to the slow attachment between the cellular membrane and the permeabiliser PP-50, followed by the slow movement of trehalose across the cell membrane[143]. Additionally, the loading protocol showed no effect on GR and LDH activities, which suggests that no cellular damage was induced during loading, as the gradual accumulation of trehalose could contribute to protecting cells against stress[226]. Furthermore, it is unknown whether trehalose can be metabolised in RBCs, although

trehalase, the enzyme that hydrolyses trehalose, is found in mammalian cells[227]. Indeed, the measured activity of G6PDH suggests that trehalose can be degraded and utilised in the pentose phosphate pathway. Whether trehalose degradation occurs via an intra- or extracellular pathway remains unknown, however.

This study also carried out the first comparison of the effects of glycerol and trehalose on sRBC cryopreservation. This involved assessing haemolysis levels and oxidative status of sRBCs in the presence and absence of Sal in both cryo- and recovery media. Previous studies have shown that incubating RBCs in 800 mM trehalose at 37°C causes a high osmotic pressure, leading to oxidative injury and pro-apoptosis[228]. The present study indicated that glycerol and trehalose induce similar oxidative damage on proteins post thaw. Adding Sal to the media was found to reduce oxidative damage to a level comparable with the control. In addition, trehalose appeared to induce lipid peroxidation; however, incorporating Sal to the media significantly prevented lipid peroxidation. Trehalose may have distinct effects on nucleated versus anucleated cells. The data presented here showed trehalose oxidation effect on sRBCs, which conflicts with that observed in bovine calf testicular tissues[229], where trehalose exhibited a protective effect on lipids against oxidative damages. Similarly, another study suggested that trehalose has anti-inflammatory and antioxidative stress effects on subarachnoid haemorrhage[230]. These findings indicate the variation in responses among different cell types to the same agent. In contrast, the present study found that glycerol did not induce lipid peroxidation in sRBCs. Adding Sal, however, lowered the lipid oxidation to a level below that observed in trehalose alone. Additionally, GR activity was promoted in cryopreserved sRBCs and maintained in media containing Sal, which supported its antioxidant properties[206]. Further analysis of PS, the apoptosis biomarker, showed that no apoptosis was triggered in trehalose-loaded sRBCs after refrigeration with or without Sal.

The protective cold or freezing media showed a damaging effect on membrane-associated cytoskeleton proteins[231] and Hb[232]. For instance, using glycerol for RBC cryopreservation has been shown to yield a considerably low survival rate[233], which dramatically decreases within the following 48 h[234] post-thaw. In this study, adding Sal to the glycerol and SAGM media significantly enhanced cryosurvival rate and extended the stability of sRBCs from two to four days post-thaw. Moreover, it reduced the stress levels, as evidenced by the LDH readout[198]. Adding Sal to trehalose and SAGM media appeared to promote cells' survivability and stability to a greater level than those observed in trehalose alone, or in glycerol with or without Sal. This suggests that adding Sal to trehalose and SAGM offers a high protective potency.

It is important to emphasise that cryopreserving sRBCs in a low concentration (10%) of glycerol results in a similar cryosurvival rate to that obtained in 20% glycerol (Supplementary data S4.1). Reducing glycerol concentration was preferable in the presented study because it requires a less aggressive wash during the CPA removal step[235]. Finally, the present findings can potentially be applied to developing biopreservation protocols for hRBCs. The next chapter presents an investigation of the potential application of the compounds tested here in the biopreservation of human cells.

4.5 Conclusions

Survival of sRBCs was enhanced by adding CCM, Sal and Nig to the cryomedia. Further evaluation of the molecular profile of sRBCs during cryopreservation and cold storage using Sal showed a remarkable reduction in the oxidative damage to proteins and lipids that is typically observed when adding trehalose or glycerol cryomedia alone. Sal also considerably reduced the haemolysis in refrigerated sRBCs post-thawing, in comparison to those stored in conventional media with no additives. The present findings demonstrate the potential benefits of using Sal as a protective agent alongside existing compounds used in cryopreservation and cold storage. This opens up a new avenue of research in the fields of blood cells, stem cells, infertility treatment and, potentially, tissue cryopreservation. Further investigations should apply the present findings to human cells, and elucidate the targeted pathways of cryo-damage and the modulatory effect of the additive agents. The next chapter will address the application of the present study findings to human RBCs.

5. Use of novel protective agents to influence biopreservation of hRBC and optimisation of proteome analysis

5.1 Introduction

The current chapter describes experiments regarding the biopreservation of hRBCs and the influences of two novel protective agents, Nig and Sal. Blood obtained from male donors through the blood donation services centre was utilised to assess the effect of Nig or Sal incorporated in trehalose or glycerol cryomedia. The ages of donors and blood samples, as well as the collection procedures, were considered for potential influence on the performance of the commonly-used cryomedia. In addition, this chapter describes the effect of the compounds (Nig and Sal) on hRBC cold storage in SAGM medium for five weeks. Moreover, a pilot proteomic analysis of cryopreserved sRBCs, an easily accessible cellular model, is also presented here. The study aimed to assess the feasibility of analysing the proteome of cryopreserved sRBCs post-thaw. Proteome analysis can provide an assessment of multiple protein pathways and enhance our understanding of biopreservation efficiency. Such analysis can also help to pinpoint altered pathways that can be used as biomarkers for optimising the biopreservation process. The investigations presented in this chapter are proof of concept for future practical applications to improve hRBC biobanking.

5.2 Materials and experimental procedures

5.2.1 Blood description and preparation procedure

Human blood samples were obtained from 27 male donors provided by NHS blood and transplant (NHSBT) in Cambridge, NHSBT; Filton or from Cambridge Bioscience Ltd. The donors' age ranged between (20 to 60 years old). The sample descriptions, collecting procedures and storage period upon receiving them and before experiments are given in **Table 5.1**

Samples description	No. of samples	Provider	Donors gender and age (y= years)	Storing period prior experiments
hRBC collected and suspended in CPD bag	12	NHSBT, Cambridge	Male, (20-30) and (50-60) y	5 – 30 days
Whole blood collected in EDTA tubes	10	NHSBT, Filton	Male, (20- 30) and (50-60) y	5 days
Whole blood collected in Na-heparin tubes	5	Cambridge Bioscience Ltd	Male donors, (35-45) y	5 days

Table 5. 1 Experimental human blood samples descriptions*: number of samples, suppliers, donors' age and genders, collecting procedures and storing period prior experiments*

hRBC's were separated from whole blood by centrifugation. Briefly, 9-10 mL of blood was placed in a 15 mL conical tube and centrifuged at 400 x g for 20 min. This caused blood to separate into layers; plasma, buffy coat and hRBC in the bottom. The top layers were discarded following the local laboratory guidelines in compliance with the HTA. Then, the hRBC layer was washed by gently mixing the cells with 2 x hRBC volume of PBS (Sigma, UK) and centrifuged again as above. This step was repeated 3 times. Finally, the hRBC pellet was diluted in PBS buffer to make 15% haematocrit, where hRBC actual volume in the suspension solution is 15:100. The same preparation and washing step was applied on hRBC obtained from NHSBT and Cambridge Bioscience Ltd.

5.2.2 Cryopreservation and cold storage

hRBCs were cryopreserved with and without adding protective compounds as previously described in **Chapter 4**. The potential effects of the collecting tubes or bags and the gap time between blood donation and usage were taken into consideration for each of tested samples. For cold storage, hRBCs obtained from Cambridge Bioscience Ltd were stored immediately at 4 ^{o}C in SAGM solution with and without 200 µM Sal or 300 µM Nig to test the efficacy of the compounds on cold stored cells. The survival rate was assessed by measuring the haemolysis rate as described in **Chapter 3**.

5.2.3 Proteome analysis procedure for sRBC

5.2.3.1 Sample preparation – Haemoglobin depletion

Haemoglobin (Hb) of sRBCs lysate was depleted by using a HemoVoid™ kit (Biotech support group, Monmouth JCT, USA) according to the manufacture's protocol. Briefly, HemoVoid spin columns were prepared following the instruction of the manufacturer. Protein concentration was measured as described in **Chapter 3**. Then 300 µl lysate of 1.45 µg/µL proteins was loaded into the column with a similar volume of binding buffer. The mixture was vortexed thoroughly for 10

minutes followed by centrifugation for 4 min at 10,000 rpm. The filtrate, which contained Hb, was discarded and the enriched beads washed two times by adding 500 µl washing buffer and vortexing well. Then samples were centrifuged at 10,000 rpm for 4 min followed by removing filtrate. Free Hb proteins enriched on spin column beads were eluted by 300 µl elution buffer after mixing well for 10 min then centrifuged again for 4 min at 10,000 rpm. To determine the efficiency of the Hb-depletion, the elution was collected and checked on sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE).

5.2.3.2 SDS – PAGE and protein visualization

A standard SDS-PAGE gel was run in order to check the efficiency of Hb- depletion. Briefly, 50 µL of 1µg/µL protein samples were mixed with 2x sample loading buffer in 1:1 ratio and heated at 95˚C for 5 min, then placed onto ice to cool down. After that, 30 µL of each sample was loaded along with 10 µL molecular markers and 30 µL Hb- undepleted sRBC lysate onto 4-20% SDS polyacrylamide gradient gel in tank filled with 1x running buffer (Sigma-Aldrich) and left to run for 1 h at 100 V.

Separated protein bands on the gel were either processed for visualization using silver staining or transferred onto polyvinylidene difluoride membranes (PVD) for visualization by Ponceau-S staining. For transferring step, gel, membrane, sponges and filter papers were emerged in transferring buffer for 15 min prior to the transfer. Then, a gel sandwich was assembled as illustrated in **Figure 5.1.**

Figure 5. 1 Schematic diagram showing assembly of gel sandwich for electrophoretic transfer of proteins

The gel sandwich was completely submerged in the transfer buffer and any trapped air bubbles between layers were pushed out gently using a spatula to allow continuous transfer of proteins from the gel to the membrane. Following this, the cassette was locked and placed in the electrode module tank. The tank was filled with transfer buffer and the system was cooled by a frozen bag. A magnetic stir bar was placed in the tank for even temperature distribution and maintenance. Electrophoretic transfer was carried out at 100 V and a constant 350 mA for 1 h. Then, the sandwich was disassembled upon run completion. The membrane was immersed in Ponceau-S stain (0.1% (w/v) in 5% (v/v) acetic acid) (Sigma-Aldrich) and shaken gently for 5 min.

For gel staining, a silver stain kit was used following the supplier's instructions (Thermofisher). Briefly, the gel was washed twice in ultrapure water for 5 min and then incubated in fixing solution (30% ethanol: 10% acetic acid solution) for 15 min, the same step was repeated one more time. Meantime, a sensitizer working solution was prepared by mixing 50 µL sensitizer in 25 mL ultrapure water to enhance the silver ion binding to proteins. The fixing solution was removed carefully and then sensitizer solution was poured over the gel and kept for 1 min. After that, gel was washed twice with ultrapure water. A stain working solution (0.5 mL enhancer with 25 mL stain) was used to stain the gel, by submerging the gel in the solution and incubating for 30 min. After that, the gel was washed twice for 20 s with ultrapure water. A prepared developer working solution, which consisted of 0.5 mL enhancer with 25 mL developer, was used to develop the gel by pouring over the gel and kept for 2 min. When bands appeared, the reaction was stopped immediately by adding 5% acetic acid following by incubation for 10 min.

5.2.3.3 Protein identification by LCMS/MS and bioinformatic analysis

sRBC depleted proteins were digested and purified following the described procedure in **Chapter 3**. Peptides were analysed with an ESI-Orbitrap-HCD (Thermo-Scientific) coupled with a nanoelectrospray ion source (Proxeon Biosystems, Odense, Denmark)[236]. Raw data were converted to the Mascot generic format (MGF) with Proteome Discover v1.2.0.208 (Thermo-Scientific) and the data were submitted to a local MASCOT (matrix science London, UK) using the Uniprot database for Bos Taurus sequences hence it is the closest species to sheep, with a precursor mass tolerance of 5 ppm, fragment ion mass tolerance of ±0.1 Da, trypsin specificity missed cleavage allowance of two, carbamidomethyl modification on cysteine residues as fixed modification, and oxidation of methionine residues, phosphorylation of serine, threonine and tyrosine residues. MASCOT searches were then repeated with the same conditions except for the

variable modifications, which were changed to palmitoylation on lysine and N-terminal acetylation as variable modifications. Identified proteins were considered for gene ontology cellular component, biological processes and molecular function enrichment analyses using Bos Taurus database on PANTHER[237].

5.3 Results

5.3.1 Effect of additive agents on cryosurvival rate of hRBCs

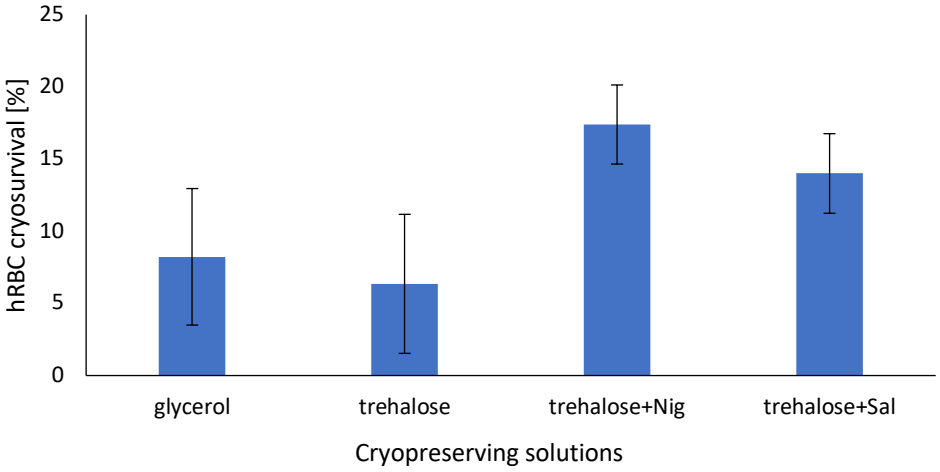


Figure 5. 2 Effects of additive agents nigerose (Nig) and salidroside (Sal) on the cryosurvival rate human RBC (hRBC) post-thaw. *hRBCs were either frozen in trehalose alone or in trehalose with Nig or Sal for additive effects; hRBCs frozen in glycerol were used as a control. After thawing frozen samples in a water bath at 37°C, haemolysis was measured using Drabkin's reagent to estimate the cryosurvival rates. Data are derived from 10 samples and expressed as mean ± SD. * and bars indicate significant variables (ps<0.01).*

The effects of the protective agents Nig and Sal were examined in combination with trehalose solution using the PP-50 loading protocol. The results presented in **Figure 5.2** demonstrate a significant increase in cryosurvival rates when hRBCs were cryopreserved in trehalose plus Nig (17.74 ±2.5%), in comparison to trehalose alone (6.3 ±4.8%) and glycerol alone (8.2 ±4.7%). A similar effect was seen when adding Sal, with the hRBC cryosurvival rate increasing to reach 13.99 ±2.7%. In all cases, the data were derived from tested hRBCs that were provided by the NHSBT in Cambridge 30 days after donation and randomised by mixing age groups during the sample examination under the specified conditions to avoid age bias. The age of the donated blood greatly influenced the cells responses to the cryopreservation; hence, the cryosurvival rates under all cryopreserving conditions were considerably low.

5.3.2 Effects of donors age and Sal on hRBC cryopreservation

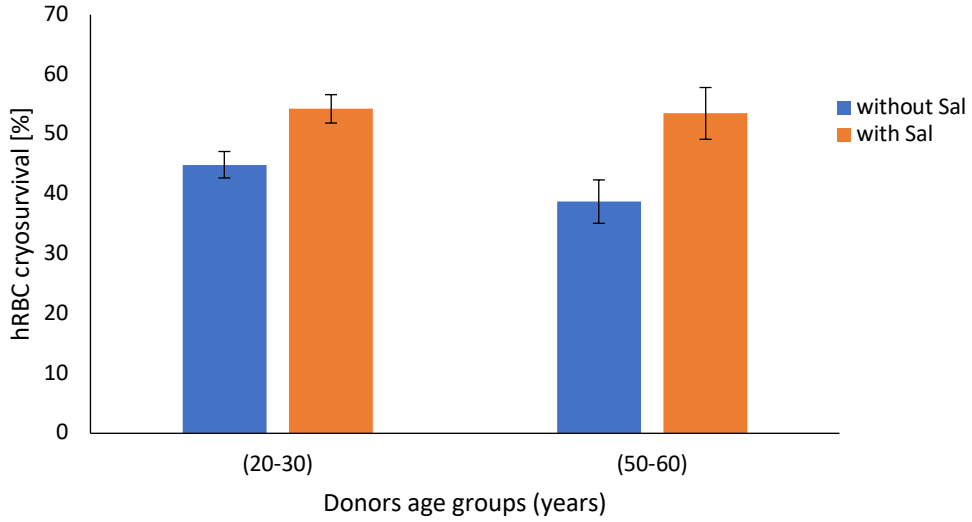


Figure 5. 3 Effects of donor age and protective agent salidroside (Sal) on human RBC (hRBC) cryopreservation. *hRBCs obtained from two different age groups, 20 – 30 years and 50 - 60 years, were cryopreserved in glycerol with and without Sal. After freeze-thaw, haemolysis was measured to determine cryosurvival rate. Data represent five tested samples in each group, and are expressed as mean ± SD. * and bars indicate significant variations ($p < 0.01$).*

hRBCs in EDTA tubes, obtained from the Filton NHSBT centre, were grouped based on the donors' ages and cryopreserved in glycerol with and without Sal. The data showed that there was a slight variation in the cryosurvival rate of hRBCs cryopreserved in glycerol only between young and old donor groups, with cryosurvival rates of 44.9±2.2% and 38±3.6 %, respectively. Adding Sal, however, improved the cryosurvival rates significantly compared to those in glycerol alone, with cryosurvival rates reaching 54±2.3% and 53.5±4.3% in both age groups. The data also showed that donor's age has an insignificant effect on RBC cryopreservation.

5.3.3 Effects of additive agents on hRBC cold storage

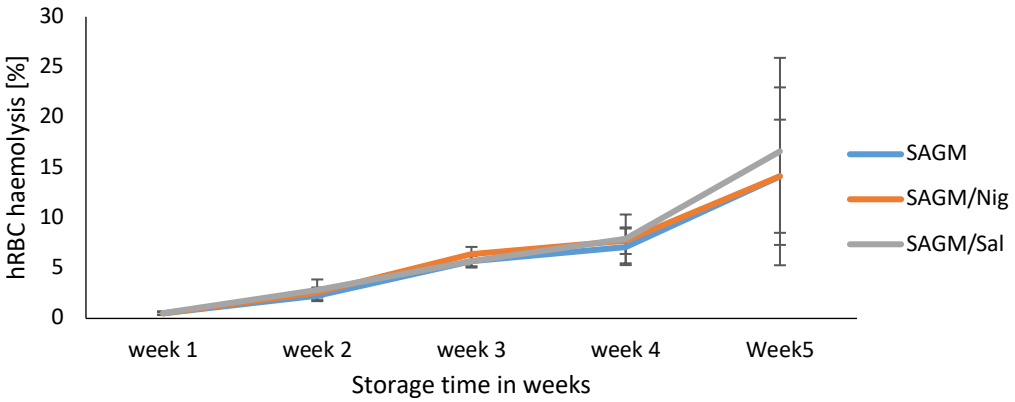


Figure 5. 4 Effects of the additive agents nigerose (Nig) and salidroside (Sal) on human RBCs (hRBCs) during cold storage in saline-adenine-glucose and mannitol (SAGM) solution. *hRBCs obtained from five individual males aged between 35 and 45 years were incubated in SAGM with and without either Nig or Sal. Samples were collected every week to measure haemolysis using Drabkin's reagent. Data are represented as mean ± SD, (all ps ≥ 0.1).*

Haemolysis was assayed for five weeks in hRBCs (obtained from Cambridge Bioscience Ltd) refrigerated in SAGM in the presence and absence of either Nig or Sal. The results showed a gradual increase in haemolysis over time (**Figure 5.4)**. This increase was estimated to be 2.5% for all three conditions (SAGM alone, SAGM/Nig and SAGM/Sal), until it has finally reached an average rate of 14.9±7.9% on the fifth week. Under these conditions, the additive agents showed no significant effects on refrigerated hRBCs (all ps ≥ 0.1).

5.3.4 The correlation between hRBC age *ex-vivo* and cryosurvival rates in trehalose solution

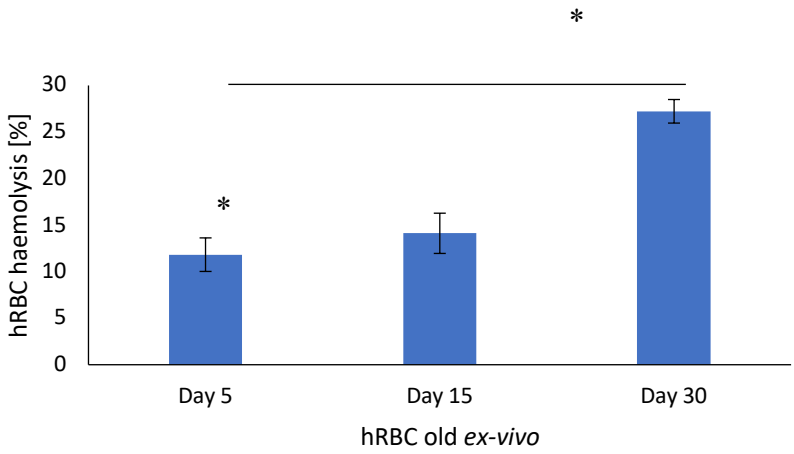


Figure 5. 5 Effects of human RBC (hRBC) age *ex-vivo* and the trehalose incubation on hRBC stability. *hRBCs obtained from the NHS were incubated in 300 mM trehalose solution with 100 µg PP-50 for 9 h at 37˚C. Induced haemolysis was measured after incubation using Drabkin's reagent. Data are derived from different experiments. Day 5 is the mean± SD of three tested samples, and Days 15 and 30 are the mean± SD of 10 samples. * and bars indicate statistical significance of (p < 0.01, r > 0).*

Here, the results show the effect of blood age *ex-vivo* on hRBC stability after trehalose loading using the polymer PP-50 loading protocol. The use of fresh hRBCs, obtained within five days of donation, resulted in a low haemolysis rate of 11.8±1.8% (**Figure 5.5**). The haemolysis rate increased with increasing time between hRBC donation and testing days. This was seen on Day 15 post-donation, where hRBC haemolysis increased after loading trehalose to reach 14.11±2.2%. A further increase was observed as hRBCs become older, with the haemolysis rate reaching its highest level, 27.2±1.2% on Day 30.

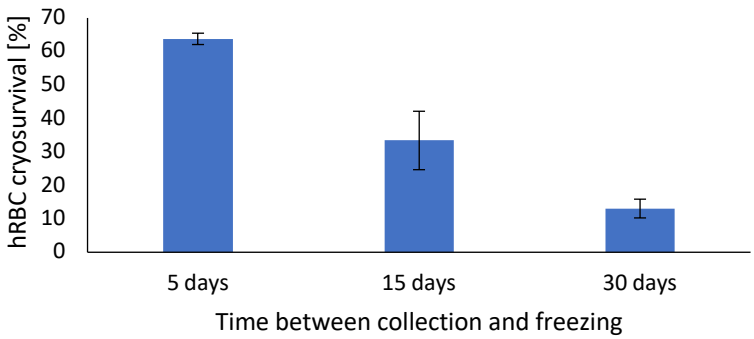


Figure 5. 6 Effect of human RBC (hRBC) age *ex-vivo* on the cryosurvival rate using the trehalose loading protocol. *After freeze-thaw, hRBC haemolysis was measured using Drabkin's reagent to determine*

*the cryosurvival rate. Day 5 is derived from three samples, and Days 15 and 30 are derived from ten samples. Data are expressed as mean± SD. bars and * indicate statistical significance (p < 0.01, r < 0).*

The cryosurvival rates after freeze-thaw in trehalose solution were inversely correlated with hRBC age. The freshness of hRBCs resulted in a high cryosurvival rate; this was clearly represented on Day 5 post-donation, when the cryosurvival rate was 63.75±1.6% (**Figure 5.6**). In contrast, the rate had decreased by nearly 30±8.72% on Day 15, and by Day 30, the lowest cryosurvival rate was reached, 13.1 ±2.8%.

5.3.5 The influence of donor age and cryomedia on cryosurvival rates of hRBCs

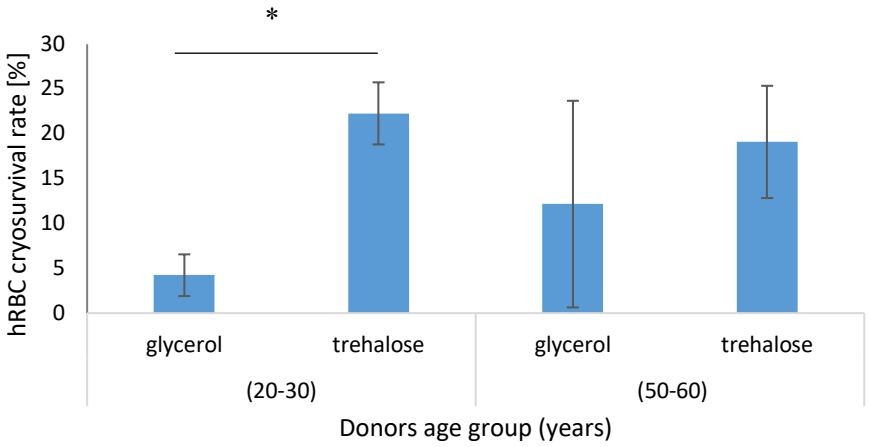


Figure 5. 7 Effects of donor age and cryomedia on human (hRBC) cryosurvival rates post-thaw. *Aged hRBCs obtained from 10 different male donors were separated equally into two groups based on donor age (20 - 30 years and 50 - 60 years) and cryopreserved in glycerol or trehalose without any further additives. The data are derived from five samples each and are expressed as mean ± SD. The * and bars indicate a statistically significant difference (p < 0.01).*

The effects of different cryomedia on hRBC survival rates obtained from different donor age groups are represented in **Figure 5.7**. Aged hRBCs cryopreserved in glycerol and trehalose cryomedia exhibited a low cryosurvival rate regardless of age group and cryomedia – no more than 25% in both conditions. Cryopreserving hRBCs in glycerol and trehalose obtained from healthy donors aged between 20 and 30 years showed a significant difference in survival rates. hRBCs cryopreserved in trehalose solution exhibited a cryosurvival rate of 22.2±3.4%, whereas hRBCs in the same age group, but cryopreserved in glycerol, showed a 5.26-fold lower rate. hRBCs obtained from elder donors (50 – 60 years) did not show any significant differences when

cryopreserved in glycerol (12.2±11.5%) compared to trehalose (19.1±6.3%), indicating that age has no effect on the cryosurvival rates outcome.

5.3.6 Proteins identification and their respective biological function of cryopreserved sRBCs

A pilot proteome study using LCMS/MS analysis of sRBC after Hb depletion is represented in **Figure 5.8.** The data obtained from triplicates sRBC cryopreserved in trehalose show only 47 functionally annotated proteins (**Table 5.2**). This low number is likely to be owned to the poor matching of sheep and Bors Taurus peptide sequences in the Uniprot database. Nevertheless, these preliminary data show that the main biological activities of identified proteins were associated with cellular process (23.3%), metabolic process (23.3%), response to stimulus (16.6%), biological regulation (16.7%), developmental process (6.7%), localization (6.7%), immune system process (3.3%) and multicellular process (3.3%). Some of the identified proteins overlapped have a multi biological activity groups. Such as thiroedoxin and cytochrome c oxidase which are involved in both biological regulation and metabolic processes. Proteins identifications (IDs), molecular weight, sequence coverage and number of match hits in the Unitprot database (http://www.uniprot.org) are represented in **Table 5.2**.

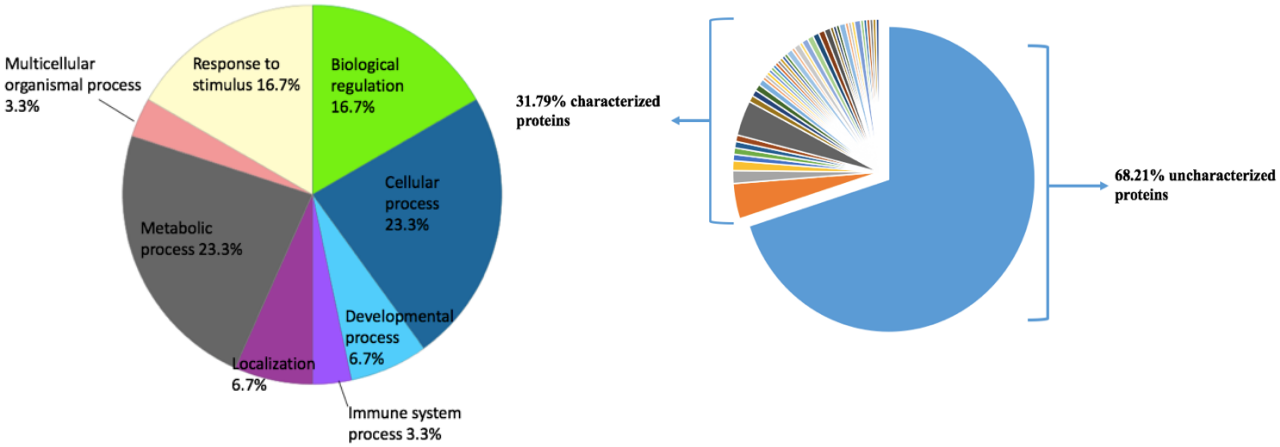


Figure 5. 8 Diagrams represent the percentage of total characterized sheep RBCs' (sRBC) proteins and thier corresponding biological functions. *Analysed proteins by LCMS/MS were identified by searching the Uniprot database. The data were then searched using the PANTHER database (www.panther.org) for biological function identification.*

Accession ID	Protein description	No of matched hits	Molecular mass (Da)	Sequence coverage	Panther biological process and protein class	Modification
1. P62262	14-3-3 protein epsilon	146	29326	10.6		
2. W5Q780	Adenylosuccinate lyase	166	56443	5.9		
3. Q28743	Alpha globin chain	34	15226	94.4		
4. I1WXR3	Alpha-1-antitrypsin transcript variant 1	30	46340	42.5	Biological regulation-Serine protease inhibitor	
5. W5QH56	Alpha-2-HS-glycoprotein	29	46371	45.4	Biological regulation-extracellular matrix	
6. W5NRF4	Amine oxidase	117	85764	13.7		deaminated
7. W5PD87	Anion exchange protein	129	104495	8.6		
8. Q2TCH3	ATP-citrate synthase	256	121769	1.4		
9. A4ZVY7	Beta-2-microglobulin	168	13734	22.9	Immune system process-major histocompatibility comples antigen	
10. Q1KYZ7	Beta-A globin chain	24	16050	97.2		
11. Q1KYZ6	Beta-B globin chain	20	16120	97.2		deaminated
12. A0A0F6YFJ0	Beta-C globin	32	15797	90.8		
13. R4R2H5	Beta-casein	265	4975	17.8	Biological regulation/localization- storage protein	
14. W5PZ55	C-X-C motif chemokine	211	12990	31.1		
15. W5PTU7	Carbonic anhydrase 2	76	29446	43		
16. A0A077JGJ6	Cathelicidin	150	18036	22.6		
17. W5P4S0	Ceruloplasmin	17	120020	27.7		
18. W5P610	Chloride intracellular channel protein	119	28443	29.9		
19. W5PLL0	Coagulation factor IX	149	51107	7.9		
20. Q29439	Complement component C4	108	14522	52.7	Cellular process/ metabloic process – complement	
21. B5B304	Complement factor H	152	13541	18.6		
22. W5P8G0	Delta-aminolevulinic acid dehydratase	278	37681	11.2		
23. W5NUX2	DNA-(apurinic or apyrimidinic site) lyase	269	34798	3.2		
24. W5QDP8	Fibulin-1	290	81118	1.6	Developmental process	
25. P00883	Fructose-bisphosphate aldolase A	272	39774	7.7		
26. A8DR93	Heat shock protein alpha	155	85077	4.8		
27. W5QGD1	L-lactate dehydrogenase	101	37098	28.2		

28.	D3G9G3	Lactotransferrin	151	79285	3.7	Cellular process-receptor/localization /transporter	
29.	W5Q0T3	Lactoylglutathione lyase	264	21253	3.7		
30.	A0A0L0CMV9	Maltase A3	303	66723	1.2		
31.	C8BKC5	Peroxiredoxin 2	23	22059	80.3		
32.	Q7M371	Plasma proteinase inhibitor	172	2238	65		deaminated
33.	W5P3R3	Plasminogen	44	93859	33		
34.	W5QIY3	Proteasome subunit alpha	246	25665	34.2		
35.	W5P500	Proteasome subunit beta	306	23067	8.9		
36.	W5QIV1	Protein S100	271	11785	8.5		
37.	W5NY22	Protein-L-isoaspartate O-methyltransferase	299	22956	4.2		
38.	C8BKD1	Prothrombin	28	71430	35.6	Metabolic process-serine protease	
39.	W5P275	Pyruvate kinase	54	64319	22.4		oxidation
40.	A0A0L0CIF5	Serine/threonine-protein kinase fused	259	91290	0.8		
41.	P14639	Serum albumin	1	71139	95.4		Deaminated
42.	P02768	Serum albumin	93				oxidation
43.	W5NQP5	Superoxide dismutase [Cu-Zn]	210	26891	5.3		
44.	P50413	Thioredoxin	138	12120	30.5	Biological regulation/metabolic process-oxidoreductase	Oxidation (M)
45.	P12303	Transthyretin	74	15875	66	Cellular process/metabolic process-transfer/carrier protein	
46.	W5NY78	Uncharacterized protein	307	19250	8.6		deaminated
47.	C9E8M7	Cytochrome b5	102	15329	47.8	Cellular process-Oxidase	

Table 5. 2 Identified proteins of sheep RBCs (sRBCs) cryopreserved in trehalose solution. *The table shows proteins biological processes and classes that identified using PANTHER database. Proteins modifications were indicated using uniprot database.*

5.4 Discussion

In the previous study, reformulating cryomedia by adding the novel protective compounds Nig or Sal improved the cryosurvival rates of sRBCs. Here, we anticipated similar results when applying the reformulated media to hRBCs during biopreservation, especially because hRBCs are more robust in comparison to sRBCs[238]. Experimentally, we have shown that hRBCs respond in a manner similar to that observed in sRBCs when introducing Nig or Sal to the cryomedia; that is to say, this resulted in an increase in the cryosurvival rate post freeze–thaw. Nevertheless, there some other factors, mainly the age of the blood samples and the biosampling procedures, interfered with the results.

Previous research did not account for the influence of donor age on hRBC cryopreservation. In the current study, the donor age showed nearly no correlation with cells' recovery rates post-thaw. Notably, the two age groups in this study, 20-30 years and 50-60 years, fall within the approved age range of blood donors in the UK and some other countries[239]. Blood donation services have strict health criteria and most of their customers who donate blood regularly are healthy individuals[239], which minimise the risk of collecting blood products of low quality. Furthermore, the findings herein are consistent with those of previous studies showing no effect of aging metabolites on blood from aged donors[240]. Likewise, the donors' ages showed no considerable effect on other healthcare products, such as cornea[241], further supporting the findings of the present study.

Conversely, the age of blood itself has a recognisable effect on hRBC cryopreservation[242]. Aged RBCs become more fragile[243,244] and cannot withstand the severe changes in temperature that occur during freeze-thaw, resulting in a significantly low rate of cryosurvival for RBCs. In the present study, hRBC age was defined by the storage duration *ex-vivo* prior to cryopreservation, which ranged from five to thirty days. RBCs aged five days or less were considered fresh.

During storage, RBCs undergo a series of changes, including loss of elasticity, protein aggregation, accumulation of metabolites and the loss of the ability to resist osmotic changes[244,245,246]. As a consequence, the overall cryosurvival rate of aged hRBCs was extremely low. Some differences in the recovery rate, however, resulted from using different cryomedia. For instance, trehalose tended to recover a relatively high rate of hRBCs post-thaw in comparison to glycerol, and this was improved further by incorporating Nig or Sal into the media.

Biobanks are commonly established to meet the needs of clinical healthcare and emergency care. For this reason, few biospecimens have been made available for research purposes, and research studies have mostly made use of low-quality or nearly-expired samples. Consequently, this affects the impact and validation of said research. The variation among hRBC samples in the current study provides an example of the limitations and tight parameters met when accessing biospecimens of high quality through biobanks for basic research validation, although there is global agreement concerning the necessity of promoting discovery and standardising clinical procedures by accessing high quality biosamples [247]. In order to improve this situation, it is necessary to establish policies and procedures facilitating access to biospecimens of good quality, either through direct purchase or collaboration, and maximising the utilisation of leftover clinical samples[248].

Here, the materials used to collect blood samples were found to be another major factor affecting the quality of cryopreserved cells. For instance, the use of EDTA tubes lowered the efficiency of trehalose loading and cryopreserving protocol (Supplementary data S5.1). EDTA is well known as a chelating agent[249], binding irreversibly to metal ions such as calcium (Ca^{2+}), magnesium (Mg^{2+}) and others to prevent blood clotting[250]. The presence of Ca^{2+} and Mg^{2+} ions are essential for achieving a successful trehalose balance around the cells for efficient cryopreservation using the membrane permeabiliser PP-50. These ions have a salting effect on proteins, peptides and similar molecules, including the biopolymer PP-50[251,252], allowing them to become embedded in the cell membrane. In this study, it was evident that increasing the ionic strength of the solution led to an increase in PP-50 solubility and interaction with the cell membrane (supplementary data S5.1). Thus, hRBCs collected in EDTA tubes did not respond well to the trehalose loading prior to cryopreservation, making glycerol more suitable for these samples.

Many studies have examined the proteome and metabolome of RBCs[253,254,255], but few have examined proteomic changes under biopreservation conditions, mainly under cold storage[256,257]. Studying the proteome of cryopreserved RBCs could improve our understanding of the mechanisms by which protective agents operate. Nonetheless, there are a number of considerations that must be accounted for. Hb and carbonic anhydrase-1 form 97% and 1% of RBC total protein, respectively; however, the abundance of these proteins makes the analysis of the remaining 2% a serious challenge[258,259]. Thus, protein fractionation should be performed to prevent false analysis resulting from the interference of abundant proteins[260]. An Hb depletion protocol developed by Walpurgis *et al*.[261] was applied in the present study to fractionate proteins in sRBC lysate and establish the proteome analysis protocol of cryopreserved RBC in trehalose only. Samples cryopreserved in glycerol were excluded because glycerol forms a shield on

proteins and prevents protein ionisation, which is a critical step in LCMS analysis[262]. The present study identified 47 proteins that were either structural (e.g. anion exchange protein) or functional (e.g. pyruvate kinase, LDH and heat shock proteins) cytosolic proteins. Protein identification was limited, however, by the lack of available data regarding the sRBC proteome, and thus the Bos Taurus database was used. Interestingly, the pilot proteomic analysis revealed a number of important proteins that were modified, including PK and thioredoxin. PK is a key enzyme involved in the RBC glycolysis pathway that provides cells with energy; any deficiency or defect of this enzyme could cause an enzymatic haemolytic anemia[263]. The present proteomic study showed that this enzyme modification under the cryopreservation condition is ascribed to the increase in ROS production[264], which consequently impairs essential energy production in the cells. Similarly, thioredoxin, an antioxidant[265] and anti-apoptotic protein[266], was found to be oxidised during cryopreservation. Here, the modification is directly linked to apoptosis, as has previously been observed in HeLa cells[267]. Such modifications explain the relatively low survival rate of RBCs and their instability after freeze–thaw in conventional cryomedia (e.g. trehalose and glycerol). Applying such a study in hRBCs, however, and using high fractionation methods to separate membrane and cytosolic proteins would be more beneficial for pinpointing cryodamaged pathways[254,260] and the modulation effect of novel CPAs. Furthermore, accessing fresh human blood via a collection protocol suitable for studying the mechanisms of protective agents remains essential for developing hRBC cryopreservation. For the current project time-frame and objective, a substitute cellular model is considered, as will be discussed thoroughly in the next chapter.

5.5 Conclusion

Accessing good quality, fresh hRBCs is essential for successful cryopreservation. Herein, hRBC cryopreservation is limited by several factors, such as the collection protocol and RBC storage time before the experiments. Nevertheless, the additive agents Nig and Sal improved hRBCs survival rates post cryopreservation. Donor age showed no effect on hRBC biopreservation, as the age range in the experiment was consistent with blood donation guidelines in the UK. Additionally, the present chapter reported the pilot proteome analysis of sRBCs cryopreserved in trehalose solution, which permitted the identification of a number of intact and modified proteins. PK and thioredoxin modifications are potentially linked with RBC apoptosis and haemolysis, and the consequent low recovery rate. The study was limited, however, by the availability of suitable blood samples for deep proteomic analysis. The establishment of such a study in collaboration with clinics or biobanks is therefore recommended to facilitate access to

high quality samples and increase the impact of the study. Moreover, the proteome profiling of hRBCs can be valuable for identifying the effect of classical protective media versus reformulated media containing novel CPAs. This is described in the next chapter using alternative peripheral blood cells.

6. The modulatory effect of novel cryo-additive agents on the molecular and biological signatures underpinning cryo-damage in human nucleated cells (HL-60)

6.1 Introduction

As a result of the limitations of hRBC biopreservation, and in light of the previous chapter's demonstrated effect of the novel CPAs and establishment of a proteome profile for sRBCs, the present chapter describes the cryopreservation of human leukaemia cells (HL-60) as an alternative peripheral blood cell module. The present work aimed to elucidate the effect of conventional cryomedia DMSO on HL-60 cells in the presence and absence of Nig or Sal.

Herein, the cryosurvival rates of HL-60 cells were measured and their biochemical profiles (e.g. enzymatic activity, oxidative damage) were investigated. Additionally, the cells were subjected to proteome profiling using LCMS, both prior to and post cryopreservation, in order to determine the damaged pathways and the modulatory effect of the two CPAs, Nig and Sal. This investigation sought to enhance nucleated-cell cryomedia formulae, raise the recovery rate post-thaw and improve cells' resistance to cryo-damage.

6.2 Materials and experimental approaches

HL-60 cells, RPMI-1640 media, foetal bovine serum (FBS), penicillin-streptomycin, dimethylsulphoxide (DMSO) and isopropanol were all purchased from Sigma-Aldrich. A Mr. Frosty™ Freezing Container was purchased from ThermoFisher Scientific. Caspase 3 was purchased from Abcam (Cambridge, UK).

6.2.1 Experimental design

The present study was divided into three arms (**Figure 6.1**). Arm 1 involved culturing HL-60 cells up to 70% confluence in RPMI-1640 media containing 10% (v/v) FBS and 50 U/mL penicillin-streptomycin. HL-60 cells were centrifuged at 100 x g for 5 min and then cell culture media was removed. Pellets of HL-60 cells were re-suspended in freezing media (10% DMSO and 90% FBS) at a ratio of 10^6 cells/mL, slowly frozen in cryogenic tubes and then stored at -80°C overnight. Next, HL-60 cells were removed from -80°C, placed in a liquid nitrogen biobank and stored

between one day and several months. HL-60 cells were then thawed in a water bath at 37°C, followed by centrifugation at 100 x g for 5 min. Next, thawed HL-60 cells were washed three times with RPMI media. Afterward, HL-60 cells were cultured in a recovery media containing RPMI, 20% FBS and 50 U/mL penicillin-streptomycin. The FBS concentration was reduced to 10% 24 h post-thaw.

For Arms 2 and 3 of this study, HL-60 cells were cultured as in Arm 1, with the addition of 300 µM Nig (Arm 2) or 200 µM Sal (Arm 3) to the cryo- and culture media. The latter media was used for 24 h prior cryopreservation and up to 48 h post-thaw incubation. The selected concentrations of the cryo-additive agents (e.g. Sal and Nig) were optimised as shown in supplementary data S6.1. During culturing time, cells were maintained in an incubator at 37°C, 5% $C0_2$ and 95% air atmosphere.

For proteomic and biochemical analyses (n = 5 per arm), unless otherwise stated, HL-60 cells cryopreserved in DMSO +/- Nig or Sal were collected (at 70% confluency) prior to freezing and then 24 h or 48 h post thawing. Proteomic analysis was performed using HL-60 protein extracts, as will be described later.

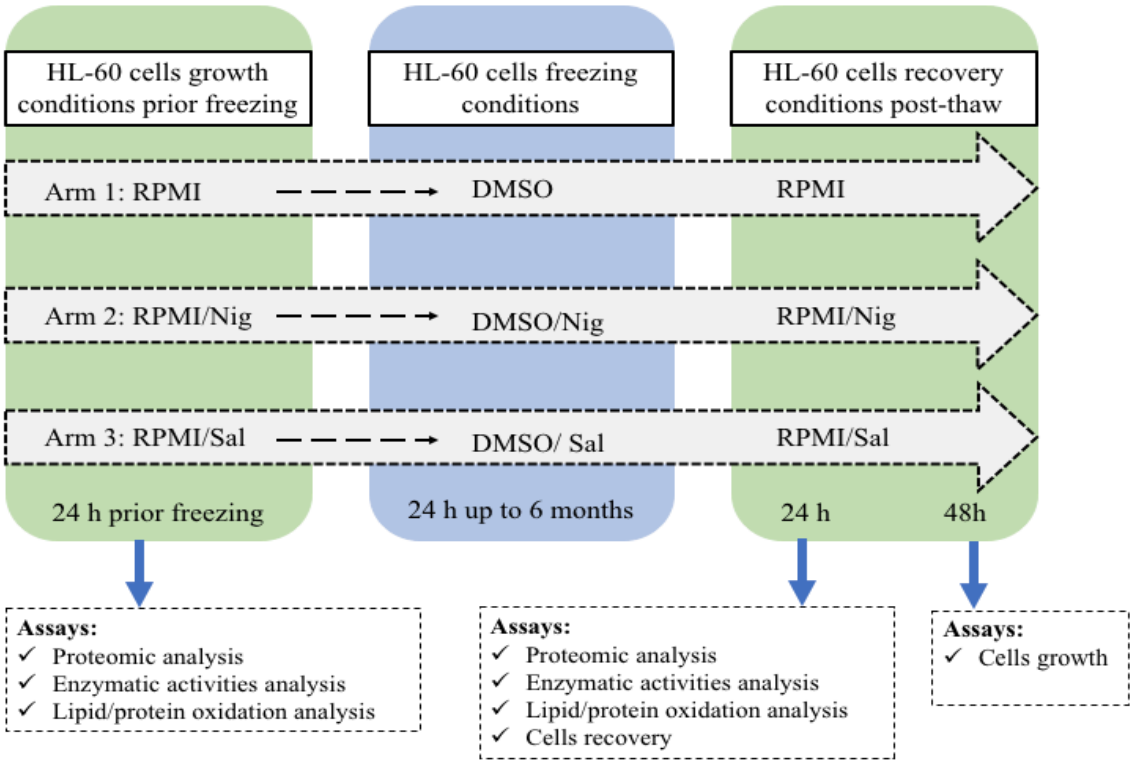


Figure 6. 1 Schematic diagram showing the experimental design for cryopreserving human leukaemia (HL-60) cells in dimethylsulphoxide (DMSO) +/- nigerose (Nig) or salidroside (Sal). Proteomic analysis and corresponding biological assays were conducted 24 h prior to and post cryopreservation. Cell growth was assessed at 1 h, 24 h and 48 h post thaw.

6.2.2 Biochemical assays

The assays in this section were conducted as described in **Chapter 3.** HL-60 cells were pelleted by centrifugation at 100 x g for 5 min, then washed in cold PBS buffer once. Pellets were lysed in 350 µl RIPA buffer and 2.85 µl protease inhibitors and kept on ice for 30 min. Cells lysate were centrifuged at 100xg for 5min and enzymatic assays GR, LDH were performed using 1 x 10^6 HL-60 (n=5).

For HL-60 oxidation assays, protein carbonylation was measured prior cryopreservation and 24h post thaw (n=4)**.** Similarly, lipid peroxidation was measured in 10^6 cells/mL lysate (n=3) following the manufacture's instruction.

6.2.3 Cell proliferation and proteomic analysis

HL-60 cell viability and proliferation were assessed at 1 h, 24 h and 48 h post thaw. HL-60 cells were mixed with trypan blue and placed on haemocytometer slides for counting under light microscope as described previously in **Chapter 3**. The cells were counted in duplicate at each time point. For proteome analysis, proteins were extracted from HL-60 cells (n=5) prior and 24 h post cryopreservation. Followed by purification and digestion (**Chapter 3)**.

6.2.3.1 Nano-high-performance liquid chromatograph-mass spectrometry proteomic analyses

Qualitative and quantitative proteomic analyses were performed in a bidimensional micro UPLC tandem nano ESI-HDMS^E platform by multiplexed data-independent acquisitions experiments[268]. 2D-RP/RP Acquity UPLC M-Class System (Waters Corporation, Milford, MA) coupled to a Synapt G2-Si mass spectrometer (Waters Corporation, Milford, MA) platform was used. The samples were fractionated using a one-dimension reversed-phase (RP) approach.

Peptide samples (0.5 µg) were loaded into a M-Class HSS T3 (100 Å, 1,8 µm, 75 µm × 150 mm (Waters Corporation, Milford, MA). The fractionation was achieved by using an acetonitrile gradient from 7% to 40% (v/v) for 95 min at a flow rate of 0.4 µL/min directly into a Synapt G2-Si. For every measurement, the mass spectrometer was operated in resolution mode with an m/z resolving power of about 40.000 FWHM, using ion mobility with a cross-section resolving power at least 40 Ω /ΔΩ. MS and MS/MS data were acquired in positive ion mode using ion mobility separation (IMS) of precursor ions (HDMS^E) in a range of 50-2000 m/z. The lock mass channel was sampled every 30 sec. The mass spectrometer was calibrated with an MS/MS spectrum of

(Glu1)-Fibrinopeptide B human (Glu-Fib) solution delivered through the reference sprayer of the NanoLock Spray source.

6.2.3.2 Data processing and analysis

Proteins were identified and quantified by using dedicated algorithms and searching against Uniprot proteomic databases of *Homo sapiens* (version 2016/09), with the default parameters for ion accounting[269]. The databases used were reversed "on the fly" during the database queries and appended to the original database to assess the false-positive identification rate. For proper spectra processing and database searching conditions, we used Progenesis QI for proteomics software package with Apex3D, Peptide 3D, and Ion accounting informatics (Waters Corporation). The label free protein quantitation was done using Hi-N (n=3) method[268]. This software starts with LC-MS data loading, then performs alignment and peak detection, which creates a list of interesting peptide ions (peptides) that are explored within Peptide Ion Stats by multivariate statistical methods. The initial ion-matching requirements were≥1 fragment per peptide, ≥3 fragments per protein and ≥ 1 peptide per protein. The following parameters were considered in identifying peptides: 1) Digestion by trypsin with at most two missed cleavages; 2) variable modifications by oxidation (M) and glycosylation (-O-GlcNac ST) and fixed modification by carbamidomethyl (C); and 3) false discovery rate (FDR) less than 1%. Identifications that did not satisfy these criteria were rejected. The shaprio-Wilk W-test analysis of variance (ANOVA) was used to identify proteins that were present at different levels. Only those findings with p-values<0.05 were considered as significant.

6.2.3.3 Functional and biological classification of differentially expressed proteins

Proteins expressed differentially at significant rates in HL-60 cells cryopreserved in DMSO alone, DMSO/Nig or DMSO/Sal (n=5) were classified according to their biological and functional pathways. The Uniprot accession genes' code of differentially expressed proteins were mapped to Gene Ontology Annotation using a software linked to Funrich database. (http://www.funrich.org)[270]. Unique and overlapped differentially expressed (e. g. up/down-regulated) proteins of HL-60 cryopreserved in DMSO +/- Nig or Sal were analysed.

6.2.4 Statistical analysis

All enzymatic assays were performed in five biological replicates except for caspase-3 activity. Caspase-3 activity and lipid peroxidation and protein carbonylation assays were performed in triplicate, protein carbonylation test for controls were performed in duplicates. Results were presented as mean ± standard deviation. Significance differences between groups were determined using Student's t-test for paired and unpaired observations, p values <0.05 were considered significant.

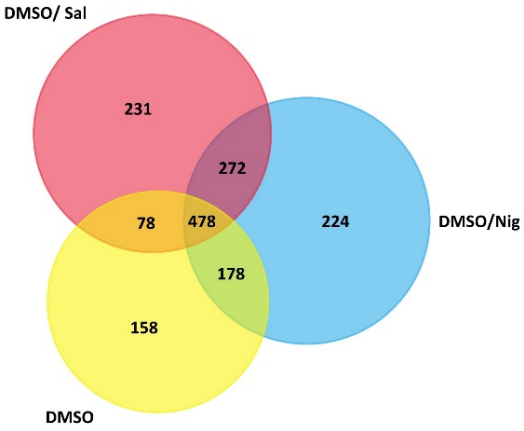
6.3 Results

6.3.1 Proteomic analysis

A

Conditions	DMSO only	DMSO/Nig	DMSO/Sal
No. of identified proteins	887	1140	1032
No. of identified genes	892	1152	1059

B



C

	DMSO only	DMSO/Nig	DMSO/Sal
⇧	484	536	491
⇩	403	604	541

Figure 6. 2 Total number of differentially-expressed proteins in human leukaemia (HL-60) cells after cryopreservation in dimethylsulphoxide (DMSO) +/- nigerose (Nig) or salidroside (Sal). *A) Table representing the number of proteins identified versus the number of genes under each cryopreservation condition. B) Venn diagram illustrating the number of unique and shared proteins that were significantly changed in HL-60 cells 24 h post thaw. The numbers in the circles represent the number of corresponding genes that significantly changed in HL-60 cells post cryopreservation in DMSO alone, DMSO/Nig or DMSO/Sal (n = 5). C) Table representing the total number of upregulated versus downregulated identified proteins in HL-60 cells under each of the tested conditions; blue arrow indicates upregulation and red arrow indicates downregulation.*

Label-free quantitative shotgun proteomic analysis was used to identify differentially-expressed proteins in HL-60 cells post cryopreservation in DMSO alone, DMSO/Nig or DMSO/Sal. After cryopreservation, significantly higher numbers of proteins were quantified in cells preserved in DMSO/Nig (1140 proteins) and DMSO/Sal (1032 proteins), with only 887 proteins found in DMSO alone (**Figure 6.2A**). In addition, the numbers of uniquely-expressed proteins were 158, 224 and 231 in DMSO, DMSO/Nig and DMSO/Sal, respectively (**Figure 6.2B**).

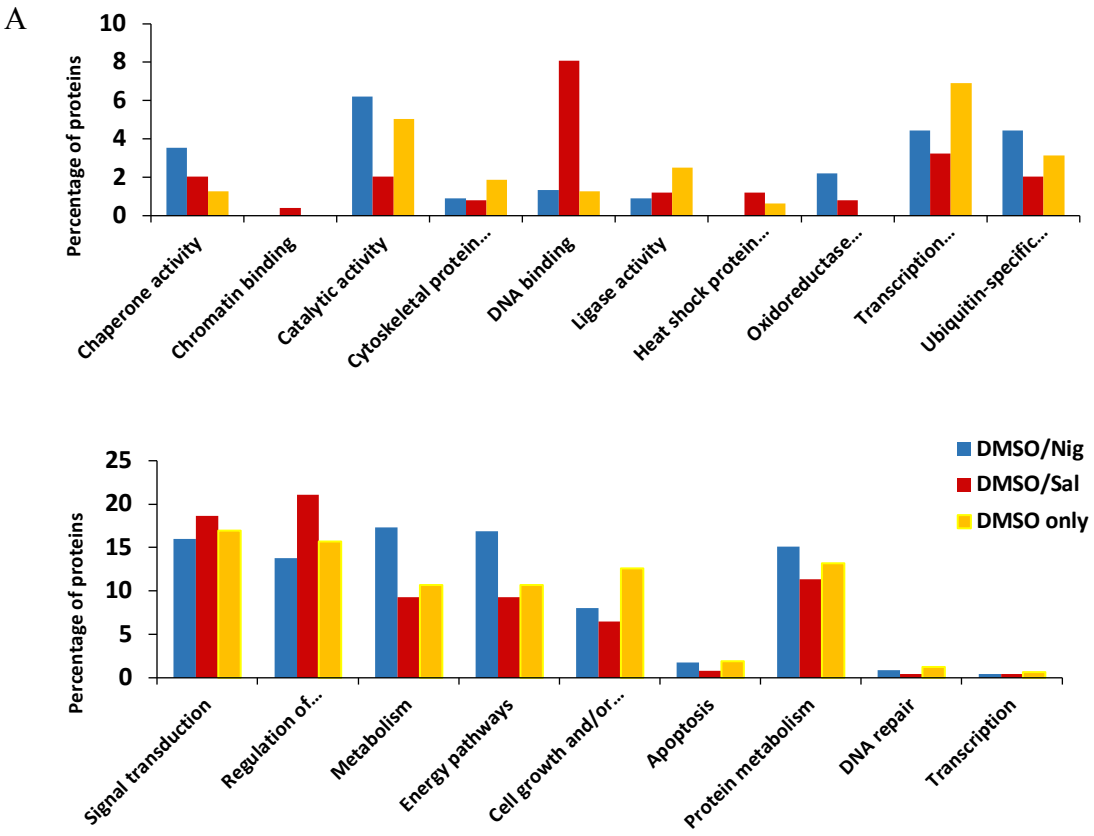


Figure 6. 3 Comparison of proteins identified in human leukaemia (HL-60) cells cryopreserved in dimethylsulphoxide (DMSO) +/- nigerose (Nig) or salidroside (Sal), according to their (A) biological processes and (B) functional classifications. *The biological processes and functions of proteins extracted from HL-60 cells cryopreserved in DMSO alone, DMSO/Nig or DMSO/Sal were identified using FunRich software.*

In silico functional analysis of the proteome revealed the nature of biological pathways associated with cryodamage in HL-60 cells cryopreserved in DMSO alone and in those differentially modulated by the CPAs post thaw. A considerably high number of proteins (21.05%) engaged in nucleotide and nucleobase regulation or DNA binding were identified in HL-60 cells cryopreserved in DMSO/Sal. In contrast, cells in DMSO/Nig exhibited high levels of proteins associated with energy pathways and protein metabolism (16.8%) as shown in (**Figure 6.3A**). Supplementing DMSO with Nig or Sal also resulted in increased levels of oxidoreductase activity, mainly in the presence of Nig (**Figure 6. 3B**). Levels of proteins linked to cell maintenance were highest in HL-60 cells cryopreserved in DMSO alone (12.5%), in comparison to those in DMSO/Nig (8%) and DMSO/Sal (6.4%).

The percentage of DNA binding proteins were 8.09% for cells cryopreserved in DMSO/Sal, while it did not exceed 2% in DMSO/Nig and DMSO alone **(Figure 6.3 B).** HL-60 protease activity associated proteins were estimated at 4.4% in DMSO/Nig, 3.1% in DMSO alone whereas it only

reaching 2.02% in DMSO/Sal **(Figure 6.3 B)**. In respect to cryo-stress, heat shock proteins were differentially expressed in HL-60 cryopreserved in DMSO/Sal (1.2%) and DMSO only (0.6%), whereas these proteins were undetected in the presence of Nig. The differentially expressed proteins level reflecting the effect of freeze-thaw cycle on HL-60 cryopreserved in DMSO alone and DMSO+/- Nig or Sal are summarised in **table 6.1**.

Uniprot entry name	Protein name	DMSO alone		DMSO/Nigerose		DMSO/Salidroside	
		UP (PC/PT)	FC	UP (PC/PT)	FC	UP (PC/PT)	FC
Redox pathways							
Q99497	Protein deglycase DJ-1	ND	-	ND	-	12	1.4
P00338	Lactate dehydrogenase chain A	11	- 1.6	ND	-	11	1.6
P00390	Glutathione reductase	7	3.2	ND	-	ND	-
P00441	Superoxide dismutase [Cu-Zn]	8	1.4	ND	-	ND	-
Q16881	Thioredoxin reductase 1	2	14.6	2	35	2	15
P28331	NADH-ubiquinone oxidoreductase 75 kDa subunit	4	4.9	4	46	4	16
Q9Y2Q3	Glutathione S-transferase kappa 1	2	-8	2	-13.6	2	-3.5
P30048	Thioredoxin-dependent peroxide reductase, mitocondrial	2	- 3	2	-5.2	2	-8.8
P30041	Peroxiredoxin	ND	-	17	-2.04	17	-3.5
C9J0G0	Acyl-coenzyme A oxidase (ACOX)	2	32	2	16.8	2	42.7
P49748	Very long-chain specific acyl-CoA dehydrogenase	5	- 2.7	5	- 11.6	5	- 14.8
P16152	Carbonyl reductase	ND	-	ND	-	5	-1.5
P49368	T-complex protein 1 subunit gamma	ND	-	17	1.2	ND	-
P40227	T-complex protein 1 subunit zeta	ND	-	7	1.4	ND	-
Q9NZL4	Hsp70-binding protein 1	3	14.4	3	-71	3	-77
P48723	Heat shock 70 kDa protein 13	ND	-	2	15.8	ND	-
P34932	Heat shock 70 kDa protein 4	ND	-	ND	-	17	1.3
Q53EL6	Programmed cell death protein 4	ND	-	ND	-	4	-1.6
P08758	Annexin A5 (Annexin-V)	6	-6.6	6	-9.2	6	-4.5
Q5VT06	Centrosome-associated protein 350	29	88.9	29	61.2	29	81.2
P25787	Proteomsome subunit alpha type-2 (PSAT2)	ND	-	3	34.4	ND	-
Nuclear activities regulation							
Q9BTE3	Mini-chromosome maintenance complex-binding protein	ND	-	2	11	2	70
P33993	DNA replication licensing factor MCM7	ND	-	ND	-	9	2.4

P35658	Nuclear pore complex protein Nup214	ND	-	ND	-	6	1.6
Q86YP4	Transcriptional repressor p66-alpha	ND	-	ND	-	11	2.5
Q5T890	DNA excision repair protein ERCC-6-like	4	-14.4	ND	-	4	8.8
Q99973	Telomerase protein component 1	ND	-	3	-2.3	3	-2.3
Q8WXI9	Transcriptional repressor p66-beta	4	-2.6	ND	-	ND	-
O14980	Exportin-1	5	3	ND	-	5	3.7
A6H8Y1	Transcription factor TFIIIB component B	9	2.1	9	7.9	9	10.6
Q15054	DNA polymerase delta subunit 3	2	3.4	2	30	2	22.3
Cell growth and function							
P00533	Epidermal growth factor receptor	ND	-	ND	-	4	2.1
Q14676	Mediator of DNA damage checkpoint protein 1	ND	-	5	17	5	21.4
Q6ZUM4	Rho GTPase-activating protein 27	2	13.7	2	39.5	2	75.4
Q9BYX2	TBC1 domain family member 2A	ND	-	3	11.2	3	39
O14976	Cyclin-G-associated kinase	4	4.1	4	8.5	4	9.8
Q8N163	Cell cycle and apoptosis regulator protein 2	ND	-	9	1.8	9	2.3
O94986	Centrosomal protein 152 KDa	ND	-	7	59.8	7	19
Q13576	Ras GTPase-activating-like protein IQGAP2	4	15.2	4	65.7	4	40.9
Q14789	Golgin subfamily B member	14	18.9	14	37.2	14	21.3
P49327	Fatty acid synthase	ND	-	39	10.4	39	9
Q01484	Ankyrin-2	17	32	17	39.7	17	48.8
O00423	Echinoderm microtubule-associated protein-like 1	4	23	4	42	4	32.22
A0A0U1RR07	Synaptotagmin-like protein 2	4	4.1	4	9	4	22
Q15691	Microtubule-associated protein RP/EB family member 1	10	7.1	10	3.2	10	7.1
E9PNZ4	Microtubule-actin cross-linking factor 1, isoforms 1/2/3/5	2	12.6	2	12.3	2	4.4

Table 6. 1 Significantly changing proteins identified using label-free LCMS/MS profiling of the Human cells HL-60 cryopreserved in dimethylsulphoxide (DMSO) +/- nigerose or salidroside (n=5 per condition). *All expressed proteins post cryopreservation of HL-60 in comparison with HL-60 proteins prior freezing shown here are statistically significant with ps<0.05.*

Abbreviations: UP = unique peptides, FC = Fold Changes indicating the ratio of differentially expressed proteins identified prior cryopreservation (PC) and post thaw (PT).

6.3.1.1 Redox functions

Reduction of cryo-oxidation in HL-60 cells was determined based on 3.2-fold and 1.4-fold increases in the expression levels of glutathione reductase (GR) and superoxide dismutase (Cu-Zn), respectively, in DMSO alone. No changes were detected, however, in the levels of either of these markers in cells preserved in the presence of Nig or Sal. In contrast, the expression level of thioredoxin reductase-1 was increased 35-fold when cryopreserved with Nig and 15-fold in the presence of Sal. A similar pattern was seen with the NADH-ubiquinone oxidoreductase 75 kDa subunit. Decreases were observed in the levels of pro-oxidative enzymes such as peroxiredoxine (not detected in DMSO and downregulated 2-fold in DMSO/Nig and 3.5-fold in DMSO/Sal), glutathione S-transferase Kappa-1 (decreased 8-fold in DMSO, 13.6-fold in DMSO/Nig and 3.5-fold DMSO/Sal) and thioredoxin-dependent peroxide reductase (decreased 3-fold in DMSO, 5.2-fold in DMSO/Nig and 8.8-fold DMSO/Sal).

Very long-chain specific acyl-CoA dehydrogenase (involved in fatty acid ß-oxidation) showed a 4-fold decrease in expression levels in HL-60 cells cryopreserved in DMSO with Nig and a 5-fold decrease in DMSO with Sal, compared to the levels in HL-60 cells cryopreserved in DMSO alone. In the presence of CPAs, a similar anti-oxidative pattern was observed regarding increased levels of acyl-coenzyme A oxidase (16.8-fold in DMSO/Nig and 42.7-fold in DMSO/Sal) and carbonyl oxidase (5.5-fold in the presence of Sal).

A differential response to cryo-stress in HL-60 cells was identified when Nig or Sal were added to culture media prior and post cryopreservation, and to DMSO during cryopreservation. For example, the stress-related protein Hsp 70-binding protein 1 was increased 14.4-fold in HL-60 cells cryopreserved in DMSO alone. In contrast, it was downregulated 71-fold and 77-fold in the presence of Nig or Sal, respectively. Conversely, a protein indicating cytosolic stress response, the heat shock 70 kDa protein 4, was not detected in DMSO +/- Nig and was increased 2.3-fold in DMSO/Sal. Finally, microsomal Hsp 70 protein-13 was not detected in HL-60 cells cryopreserved in DMSO +/- Sal, but it was detected in HL-60 cells cryopreserved in DMSO/Nig, where its expression levels increased 15.8-fold.

6.3.1.2 Nuclear and cellular functions

Twenty-four hours post thaw, Sal remarkably boosted nuclear activities in HL-60 cells (**Table 6.1**). The levels of proteins associated with DNA repair increased when Sal was present. For example, DNA excision repair protein ERCC-6-like increased 8.8-fold after cryopreservation with

Sal, was not detected in the presence of Nig and decreased 14.4-fold in HL-60 cells cryopreserved in DMSO alone. Mini-chromosome maintenance complex-binding protein, known to regulate DNA replication, increased 71-fold in DMSO/Sal and 11-fold in DMSO/Nig, but was not detected in DMSO alone. Sal also enhanced the levels of proteins associated with transcriptional regulation, such as transcription factor TFIIIB component B protein, which was upregulated 11-fold in Sal, 8-fold in Nig and 2-fold in DMSO alone.

In the presence of CPAs, the significantly changed levels of proteins associated with nuclear activity were also reflected by the changes in HL-60 proteins associated with cell growth and cytosolic functions. For example, the presence of Sal and Nig doubled the fold change of cyclin-G-associated kinase, involved in the cell cycle, from a 4-fold increase in DMSO alone, to 8- and 9-fold increases in Nig and Sal, respectively. TBC1 domain family member 2A, known to be involved in the regulation of GTPase activities and vesicle fusion, was not detected in HL-60 cells cryopreserved in DMSO alone, but was increased 11.2-fold in Nig and up to 39-fold in Sal. The levels of cytoskeleton proteins were also upregulated by Nig and Sal. Ankyrin-2, microtubule-associated proteins and Echinoderm microtubule-associated protein-like 1, which are known to be associated with cell shape, function (e.g. cell re-organisation) and division, were also increased in the presence of Nig and Sal in comparison with DMSO alone (**Table 6.1**).

6.3.2 HL-60 cell proliferation post thaw

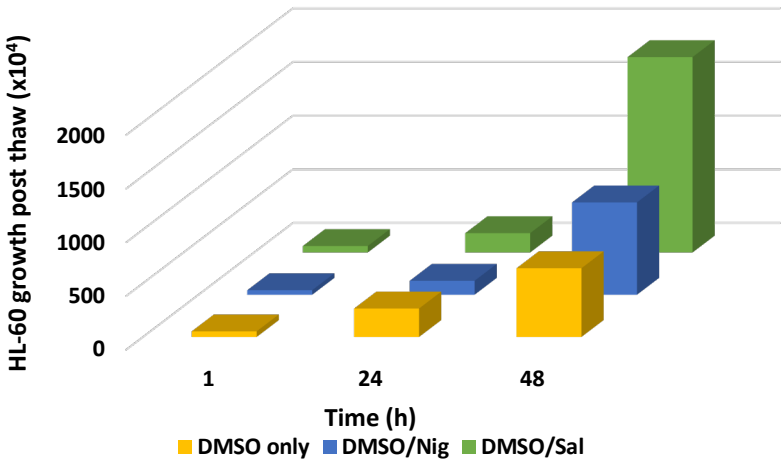


Figure 6. 7 Human leukaemia (HL-60) cells growth and proliferation at 1, 24 and 48h post freeze-thaw with and without additive agents Nigerose (Nig) or Salidroside (Sal). *Cells were cryopreserved in dimethylsulphoxide (DMSO) alone versus DMSO+/- (300 µM) Nig or (200 µM) Sal. Afterward, cells were thawed and cultured in RPMI-1640 media alone or containing Nig or Sal for up to 48 h. Data are obtained from duplicates for each condition and expressed as mean.*

The number of HL-60 cells 24 h post thaw was estimated at 265x10⁴ cells/ml 130x10⁴ cells/ml and 180x10⁴ cells/ml for DMSO alone, DMSO/Nig and DMSO/Sal respectively **(Figure 6.4)**. At 48 h, Sal greatly increased HL-60 cells proliferative rate by 2.84-fold compared to cells cryopreserved in DMSO alone. Cell proliferation rate at the same time point in DMSO/Nig showed a slight increase in proliferation (1.3 fold) in comparison to cell cryopreserved in DMSO alone. The direct comparison between the effect of Nig and Sal on cell growth at 48 h showed that the number of HL-60 cells in the presence of Sal was at 1820 x10⁴ cells/ml while it only reached 860 x10⁴ cells/ml in the presence of Nig. Such an increase in the HL-60 proliferative rate post thaw in the presence of Sal was also linked to the increase in the expression level of epidermal growth factor receptor (2.1 times) and Cyclin-G-associated kinase (9.8 times) (See **table 6.1**).

6.3.3 Caspase activity

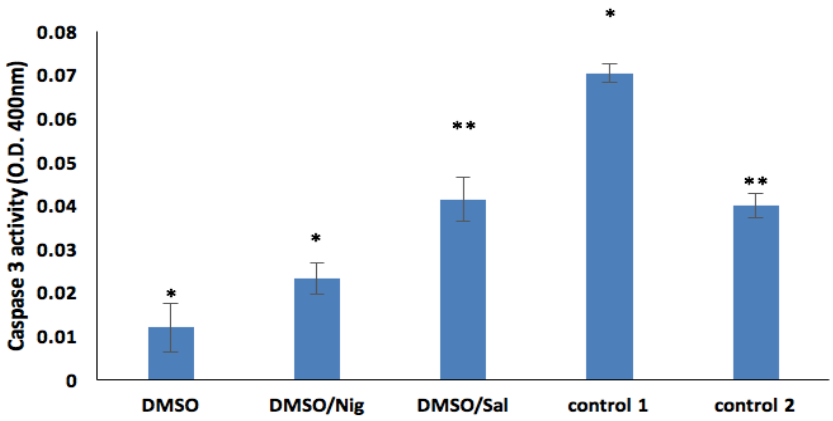


Figure 6. 8 Effect of additive agents nigerose (Nig) and salidroside (Sal) on caspase-3 activity in cryopreserved human leukaemia (HL-60) cells post thaw. *The activity of caspase 3 in HL-60 cells cryopreserved in dimethylsulphoxide (DMSO) alone, DMSO/Nig and DMSO/Sal was measured post thaw. Control 1 and 2 represent caspase-3 activity in HL-60 cells prior to freezing and after insulting by 0.5 mM H_2O_2, respectively. Data are derived from triplicates and represented as mean ± SD. Results were analysed using a one-way ANOVA followed by Tukey's test for multiple comparisons. * denotes statistical significance (* P < 0.01), ** denotes insignificance.*

The enzyme caspase-3 showed a differential response to the cryopreservation conditions. The measured activity of the enzyme in cryopreserved HL-60 cells in DMSO+/-Nig or Sal was lower than its activity in HL-60 cells insulted by H_2O_2 (control 1), which was 0.07 ± 0.002 mU/mL. In HL-60 cells cryopreserved in DMSO alone or with Nig post thaw, caspase-3 exhibited lower

activity rates (0.012 ± 0.005 and 0.02 ± 0.0035 mU/mL, respectively) than those in unfrozen HL-60 cells (control 2). In contrast, caspase-3 activity in HL-60 cells cryopreserved in Sal was similar level to that measured in control 2 (0.04 ± 0.0049 and 0.04 ± 0.002 mU/mL, respectively).

6.3.4 Biological profiles of HL-60 cells cryopreserved in DMSO +/- Nig or Sal

Intracellular GR activity in HL-60 cells was measured prior to freezing and 24 h post thawing. GR activity exhibited a significant increase in HL-60 cells cryopreserved under all cryopreservation conditions. Activity levels were 0.0005 ± 2E-04 mU/mL in DMSO alone, 0.0013 ± 6E-05 mU/mL in DMSO/Nig and highest in DMSO/Sal (0.0016 ± 3E-05 mU/mL), three times higher than that in HL-60 cells cryopreserved in DMSO alone.

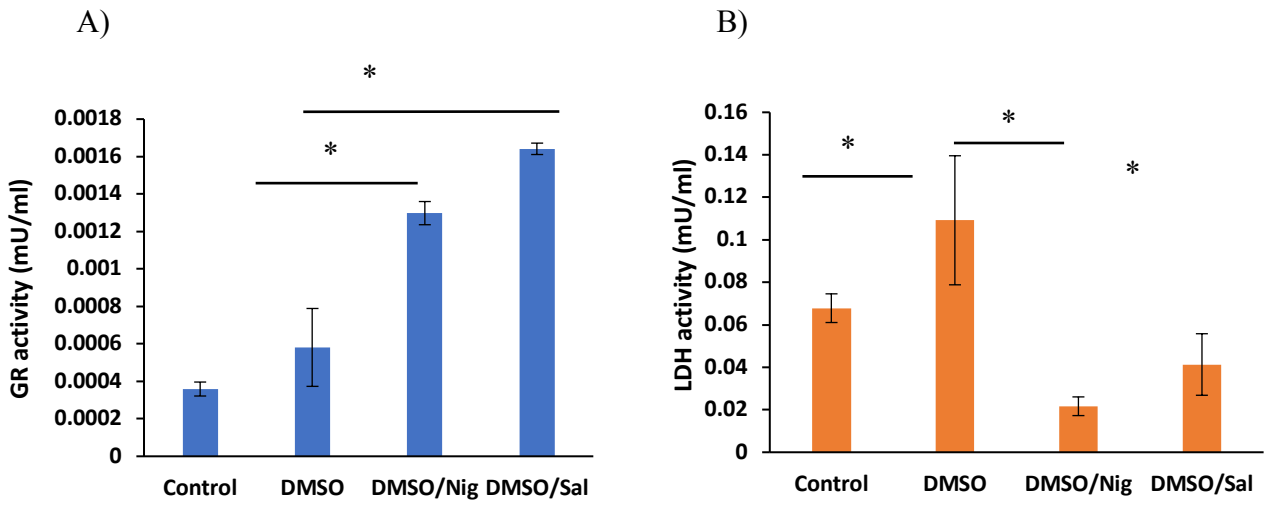


Figure 6. 9 Redox enzymatic activity in human leukaemia (HL-60) cells cryopreserved in dimethylsulphoxide (DMSO) +/- nigerose (Nig) or salidroside (Sal). *Intra-cellular enzymatic activities of HL-60 cells were measured prior to freezing (Control) and 24 h post freeze–thaw in media +/- Nig or Sal.* ***A)*** *shows glutathione reductase (GR) activity and* ***B)*** *shows lactose dehydrogenase (LDH) activity. Enzymatic activity is expressed as mU/mL. Data obtained from five replicates and presented as mean ± SD. Bars and stars dented significant variations between data (P value < 0.05).*

LDH intracellular activities were also measured prior to freezing and 24 h post thawing. Adding Nig or Sal to the culture media or cryomedia slightly lowered LDH activity. In DMSO alone, the LDH activity level was 0.1 ± 0.03 mU/mL. This was reduced to 0.04 ± 0.01 mU/mL in the presence of Sal, and further reduced to 0.02±0.044 mU/mL (three times lower than prior cryopreservation, five times lower than in DMSO alone and two times lower than in DMSO/Sal in the presence of Nig.

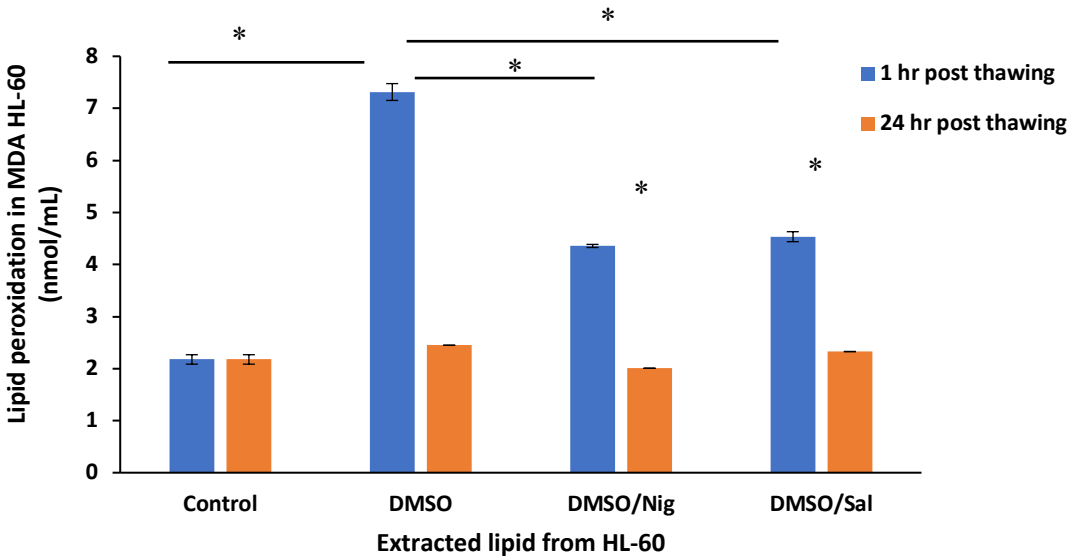


Figure 6. 13 Lipid peroxidation (MDA) in human leukaemia (HL-60) cells incubated and cryopreserved in media with and without nigerose (Nig) or salidroside (Sal). *Lipid peroxidation was measured in HL-60 cells 24 h before and after cryopreservation in dimethylsulphoxide (DMSO) +/- Nig (300 µM) or Sal (200 µM). Cells were continuously maintained in RPMI-1640 media with and without the corresponding agent, Nig or Sal before and after cryopreservation. Data are obtained from triplicates and represented as mean ± SD. Bars and stars denoted significant variations in data 1 hr post thawing (P value < 0.05). The length of the bars indicate an intercomparison between two sets of data.*

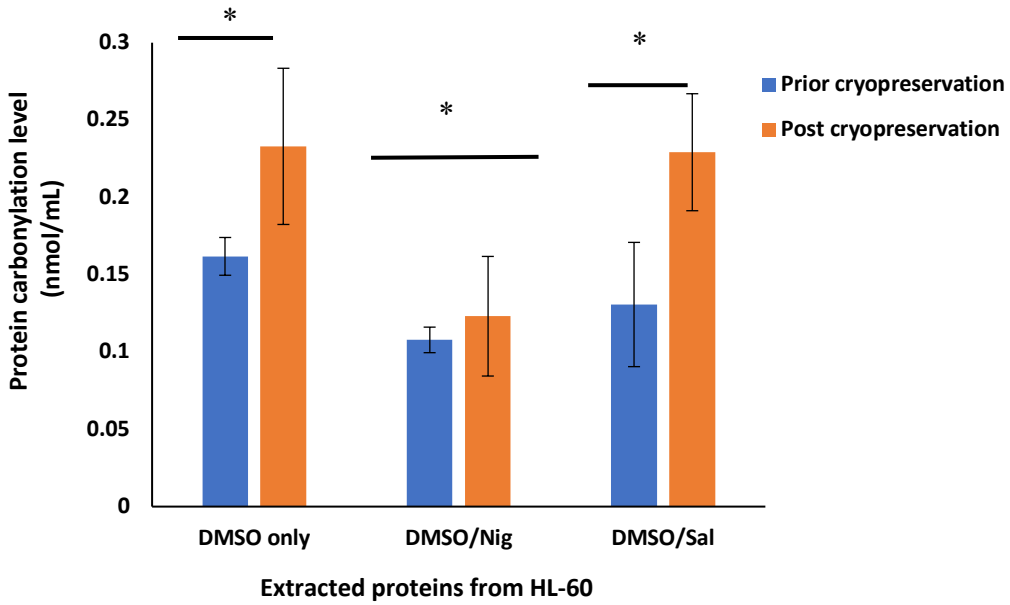


Figure 6. 14 Protein carbonylation in cryopreserved human leukaemia (HL-60) cells with and without the additive agents nigerose (Nig) and salidroside (Sal). *Protein carbonylation was measured in HL-60 cells prior to freezing when maintained in RPMI-1640 media +/- Nig or Sal. HL-60 cells cryopreserved in dimethylsulphoxide (DMSO) +/- corresponding agents were measured for additive effects 1 h post thawing. Data obtained prior to freezing and post-thawing are from duplicate and triplicate test samples, respectively. Data are expressed as mean ± SD (* P value < 0.05).*

Lipid peroxidation levels in HL-60 cells were measured in triplicate prior to freezing (control), and 1 h and 24 h post thaw in the presence and absence of Nig or Sal. There was a significant increase (7.31 ± 0.16 nmol/mL) in MDA levels 1 h post thaw in HL-60 cells cryopreserved in DMSO alone (**Figure 6.7**). In contrast, a 41% reduction in the MDA level was observed in the presence of Nig (4.35 ± 0.02 nmol/mL) or Sal (4.53 ± 0.09 nmol/mL). In the recovery phase (e.g. 24 h post thaw), lipid peroxidation levels in HL-60 cells were reversed under all cryopreservation conditions to reach the control level (e.g. prior to cryopreservation, ~ 2.1 nmol/mL).

The results showed that protein carbonylation levels in HL-60 cells cryopreserved in DMSO/Nig remained at the same level observed in cells prior to freezing (0.107 ± 0.007 nmol/mL; **Figure 6.8**), whereas Sal showed no protective effect on HL-60 cell proteins against carbonylation (~ 0.23±0.048 nmol/mL). Similar results were observed in HL-60 cells cryopreserved in DMSO alone, where the level of protein carbonylation post freeze–thaw in DMSO was 0.26 ± 0.016 nmol/mL (**Figure 6.8**).

6.4 Discussion

This is the first known study with the objective of establishing the proteomic and biological responses of HL-60 cells cryopreserved in DMSO with and without the novel CPAs, Nig and Sal. The major issue with most commonly-used CPAs, such as DMSO, is their cytotoxicity[271], which leads to cell damage and low recovery rates post-cryopreservation. Many of the pertinent proteomic studies have been validated by carrying out functional assays targeting identified protein pathways and ignoring other affected pathways. In addition, the bulk of previous proteome profiling studies investigating nucleated cell lines have been performed either using cells without cryopreservation[272], assessing the pharmacological agents' effects on specific cells[273] or comparing cellular proteome profiles of healthy versus unhealthy individuals[274].

In the current work, HL-60 cells were incubated in media including novel CPAs, either Nig or Sal, prior to and during cryopreservation. Subsequently, the differential effects of novel CPAs were identified based on the proteomic and functional profiles of the preserved cells. The highest number of differentially-expressed proteins was found in HL-60 cells cryopreserved in DMSO/Nig (37%), followed by 34% in DMSO/Sal and only 29% in DMSO alone**.** This suggests that the new additive agents, Nig and Sal, improved cellular protein preservation.

The data revealed that HL-60 cells cryopreserved in DMSO exclusively exhibited increasing levels of proteins associated with cryo-stress (e.g. superoxide dismutase, acyl coA oxidase, Hsp 70 binding protein 1). Interestingly, most of these observations were reversed to extremely downregulated levels by adding Nig or Sal. Moreover, proteins Hsp 13 and 4 were undetectable in DMSO alone but were found to be upregulated in the presence of Nig and Sal, respectively. The enhanced expression of Hsp 70 was involved in mitochondrial protection against oxidative damage and the removal of incorrectly folded proteins[275]. This corresponded with a biochemical profile such that oxidative protein levels were maintained under Nig conditions. The reason for such differential expression patterns in terms of Hsp is unclear, but it could be based on post-translational modifications and differential interactions with co-chaperones that may alter their functions[276], consequently causing them to become undiscernible. In such cases, it is useful to employ a labelling approach and enrichment combined with advances in mass spectrometry analysis to detect carbonylated proteins[277].

Furthermore, protein deglycase, which plays an important role as an oxidation sensor and features prominently in cell proliferation[278], was undetected under the DMSO+/- Nig condition, but was upregulated in DMSO/Sal. This suggests the promotion of an anti-oxidative intracellular environment. These findings are in line with reports of putative stress factors related to cryopreservation[279]. Further data analysis established that NADH-ubiquinone oxidoreductase's 75 kDa subunit, a protein known to be involved with cellular oxidative metabolism[280], was influenced by CPAs. It was upregulated in DMSO alone (4.9-fold) and DMSO/Sal (16-fold), and reached even greater levels in DMSO/Nig (46-fold higher), suggesting that the Nig effect is more likely targeting mitochondrial machinery and reducing apoptotic effects, as suggested by Ricci *et al.*[281]. A similar impact was observed regarding the higher levels of T-complex protein 1 subunits gamma/zeta exhibited under Nig conditions. These proteins are known to be crucial for chaperone-mediated protein folding and carbonylation[282,283], which often results in the loss of protein function[284] and can lead to apoptosis[285]. The expression of these proteins can be used as an indication of the anti-oxidative properties of CPAs.

The data also revealed a differential effect of Sal and Nig (when added to DMSO) on key enzymes associated with cryo-stress. For example, LDH protein levels were diminished when HL-60 cells were cryopreserved in DMSO alone and the addition of Sal reversed this by elevating their levels 1.6-fold. The differential effects of CPAs on the outcomes of HL-60 cell cryopreservation was also reflected in the correlation between the increased levels of GR in the presence of DMSO alone. Glutathione reductase is a critical enzyme known to promote a reductive environment and protect cells against the damaging effects of free radicals. Surprisingly, its protein levels were not

correlated with its activity, which was increased in the presence of Nig and Sal. Similar findings of a poor correlation between GR or LDH activities and protein expression levels have been reported elsewhere by Glanemann *et al*.[286]. In addition, Nig promoted antioxidative status by raising the level of thiordoxin reductase 1 (TXNRD1) in HL-60 cells. TXNRD1 overexpression has been reported to affect various cellular activities, including gene regulation, growth rate and increasing cellular resistance to toxicity[287], as well as reducing oxidative stress[288]. PAST2 is another redox enzyme that was detected under the Nig-only condition. The major part played by this enzyme is breaking down dysfunctional and damaged proteins [289].

One of the most defined outcomes of the present proteomic study is the increased levels of protein expression associated with nuclear cell activity when HL-60 cells were cryopreserved in DMSO/Sal**.** For instance, the mini-chromosome maintenance complex-binding protein MCM7 and DNA replication licensing factor are essential for synthetic phase activation, when DNA replication and elongation take place[290]. These proteins increased in HL-60 cells treated by Sal but were undetected in the presence of DMSO alone. In addition, Sal reversed the effect of DMSO on the DNA excision repair protein ERCC-6-like. The increase in activity of ERCC-6-like may facilitate transcription-coupled DNA excision repair and the de-blocking of the stalled RNA polymerase II[291] during cryo-stress. The current findings support the role of Sal in reducing oxidative damage by promoting DNA repair, as shown in hematopoietic stem cells via the regulation of the base excision repair pathway (e.g. poly [ADP-ribose] polymerase-1)[292].

Post-thaw, the levels of expression of proteins associated with transcriptional activities, such as Rho GTPase-activating protein 27 and Ras GTPase-activating-like protein IQGAP2, were also upregulated by Sal in comparison to cells cryopreserved in DMSO alone. This upregulation was associated with DNA repair/replication and transcriptional activities in the presence of CPA, and also appeared to be mirrored by an increase in the levels of proteins associated with cellular growth. For instance, the expression levels of epidermal growth factor receptor rose by 2.1-fold in the presence of Sal, although it was undetected in the recovery phase of HL-60 cells cryopreserved in DMSO +/- Nig. This receptor is known to be crucial in DNA replication and cell division[293]. Similarly, the mediator DNA-damage checkpoint protein 1 was highly elevated in terms of concentration under Sal and Nig conditions but was not observed in DMSO alone. Such regulatory elements of the DNA damage signalling pathway are paramount for cell survival by controlling passage from the S to the G2/M phases of the cell cycle[294]. In keeping with our proteomic findings, Sal exhibited a noticeable promotional impact on HL-60 cell proliferation during recovery phase. A similar elevation in proliferative proteins has been found in hepatocyte cells in response to the proliferation promoter compound perfluorooctane sulphonate[295].

Conversely, this result conflicts with the reported effect of Sal in inducing breast cancer cell-cycle arrest[296]. Such an anti-proliferative effect was previously attributed to Sal being used as an anti-hypoxia agent, leading to suppression of hypoxia-induced cell proliferation[297]. Furthermore, in the present study, we also identified an additive effect of DMSO with Sal or Nig in enhancing particular cellular functions by raising the level of cytoskeleton proteins, such as ankyrin-2, synaptotagmin-like proteins or microtubules (**Table 6.1**), resulting in favourable HL-60 cell recovery and growth post-thaw.

Finally, a further assessment of the activity of caspase-3, an enzyme connected with apoptosis and cellular proliferation[298,299], demonstrated that an increase in enzyme activity strongly corresponded with cellular proliferation. For instance, the greatest level of activity of caspase-3 post-thawing was determined in HL-60 cells cryopreserved and maintained in DMSO/Sal. This is attributed to Sal's anti-apoptotic and proliferative properties[217]. Moreover, this result is supported by previous studies that have found a link between knocking down caspase-3 gene expression and a decline in cellular growth[300,301,302]. Nevertheless, because caspase-3 possesses multiple functions, it would be highly useful to observe such activity, along with the growth of cells, for the sake of ensuring accuracy of data interpretation.

6.5 Conclusion

This is the first and largest study to focus on deciphering the molecular and biological effects of cryopreservation on the nucleated human cell line HL-60 proteome with and without novel cryo-additive agents (Nig and Sal), and to correlate the findings with corresponding biological function observations.

For the most part, cryopreservation of HL-60 cells led to oxidative damage, which was validated by biological features linked with cryo-stress in the presence of DMSO alone. The addition of modulatory novel CPAs led to differential effects. This study is unprecedented because it has clearly shown that Nig specifically diminished protein oxidation, while both Nig and Sal reduced lipid cryo-oxidation. The most striking finding generated by the current proteomic profiling study is that post-thaw, Sal raised the level of protein expression in HL-60 cells associated with nuclear activity (e.g. DNA repair/replication, transcriptional activities) and subsequently increased cell proliferation during the recovery phase. In summary, identifying the relevant molecular and biological pathways affected by cryopreservation and successfully targeting these compromised

pathways with novel CPAs is a way to enhance cryomedia formulation and limit cryodamage, which potentially resulting in improvements in cell therapy.

7 Conclusion, limitations and further research

7.1 Conclusion

Evidently, there is a need for high-quality biopreservation outcomes combined with a greater understanding of the protective mechanisms of cryomedia ingredients along with identifying powerful protective compounds to enhance cryomedia performance. These are particularly important for CTMP industries and end-users at clinics, such as those with cancer and diabetes or requiring blood transfusion, organ transplantation and infertility treatments.

This thesis has made a substantial contribution toward improving the biopreservation of CTMPs considering the aforementioned points. The discovery of Nig's and Sal's protective properties herein were exceptionally potent with anucleated as well as nucleated cell models in various cryomedia formulae and freezing modes, which verified their efficacy as powerful CPAs. The efficacy of these compounds was evidenced at low concentrations (200-300 μM) of Sal and Nig, respectively, which eliminates the risk of introducing osmotic pressure. In fact, these compounds have been found to mitigate the stress associated with adding and removing conventional cryomedia. That was clearly reflected by the level of oxidative damage induced during freeze-thaw processes and the recovery rate in the presence and absence of Nig and Sal. In addition, obtaining these compounds from natural sources, including plants and honey, consumed by humans historically with no side effects ensures their safety for biological applications.

Uncovering the protective properties of the novel compounds, Nig and Sal, demonstrated that they overlapped with regards to their protective mechanisms. Both compounds boosted the activity of GR, which enhanced the intracellular environment and promoted cellular function, diminishing stress levels as seen in LDH activity and the oxidation of proteins and lipids. Sal exhibited a high protective potency on sRBCs during and post-cryopreservation. The incubation of survival cells in a SAGM solution plus Sal for 10 days showed remarkable stability, where the cellular haemolysis rate was low and the levels of protein and lipid oxidation after storage were significantly mitigated. This phenomenon has promising applications for blood biobanks with the aim of extending cellular shelf-life and enhancing their quality during storage.

The unprecedented comprehensive analytical approach of this study involving the OMIC technology enabled us to establish the cryodamaged pathways and the modulatory effect of Nig and Sal. Each compound was observed to have a demonstrably unique effect on the proteome pattern of cryopreserved HL-60 cells. Here, Nig was strongly engaged with cell maintenance, energetic and metabolic pathways, whereas Sal influenced proteins associated with DNA binding and nuclear activities. Both overlapped with regards to influencing proteins associated with redox pathways. Moreover, the damaging effects of classical cryomedia was modulated by the reformulated media comprising the novel protective agents. The protective mechanisms of the compounds on the proteomic level was strongly compatible with the biochemical analysis of the cells cryosurvival rate and their resistance to stressors. These findings can have far-reaching applications beyond the current scope of this study, where specifically targeted media for cell-based therapy and drug production can be designed.

The utilisation of peripheral blood cells, mainly RBCs, when establishing the present study was of a great advantage because primary cells were taken directly from donors with almost no variations in their basic traits. Therefore, they are more representative functionally and characteristically of cryopreservation conditions' impact. The research was easily conducted on RBCs obtained from animals compared to human RBCs. This was because of the biobank policy that limited the access to fresh human blood and only permitted the use of nearly expired samples that were refrigerated for more than 15 days, which in turn affected the outcome of cryopreservation since RBCs age fast *ex vivo* and became more vulnerable to oxidative stress. In addition, the variation in the collecting procedure of the samples impacted the outcomes of the research.

Furthermore, we showed that employing trehalose as a base cryomedia exhibited greater efficiency in cryopreserving RBCs in comparison to glycerol. It reduced the cells' haemolysis and enhanced their stability post-thawing, which was promoted by adding Sal. This is consistent with previous studies suggesting trehalose as a universal protein stabilizer. Therefore, using trehalose to replace glycerol and DMSO media can be more advantageous for CTMP biopreservation. Because it has a low permeability into mammalian cells, however, a permeabilser was needed to achieve intracellular protection. Here, PP-50 polymers developed in Prof. Salter's lab were used to disturb the RBCs' membranes and create pores, thereby allowing trehalose to escape into RBCs. In spite of the successful application of PP-50 polymers mediating trehalose uptake in RBCs, the attachment mechanisms between PP-50 and the cell membrane remains unclear. Our findings indicated that PP-50 requires ionic solution for embedding into the cell membranes and disrupting them. Although trehalose loading protocols using PP-50 considered a lengthy nine

hours of incubation, examining the cells biochemical status prior to and after the incubation confirmed that the protocol does not exert any detrimental effects on cell quality, which proved that this protocol is a safe procedure and can be developed to mediate drugs and CPAs within other mammalian cells.

Finally, there is much in the way of developing research that can be carried out to enhance the biopreservation of biospecimens by adapting the findings of the present study. The application of the novel CPAs is not limited to CTMPs but can also be extended to tissue-engineered, food and cosmetics products.

7.2 Limitations

The utilisation of RBCs for screening protective compounds and evaluating their protective mechanisms leads to significant results regarding cellular biochemical profiles. Nevertheless, profiling the proteome of cryopreserved RBCs with LCMS was challenging owing to technical limitations. First, Hb is highly abundant in RBCs - it constitutes 97% of the total cellular proteins and should be depleted in order to profile other proteins. Secondly, analysing RBC cryopreserved in glycerol was not possible because glycerol masks proteins and interferes with proteins detection, leading to inaccurate readouts. Thirdly, it was not possible to run the study employing hRBCs because of the variations in the obtained samples. Finally, the identification of sRBC proteins was carried out using the Bos Taurus database. These reasons limited the efficiency of studying the cryopreserved RBCs. Therefore, it is highly recommended to utilise hRBCs for OMIC investigation based on the availability of the database. In addition, in-depth analysis of RBC proteins with optimising the depletion process and protein separation from cytosol and membrane is advisable.

The limitation of hRBC cryopreservation emanated from the variability of obtained hRBC biospecimens as mentioned earlier. As such, there were differences in the cryopreservation outcomes, rendering the data of hRBC under different conditions incomparable. This also limited the study of hRBC proteomes as a number of samples were only suitable for the glycerol cryopreservation protocol as discussed in Chapter 5.

7.3 Further research

Significant research and development opportunities continue to emerge in the growing and expanding field of biobanking and biopreservation. There are many issues that must be addressed in order to improve the biopreservation of CTMPs and other ATMPs. It would be of interest to investigate the effect of novel CPAs in the present study on other CTMPs (e.g., reproductive cells, stem cells) considering multiple factors that impact diverse biospecimens quality (e.g., cell biology, biophysics, biochemistry, chemistry). The work herein also underscored other possible future investigations such as:

7.3.1 OMICs and cryopreservation

Further analysis of the cellular compartments, such as lipids and genome, under different cryopreservation conditions is essential. Utilising OMICs techniques in the field of biopreservation and cryopreservation will improve our understanding of the biopreservation effects on cells' response to environmental stressors and protective agents on molecular level. Such investigations will expand the research scope for drugs delivery, biomarkers discoveries, and will assess the induced damages and the possibility to reverse or even eliminate such alterations by the tested compounds.

7.3.2 Translational research

The present study is just a step forward in the discovery of CPAs building upon basic research for a proof of concept. Follow-up translation and pre-clinical studies are required to solidify the findings and move on to the next phases in order to benefit humans. It is recommended carry out such studies on animals for toxicity and efficacy assessment *in vivo* as this will also allow establishing good manufacturing practices (GMPs) protocols for clinical applications.

7.3.3 Trehalose fate in mammalian cells

Mediating trehalose within cells and its fate internally would be of major interest for developing cryopreservation protocols for other types of mammalian cells. In a previous study by Lynch *et al.*[303] , it was suggested that intracellular trehalose can be unloaded from RBC, however this was contradicted by the findings of the present study where G6PDH activity remained constant in the presence of trehalose compared to deprived RBCs, suggesting that trehalose can be degraded

inside cells and used for glycolytic pathways. Yet, further studies are also necessary to elucidate the intracellular fate of trehalose in mammalian cells.

7.3.4 High-throughput research

Limited sample volume available for biomarker research, drugs discovery and development and subsequence research applications, is a real issue. Therefore, scaling up the stored CTMPs by applying the findings of the present study will be of serious benefit. At present, the use of trehalose as an alternative to DMSO and glycerol has not been experimentally automated on a large scale. With this, in our study, we have demonstrated its efficacy in combination with novel CPAs to improve cell stability and viability post-thaw. It is recommended beyond this research to establish protocols for thigh-throughput cryopreservation using trehalose and examining repetition feasibility.

7.3.5 Improving polymer- loading technology

This study highlighted the limitations of the currently used polymer, PP-50, loading technology. Until now, the loading protocol employing PP-50 is restricted to RBCs. Future studies seek improvements should take place, such as enhanced protocols, focusing on developing the biopolymer features to be applicable to other nucleated cells to modulate drugs and cryoprotectants.

7.3.6 Biospecimen process prior to and post- cryopreservation

Investigating the effect of biospecimen processing on biomarker discoveries associated with the outcome of cryopreservation is essential for the discovery of CPAs and leads to better outcomes for cellular biobanking and cell-based therapies. Establishing the best bioprocessing protocols for GMPs, therefore, is another important research domain to be optimised. This will significantly improve downstream utilisation (e.g. in the pharmaceutical research and development or clinically).

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Appendices

Appendix i

Statistical analysis:

Ex. Effect of additive agents on cryosurvival rate of hRBCs

Treatment		Raw Data (540) of haemolysed blood post freeze-thaw				
	Number of samples	**1**	**2**	**3**	**4**	**5**
Glycerol	supernatant	2.11	1.999	1.992	2.027	2.196
	total blood	2.457	2.231	2.079	2.082	2.434
Trehalose	supernatant	2.145	1.783	1.842	1.729	2.069
	total blood	2.23	1.946	1.842	1.983	2.219
Trehalos+Nig	supernatant	1.85	1.84	1.75	1.61	1.969
	total blood	2.289	2.283	2.096	2.006	2.287
Trehalose+Sal	supernatant	1.892	1.732	1.61	1.59	1.925
	total blood	2.213	2.072	1.895	1.866	2.121

Glycerol	**Haemolysis%**	85.877	89.601	95.815	97.358	90.221
Trehlose		96.188	91.623	100	87.191	93.240
Trehalose+Nig		80.821	80.595	83.492	80.259	86.0953
Trehalose+Sal		85.494	83.590	84.960	85.209	90.759

							Mean	**SD**
Glycerol	**Cryosurvival %**	14.122	10.398	4.184	2.641	9.778	8.225	4.728
Trehalose		3.811	8.376	0	12.808	6.759	6.351	4.815
Trehalose+Nig		19.178	19.404	16.507	19.740	13.904	17.747	2.503
Trehlaose+Sal		14.505	16.409	15.039	14.790	9.240	13.997	2.757

Anova: Single Factor

SUMMARY

Groups	*Count*	*Sum*	*Average*	*Variance*
Glycerol	5	41.1263762	8.22527524	22.3552309
Trehalose	5	31.7564926	6.35129851	23.188996
Trehalose+Nig	5	88.7360631	17.7472126	6.26773502
Trehalose+Sal	5	69.9859617	13.9971923	7.60373757

ANOVA

Source of Variation	*SS*	*df*	*MS*	*F*	*P-value*	*F crit*
Between Groups	412.354	3	137.45	9.253	0.00087	3.23
Within Groups	237.662	16	14.853			
Total	650.016	19				

t-Test: Paired Two Sample for Means

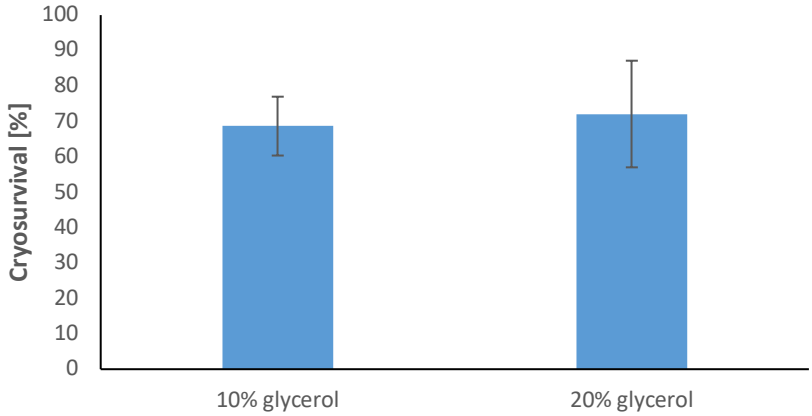
	Trehalose	*Trehalose +Nig*
Mean	6.351	17.747
Variance	23.188	6.267
Observations	5	5
Pearson Correlation	0.391	
Hypothesized Mean Difference	0	
df	4	
t Stat	-5.696	
*P(T<=t) one-tail	0.0023	
t Critical one-tail	2.131	
P(T<=t) two-tail	0.00469	
t Critical two-tail	2.776	

t-Test: Paired Two Sample for Means

	Glycerol	*Trehalose +Nig*
Mean	8.225	17.747
Variance	22.355	6.267
Observations	5	5
Pearson Correlation	-0.00095	
Hypothesized Mean Difference	0	
df	4	
t Stat	-3.978	
*P(T<=t) one-tail	0.0082	
t Critical one-tail	2.131	
P(T<=t) two-tail	0.0164	
t Critical two-tail	2.776	

Appendix ii

S4.1 Glycerol concentrations effect on sRBC cryosurvival rates post-thawing



S4.1 Glycerol concentrations effect on the cryosurvival rate of sRBC. The graph shows the cryosurvival rate of sRBC suspended in 10% vs 20% glycerol solutions and cryopreserved in liquid nitrogen for 24 h. sRBC heamolysis was measured using Drabkin's reagent ($p>0.01$).

S5.1 The effect of metallic ion Mg^{2+} on sRBC's membrane and PP-50 attachment

Brief background and pilot tdy design

The attachment mechanisms between the polymer PP-50 and RBC membrane is unclear. However, coincidently the lack of Mg^{2+} or Ca^{2+} in PBS buffer that was used to prepare trehalose and the PP-50 solution lead to failure cryopreservation. After several attempts to cryopreserve the cells, it was found that there is a clear correlation between Mg^{2+} and Ca^{2+} ions absence in PBS buffer and PP-50 action. Therefore, the ions influence on the cells membrane and PP-50 interaction was investigated. PBS buffer without Mg^{2+} and Ca^{2+} and $MgCl_2$were purchased from Sigma-Aldrich (Poole, UK). $MgCl_2$ was dissolved in PBS buffer to prepare a series of concentration ranged between 0,100 and 500 mM. Then, PP-50 conjugated with FITC was added to each solution to make final concentration of 100µg/mL. sRBCs were washed with PBS buffer. Then, incubated in the PP-50/Mg^{2+} solution for 0-3 h at 37˚C protected from light. Then, the incubated sRBCs were centrifuged at 700 x g for 5 min. The supernatant was discarded and the sRBCs suspended in PBS buffer containing Mg^{2+} and Ca^{2+}. This step was repeated twice. After that, sRBCs were diluted 30 times in PBS buffer to obtain an appropriate cells density for imaging. Cell suspensions were loaded on microscope slides and covered with a glass cover slip. Finally, the cell images were taken using an inverse oil immersion objective lens and a 488 nm excitation line on a Leica DMI 6000 CS confocal unit (Wetzlar, Germany) at x63 magnification.

Results and discussion

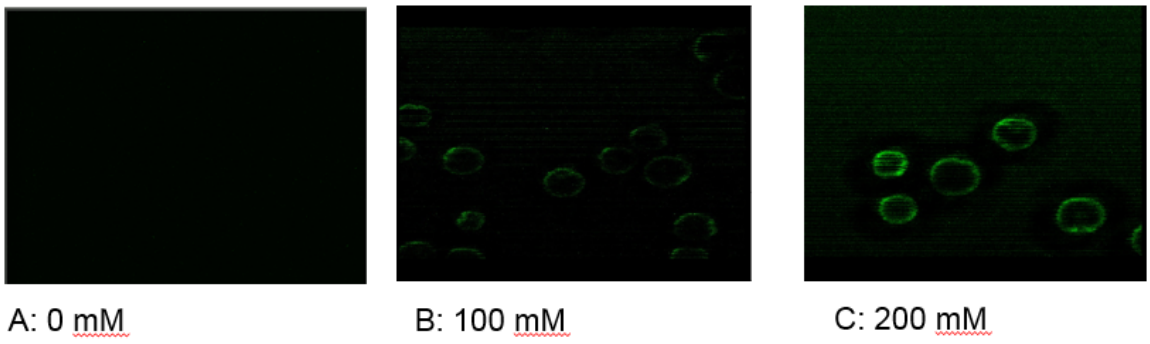


Figure S5.1. Effect of Mg^{2+} concentration on percentage of FITC labelled PP-50 attachment to RBC membranes post incubation. *Images were acquired after 3 h incubation at 37˚C in dark using Leica TCS SP5 confocal unit with a 68x oil magnification objective*

As shown in **Figure S1**, the PP-50 label was only attached to cells in the presence of Mg^{2+} ions. The intensity which reflected the amount of PP-50 attachment to cell membranes was correlated to the Mg^{2+} concentration. The attachment was only detected after 3 h of incubation. This phenomenon could be attributed to the salting effect of the ions that allow proteins and similar molecules such as the biopolymers to integrate with the cell membrane. This integration is crucial

for trehalose permeabliseing into RBCs. To further validate this effect, trehalose intracellular level quantitiy in sRBC was measured. The data confirmed the influence of Mg^{2+} on PP-50 integration with the cell membrane and, subsequently, trehalose uptake by the cells as shown in **Figure S5.2**. The intracellular trehalose was assayed using Anthrone assay[114].

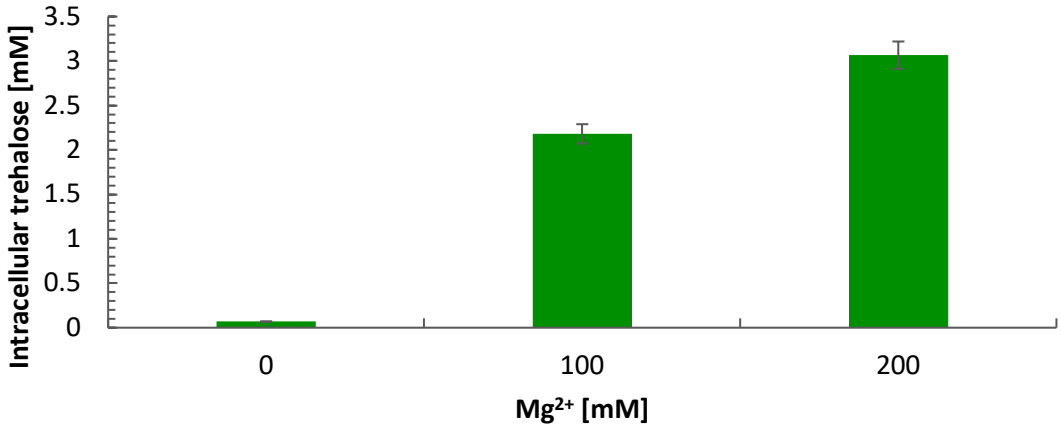


Figure S5.2. Effect of Mg^{2+} concentration on permeability of trehalose into RBCs 3 h post incubation. Trehalose was measured in 15% haematocrit RBC using the anthrone assay. The data are obtained from triplicates and expressed as mean ± SD.

S3.3. sRBC Hb-depletion using the HemoVoid Kit

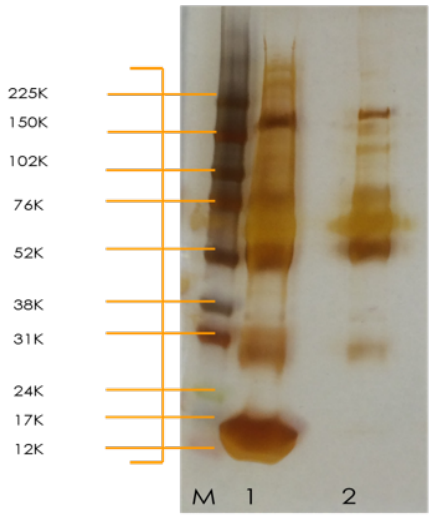


Figure S5.3A. Haemoglobin depletion in RBC lysate. M = molecular weight marker (indicated in K [kilodaltons]). Lane 1 = total RBC lysate. Lane 2 = RBC lysate after haemoglobin depletion using the Hemovoid kit. The volume loaded into each lane was 30 µL. The gel protein bands were identified post-electophoresis using the silver staining method.

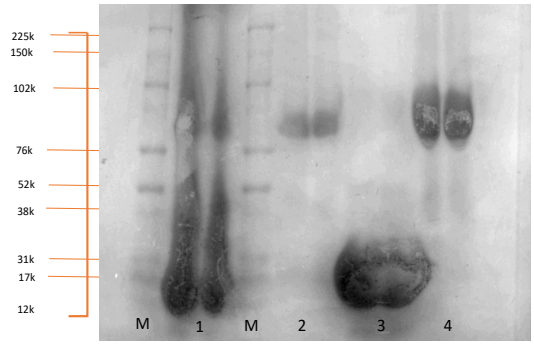


Figure S5.3B. Haemoglobin depletion is sRBCs lysate following incubation at different hcrt%. Lanes 1 and 3 are total RBCs at 40 and 15% hcrt, respectively. Lanes 2 and 4 are sRBC lysates after haemoglobin depletion using Hemovoid kit. The volume loaded in each lane was 30 µL for lanes 1-3 and 90 µL for lane 4.

S6.1 The effect of Nig and Sal concentrations on HL-60 viability post-thawing

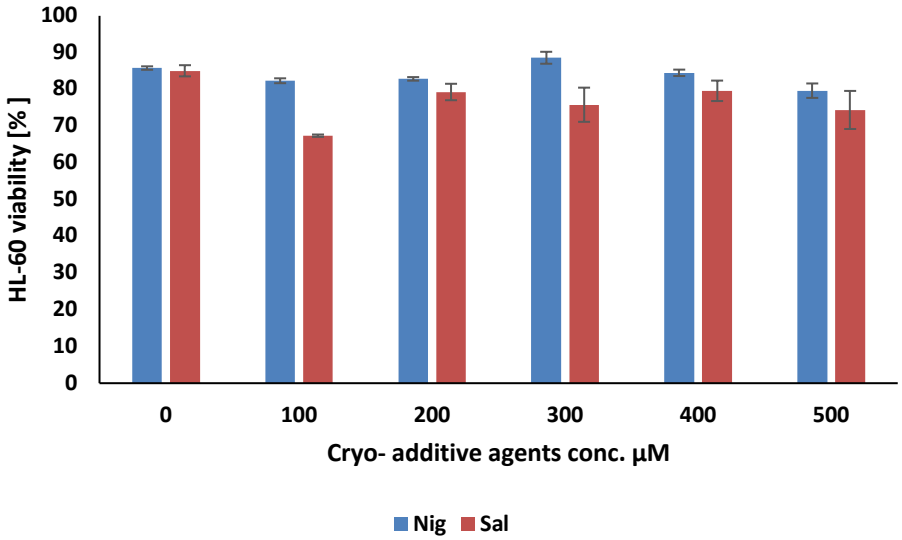


Figure S6.1. The effect of Nig and Sal at different concentrations on HL-60 cell viability post cryopreservation in 10% DMSO +/- Nig or Sal. HL-60 cryosurvival cells were measured in triplicate using trypan blue in triplicate. Data are represented as mean value ± SD (* P value <0.05)