

CRYOPRESERVATION OF HUMAN EMBRYONIC STEM CELLS AND HEPATOCYTES

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Abstract

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Cell cryopreservation has been used for cell banking, but has proven to be challenging concerning some important cells such as human embryonic stem cells (hESCs) and human primary hepatocytes. Cells can lose their viability or functions following cryopreservation. It is the aim of this study to develop novel methods and to optimise the common protocols to improve the outcome of cryopreservation of hESCs and human primary hepatocytes.

Experiments were carried out to establish the cell culture and functional assays of hESC and isolation, purification and functional assays of human primary hepatocytes. Three-dimensional encapsulation using RGDS-alginate and cryopreservation were applied to hESC as well as freshly isolated human hepatocytes and the results were compared to conventional cryopreservation of these cells in suspension. Important parameters, such as the concentration of cryoprotective agents (CPAs), freezing rate, loading time, thawing protocols, subsequent culture methods and their effect on cell viability and functions were all studied experimentally. Electroporation and Sonoporation were explored as possible methods to promote and mediate cell membrane transport of non-permeable CPAs, i.e. trehalose, to protect the intracellular components of the cells.

It was concluded that the RGDS-alginate encapsulation improves the hESC cryopreservation outcome. The human primary hepatocytes, successfully isolated from liver tissue samples, can be cryopreserved with fairly high cell survival rate and

functionality. Challenges and future research areas were identified, including optimization of 3D cryopreservation of human primary hepatocytes, effect of RGDS-alginate encapsulation on cell apoptosis of cryopreserved hESC and application of electroporation and sonoporation to the preservation of the hESCs and human hepatocytes.

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Chapter 1 Introduction

Long-term cell preservation is critically important to modern biotechnology and regenerative medicine. Cryopreservation is the most practical method at present time. It relies on the use of ultra-low temperature to effectively shut down all biochemical reactions and the use of suitable protocols to prevent cell death. Many types of human and animal cells have been cryopreserved successfully.

However, there are some important types of cells which have been proven to be difficult to cryopreserve, for example, human embryonic stem cells and human primary hepatocytes. Human embryonic stem cell (hESC) is one of the most important potential sources of cells for cell therapy and regenerative medicine. But hESC cryopreservation usually results in a low recovery, often less than 5%. Moreover, the low viability and low recovery are also coupled with a vital problem, the high incidence of short-term spontaneous differentiation after thawing. Another example is human hepatocytes. Human primary hepatocytes are treasured products for biomedical and pharmaceutical research, therapeutics and cell transplantation. Primary hepatocytes and developed hepatocyte cell lines have been widely used to study cell xenobiotic metabolism. The challenges of cryopreservation of human hepatocytes are huge. Often, the cryopreserved hepatocytes lack the primary key functions of healthy primary hepatocytes and have a high tendency of cell apoptosis.

The common features of hESC and human primary hepatocyte cryopreservation are the low survival rate and functional loss. This thesis aims to improve the survival and preserve functions of hESCs and human hepatocytes by optimizing the cryopreservation protocols,

applying 3D encapsulation technique to cell cryopreservation and using electroporation and sonoporation technique to deliver non-permeable cryoprotective agent (CPA), such as trehalose, into cells.

This thesis consists of 7 chapters:

Chapter 2 presents a literature review that describes the fundamentals and progress in cryopreservation technology. First of all, the mechanisms of cryoinjury induced by cryopreservation process was introduced, related to the cooling and thawing protocols, intracellular and extracellular ice formation and freezing rate. Following this, a variety of cryoprotectant agents (CPAs) were discussed and their protective mechanisms were described. Moreover, the technique of 3D encapsulation and its application to cell cryopreservation were analysed. Finally, the theories and potential applications to cryopreservation of electroporation and sonoporation were presented. The parameters of both techniques were introduced and discussed.

Chapter 3 demonstrates the effects of 3D encapsulation using functionalised alginates on cell cryopreservation. The RGDS (Arg-Gly-Asp-Ser) modification protocol to alginate was established and the bonding efficiency was determined using FTIR and combustion analysis. The application of RGDS-alginate encapsulation to hESC cryopreservation was tested and the outcome was compared to standard cryopreservation protocols, i.e. cells in suspension. In addition, the ROCK inhibitor was introduced to the cryoprotectant medium and its effect was analysed.

In Chapter 4, the protocols for separating and isolating human primary hepatocytes were developed and optimized. The efficiencies of different protocols with different types of liver tissue samples were tested and compared. The viability and cell function of isolated human hepatocytes were measured and presented. The outcome of the chapter provides the basis for the study of cryopreservation of human hepatocytes. The cells that adopted by each cryopreservation experiment were from the same liver tissue sample, which has the benefit of cell uniformity that the commercially available samples cannot provide.

Chapter 5 reports the experimental results of cryopreservation of freshly isolated human hepatocytes. The optimum parameters of cryopreservation of human hepatocytes, regarding formula of recovery medium, FCS concentration, usage of sucrose and glucose, cell density and effect of ROCK inhibitor were studied and selected. The feasibility of applying 3D encapsulation to human hepatocytes cryopreservation was also assessed.

Chapter 6 describes the techniques of electroporation and sonoporation mediated cell membrane transport. The experimental results of application of electroporation to cell cryopreservation were presented and compared to those using conventional cell preservation protocols. The principle and system design of sonoporation was demonstrated. A prototype sonoporation system was designed and assembled. The feasibility of its application to cell cryopreservation was analysed.

Chapter 7 presents a summary of the thesis contributions and conclusions derived from this study. The outline of future work, in particular concerning the 3D human hepatocytes cryopreservation and application of electroporation and sonoporation to hESC and human hepatocytes cryopreservation, was discussed.

Chapter 2 Literature Review

2.1. Introduction

The need for cryopreservation of both cells and engineered tissues becomes urgent and critical in the emerging field of tissue engineering and stem cell therapy to ensure the off-the-shelf availability of the bio-products, and the challenges becomes ever increasing too. A typical cryopreservation process consists of the following steps: loading of cryoprotective agents (CPA), reducing temperature to -80 C or liquid nitrogen temperature in a controlled manner, low temperature storage, thawing in a controlled manner, and removal of the CPA before application. Each step can bring stress to the cells and even lead to cell death. The related heat and mass transfer rates have become increasingly difficult to assess as the size and complexity of the investigated specimens increase. On the other hand, great progresses have been made in other related areas, for example, culturing cells within a biomaterial matrix with well-defined functions, separating cells with a much reduced risk of contamination, high cell density culture, scaffolding design, bioreactor technology etc.. Cell and tissue cryopreservation becomes a bottle neck for clinical and industrial applications of cellular and tissue products.

While engineered tissues are in a 3D structure and their cryopreservation has to be in a three dimensional construct. Three dimensional construct may offer cells within an

additional protection during cryopreservation, and this is worth serious exploring, although this does increase the process complexity at the same time.

Much research has been directed to selection and characterisation of CPAs, optimisation of freezing and thawing protocols, and mathematical modelling. In cryobiology, attempts have been made to understanding the mechanism of cryoinjury, induced apoptosis, and design cryoprotective agents to prevent cryoinjury and cell death.

This chapter presents the current understanding on cell cryopreservation based on the study of literature review. The need for new technologies to cryopreserve important cells, human embryonic stem cells and human primary hepatocytes, will be identified.

2.2. Cryopreservation

Cryopreservation is the process of storing live cells at low temperatures to maintain cell viability for varying periods of time [1] in biobanks. Initial adoption of this method dates back to the early 1940s when the recognised technique was to freeze red blood cells to replace lost blood during the World War [2]. The majority of techniques established during that period, particularly in the field of preserving red blood cells, have generally remained unchanged [3]. As scientific investigations progressed, animal cells, namely bovine spermatozoa, were preserved and were utilised in areas such as artificial insemination [4, 5]. In recent years, advances in cryopreservation have been influenced greatly by emerging technical advancements, including tissue engineering and stem cell technology. It is

anticipated that including tissue engineering and stem cell therapy, interventional medicine may well become part of accepted mainstream medical practice [6, 7]. Research concerning genetic bases involved with disease pathologies has resulted in cell banking becoming a vital factor of modern research techniques [8]. Coupled with the advancements concerning nuclear transfer along with cloning technology, any capability of conserving or even regenerating lost biodiversity brought about increased significance and potential usage of cryopreservation [9]. In tissue engineering, cryopreservation's unique importance is regarding the banking of bio-artificial cells, organs and tissues for clinical use. Therefore, cryopreservation allows the scale up of processes in producing tissue engineered products [10, 11].

A point to consider is that cryopreservation, in the context of tissue engineering, is not without its competitors, such as the simple maintenance of tissue in culture. Any loss of a functional, live cell population in the recognised freeze-thaw process has, on the other hand, made the idea of "Just-In-Time" (JIT) culturing of engineered tissue medical products for use in transplantation a competing modality in this context. Any loss in cellular functions due to cryopreservation cannot be tolerated. However, JIT culturing has its own significant drawbacks when compared to cryopreserved alternatives, for example a limited shelf-life, expense, increased risks of contamination and genetic drift [12, 13].

2.3. Challenges in Cryopreservation Process

Cell cryopreservation has a number of uses including storing human eggs (oocyte), fibroblasts (skin), blood products, sperm, embryo, and stem cells. On the whole, cells are easier to store in small quantities or in thin layers, since this requires less concentration of unnatural cryoprotective agents or cryoprotectants. These frozen samples must be stored in a -80°C freezer or liquid nitrogen at temperatures of down to -196°C . In the absence of cryoprotectants, the cells die from dehydration and there is a possibility that extracellular and intracellular ice crystals may form. Extracellular ice formation may well be harmful to these cells because of cell dehydration caused by the hyperosmotic environment when ice crystals are formed. Intracellular ice formation may be even more harmful as ice crystals will tear or pierce the cells. Both thawing and freezing after ice crystallization and hence are vital stages of the process. Therefore, it is very important to use cryoprotectants to avoid these effects occurring and maintain high cell viability. Common cryoprotective agents (CPAs) are glycerol and dimethyl sulfoxide (DMSO), due to their proven cryoprotective properties and low toxicity.

However, DMSO can be lethally toxic in high doses and therefore must be removed from cells immediately following thawing to ensure higher viabilities are achieved. Considering this, cryoprotectants with low or none toxicities are much more desirable. Many experimental studies have been carried out on the usage of trehalose as a cryoprotectant. Chemically, Trehalose is a non-reducing disaccharide with important biological functions. Trehalose may be present in some animals, insects and plants but this is quite rare. Its presence is considered to improve the ability for many organisms to survive lengthy periods

of desiccation. Trehalose can also prevent potential damage to cells from dehydration since it has strong water replacement and vitrification functions. As trehalose naturally occurs in these organisms that can survive long periods of desiccation, but human cells are not capable of producing this sugar.

Using trehalose to protect human cells has been proposed, but trehalose is impermeable through cell membrane. Therefore, experiments have been carried out introducing trehalose into cells as a lyoprotectant. In most cases, experiments have proven that trehalose has strong lyoprotectant characteristics when it is introduced into cells. Differing methods have been used to introduce trehalose into cells, including; thermal and, osmotic shock, -, electroporation, micro-injection and use of engineered switchable membrane pore [14]. These methods are complex and it is a difficult task to achieve high cell viability results other than the “engineered switchable pore membrane” method. However, this method makes use of an expensive bacterium called staphylococcus aureus and α -hemolysin so it would be very costly to perform on a large scale basis. Moreover, whether such processed cells can still be used for biomedical application is not certain.

The expected outcome following a proper execution of the cryopreservation process through to the thawing and re-incubation should be predictable and precise. These expectations are achievable if the process is mechanized or at least merchandisable. There is mounting evidence that processes that match these needs to a solution are well received [15, 16]. However, some methods in cryopreservation are not easily merchandisable. For instance, controlled-rate freezing has several features that make it harder to predict outcomes when one of them is altered. Examples of these complexities include the high

number of variables, interdependence among process variables and a high sensitivity of cells to changes in the process and high variability in technique [16]. Cell sensitivity has also contributed to the observed low viability and poor outcome of the cryopreservation of primate embryonic stem cells [17]. In addition to these field-specific aims, there are also clinical targets to be met besides the researcher's own. For instance, the cryopreservation process should simplify the transitions from explantation to implantation. The form adopted in preserving these cells whether they are suspended, embedded, adherent or immobilized should shorten the cumulative duration between explantation-to-storage and storage-to-implantation. Clinical expectations will also put restrictions on the components of cryoprotective agents (CPAs) used and techniques employed in maximizing post-thaw cellular viability [18-20]. For example, usage of sera needs to be carefully reconsidered and this is covered in Chapter 5. To achieve this, suitable substitutes, prepared with a clinically valid and recognised method, must be found. The great problem in meeting the objectives for cell therapy, in cases where the culture has been prepared in a serum-free environment, has been approached with the aim to utilise compatible donor/autologous sera [21]. A detailed biochemical analysis of this serum-substitute needs completing and to be approved by relevant bodies for clinical use. Finally, use of components in the CPA media, with a high potential of negative downstream effects, will either stop or be proven without doubt, to have been eliminated before implantation [19, 20, 22].

2.3.1. Cryoinjury

Primarily, injury to cells during cryopreservation manifests itself in the following forms:

Chilling injury

Much of the early evidence that support this theory came from the studies on membrane sensitivity to chilling of the plant cells [23]. When cells are chilled from ambient temperature to the point of freezing, without actually freezing, injury may occur. Not all cells are susceptible to such injury, but susceptible cells show symptoms only when cooling is rapid. Slowly cooled cells are not affected significantly.

When injury does occur, it is primarily in the cytoplasmic membrane and cell wall. This damage could well have resulted from the phase transitions that occur in the membrane lipids. It is most likely that the rapid transition through the melting point of the lipids causes hydrophilic channels to form in the membranes, therefore allowing leakage of solutes. In the case of slow chilling, the lipid chains are able to rearrange and prevent formation of the hydrophilic channels. This is explained by the fact that lipid perturbers, such as adamantane and 2,4-ditertiary butyl 5-hydroxytoluene (increases membrane fluidity), increase the survival rate of fast-chilled cells [24]. However, this mechanism is believed to be less significant in human cell cryopreservation.

Freezing-thaw injury

As cells are cooled to approximately -5°C both cells and surrounding medium remain unfrozen and super cooled. Between -5°C and approximately -15°C , ice crystal forms. The freeze-thaw injury occurs at this stage and the degree of injury is highly related to the cooling rate. The effect of the cooling rate has been well established by a number of researchers[25]. Most of the work has illustrated that an optimum rate of cooling, which

gives the highest level of cryosurvival, exists, although it can vary widely between cell types. Figure 2.1 shows the effect of the cooling rate on the survival of a number of cell types. An optimum rate is clearly visible for all cell types.

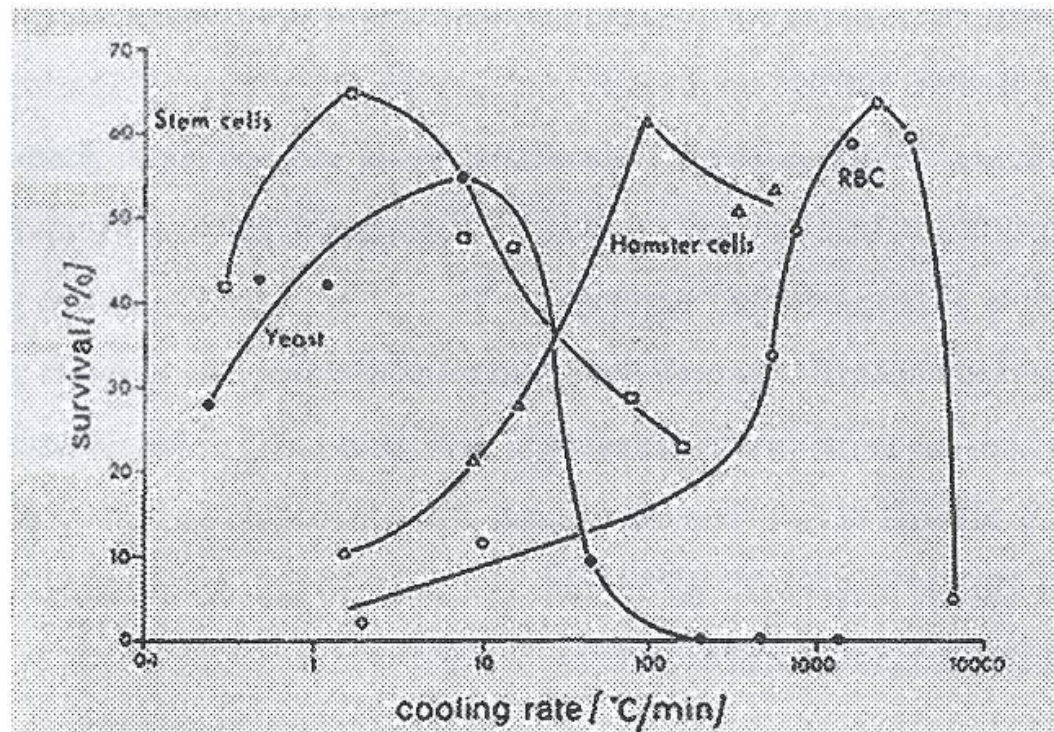


Figure 2.1 Comparative effects of cooling rate on the survival of various cell types cooled to -196°C and thawed rapidly[25].

The fact such an optimum freezing rate exists has led to the suggestion that the degree of damage at a very low and high cooling rate processed by different mechanisms involves Mazur's "two factor hypothesis"[25]. At the sub-optimal rates of cooling, cell death was a result of one single factor, termed by Mazur as "solute effect". At the supra-optimal cooling rate, cell death was a result of another factor, intercellular ice formation.

- Solute effects

During slow freezing, ice forms extracellularly, while intracellular water has time to diffuse out of the cell. This means that the extracellular medium is becoming more concentrated; the cell is thus being exposed to an increasingly hypertonic solution, and is continually losing water and shrinking. If the cell is exposed to such conditions for a prolonged period (e.g. during slow cooling), cells become dehydrated and cellular injury may well occur, especially to the cell membrane, as a result of a build-up of high stresses. This is termed as the solute effect.

- Intracellular ice formation

At very fast cooling rate, intracellular water becomes increasingly supercooled and cannot maintain equilibrium with extracellular water since it cannot diffuse out from the intracellular environment quickly enough. Thus the intracellular water freezes to achieve equilibrium. Depending on the rate of the cooling rate, the ice formed can consist of two forms: vitrified (very fast cooling) or crystallised (slower cooling). If ice crystals are formed, they may kill cells if the crystals are sufficiently large, due to the fact that many cellular sites are susceptible to mechanical damage if the size of the ice crystal exceeds a certain threshold. If vitrification occurred, a slow warming rate during thawing will result in substantial cell damage. This is because small crystals are unstable and if given time, will aggregate to form larger crystals. This re-crystallisation process will damage the cells as described above, giving the ice crystals less time to grow and thus reduce damage to the frozen cells.

Other forms of injury

Injury to cells may also occur due to the stress caused by extracellular ice. The expanding of ice field squeezes the cells and causes damage to the cell membrane, causing lysis and ultimately death.

2.3.2. Cooling

To meet all the aims and objectives of a successful cryopreservation process, it is vitally important to fully understand the thermodynamic aetiology of the given sub-processes carried out. Due to the fact that the biological media are essentially H₂O-based, the essence of this section revolves around the effects caused whilst freezing water. This action can be sub-divided into two parts with emphasis focused primarily on two vital temperature junctures. The two identified temperatures reached, of major interest, are: equilibrium melting point/ max heterogeneous nucleation temperature (T_m) (nearing 0°C); homogeneous nucleation temperature (-40°C) at 1 atmosphere pressure (protocols including vitrification that are directed at escaping crystallization introduce a further temperature point of interest, namely the glass transition temperature T_g).

Figure 2.2 illustrates the melting and homogeneous nucleation temperatures concerning the prescribed solution of the water based cryoprotectants. Both temperatures indicated merge as the cryoprotectant concentration increases until the two come together at the point of glass transition (T_g) this clearly portrays the emergence of an amorphous phase. This convergence of T_m and T_h occurs once the cryoprotectant concentrations approach the emergence level of 80%. Once there is a temperature drop, the water molecule kinetic energy falls rapidly. Once the temperature nears melting point, the anticipated reaction of

water molecules depends upon if the solution nucleators are present. If it is proven that the ice nucleators are indeed presented, freezing will commence at about 0°C as the temperature decreases. Nucleators take the role of sites for the attempted growth of ice crystal. Since the process itself is undefined, it is presumed that the nucleators produce the surface for the collection of water molecules to form an identified ice-crystal lattice [26]. This interaction coupling the molecules and the nucleator surface will accelerate the creation of ice crystals. This also may well be linked to the angle of contact between the two elements [16]. In contrast, lower volumes of water, on the whole, do not freeze approaching or once they have reached the actual melting point due to the fact that they rarely have nucleators. Cell volumes are low, so, they tend not to contain nucleators and hence their temperature can be lowered to well below that of the heterogeneous nucleation, as close as possible to -40°C avoiding ice crystallization [27, 28]. In cases where nucleators are absent, ice crystal initiation coupled with ultimate growth are greatly reduced and the initial clear phase change will usually take place at -40°C [29]. This reduced temperature is commonly known as the “homogenous” nucleation temperature (T_h) because the crystal lattice formed consists entirely of water molecules. The phase alteration at T_h is caused by the reduced energy needed for the sustenance of nascent ice crystal surfaces, as the kinetic energy present for molecular diffusion is reduced, the viscosity of the emerged solution increases [30].

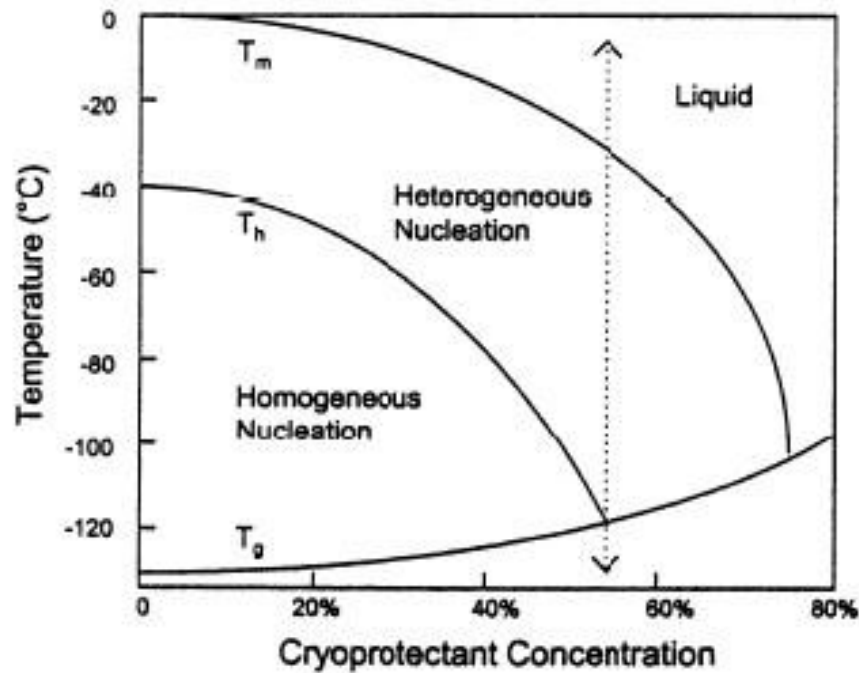


Figure 2.2 The melting (T_m), homogeneous nucleation temperature (T_h) and the glass transition temperature (T_g). Image redrawn from Dr. Sammy Sambu's work, Institute of Biomedical Engineering, University of Oxford

The T_h , Ostwald ripening is carefully analysed, along with the growth of the larger ice crystals rather than the smaller nascent ice particles [31]. Due to the fact that the smaller the particle is, the higher surface-area to volume SA/V ratio is present, these particles tend to produce a higher energy. On the other hand, bigger particles will portray a lower SA/V ratio and reduced energy. Therefore, there is observed a significant activity of water particles moving from the higher energy area (the smaller particles) directed towards the lower energy area (the larger particles). One reason for this is explained by the Gibbs-Thomson effect, related to partial pressures to a given volume/surface curvature [32]. Generally, past the T_h , alginate, sample temperature can be reduced to -80°C at a recognised

cooling rate and then stored at this particular temperature or moved to frozen nitrogen (-196°C) if a long term storage is required.

Recognising the impact of the freezing procedure on a cellular metabolism allows researchers to continually re-examine the cellular environment in the correct way. Any biochemical reactions that define cellular metabolism are initially calculated to last, until the T_h [16]. Observed water movement viewed from 0°C and -40°C has not, been slowed severely to have an effect upon the kinetics of the metabolic processes. It can be stated that using the suspension medium, hypothermic storage of any living tissues stored within this set temperature range must provide for the metabolic processes facilitated by kinetics of the movement of water [16, 33, 34]. It is, therefore, vital to provide the necessary reactants for these given processes whilst they are carried out. In all cases, metabolic processes will, in the end, stop functioning at cryogenic temperatures. At the temperature of -196°C, all metabolic reactions are detected due to the fact that any thermal energy needed for the continuation of chemical reactions are not available [12]. At this temperature, the molecular diffusion has been suspended; the only quantifiable damage sustained by cells at this temperature is damage to DNA sustained at a macromolecular level due to the ionizing radiation [1, 12].

2.3.3. Effects of Ice Formation

The location of ice crystallisation, intracellular or extracellular, is highly depended on the freezing protocols of the cryopreservation process. Controlling of ice crystal formation is vital for determine the success of the cryopreservation. Originally, the growth of

intracellular ice crystal was considered as the major mechanism attribute to cell death during cryopreservation process. But now it is generally accepted that the cells can maintain their viability when the intracellular ice growth does not exceed a certain level [35, 36]. For example, intracellular ice crystals not larger than 0.3 μm can be exist within human monocytes without affecting post-thaw survival [37]. Beyond the certain threshold, the growth of the intracellular ice becomes lethal to cells during the freezing process. When the expansion of the ice crystal is uncontrolled, the plasma membrane or the intracellular organelles can be physically damaged [16, 38]. Such damage is irreversible and certainly leading to cell death. Also, due to the crystallization of the intracellular liquid, the cytosolic salt concentration will be dramatically increased. Meanwhile, the hydrogen bond within the structures of the proteins is decreased due to loss of water molecules. Both effects will lead to protein denaturation, osmotic and solvent fluxes [39, 40]. Intracellular ice may also cause unwanted phase transition of the intracellular lipid fraction [41]. It is vital to note that these mechanisms are only a sample of the downstream effects of the intracellular ice formation. Other types of cellular damages induced by intracellular ice formation certainly exist [42].

Extracellular ice formation during cryopreservation process, as another main mechanism of cell damage, can also lead to cell death. For example, the increase of extracellular ice formation is normally accompanied by the increase of the salt concentration in the extracellular medium, which will result in cell dehydration due to the difference of osmotic pressure [43-45]. Moreover, the direct contact between extracellular ice and cells can cause rapid mechanical deformation of the cells [46]. While thawing, the extracellular ice crystal

formation will encourage the re-formation of the intracellular ice crystal hence introduce damaging effect to cryopreservation outcome [12].

2.3.4. Optimization of the Freezing Rate

Seeking the optimal freezing protocols for a given cryopreservation process is one of the primary objectives for all the researchers since it is highly related to the cryopreservation outcomes. In general, the adopted freezing rate can be classified as fast freezing and slow freezing. With a high rate of cooling, the intracellular ice formation is inevitable and is the main reason led to cell death. Since the water molecules is the main source of the hydrogen bonds that can provide support to the structures of cell organelles, the fast growth of intracellular ice crystal will result in loss of such hydrogen bonds hence cause cell membrane damage, protein denaturation and unwanted lipid phase transition [47]. Conversely, at very low freezing rate, extracellular ice will be the dominated factor. Giving the modest volume of the intracellular liquid, the nucleators that can cause water crystallization rarely exist in intracellular environments. Such lack of nucleators will greatly limits the speed of intracellular ice growth. Thus, the faster formation of extracellular ice will result in the increase of the concentration of the extracellular medium hence create a net osmotic pull. Driven by the osmotic force, the intracellular water will diffuse out and move into extracellular medium, leading to cellular dehydration [1]. A figure illustrating the effects of both fast and slow freezing is provided in Figure 2.3.

The freezing rate can be further classified into another two methods, namely controlled-rate (slow freezing) and ultra-rapid freezing. These particular methods diverge after the

extracellular ice formation is seeded and stable [12]. Typically, the slow freezing adopts -1 °C/min as the freezing rate. It can be learnt from Figure 2.3, the slow freezing is about the rate read off the leftmost side of the survival curve and possess the ability to maintain the cell survival to the highest rate. On the other hand, ultra rapid freezing will often be carried out when fastest cooling rate is available and the CPA concentration is gradually increased whilst the sample size is shrinking until the optimum cell survival rate is attained [48]. For example, the cryopreservation of oocytes was carried out by submerging a thin nylon loop with a firm containing the mixture of CPA and oocytes suspension in liquid nitrogen [49, 50]. This method adopted the ultrarapid freezing, known as cryoloop technique [51]. Consider the advantages and disadvantages of slow and fast freezing rates, a hybrid method of amalgamation of these freezing rates is being investigated. For instance, nonlinear or piecewise linear freezing rate have already adopted in the area of cryopreservation [12]. They have exhibited good cryopreservation outcomes. Generally, the freezing rate for different cell types should be carefully considered in order to obtain high-quality or desired cryopreservation outcome.

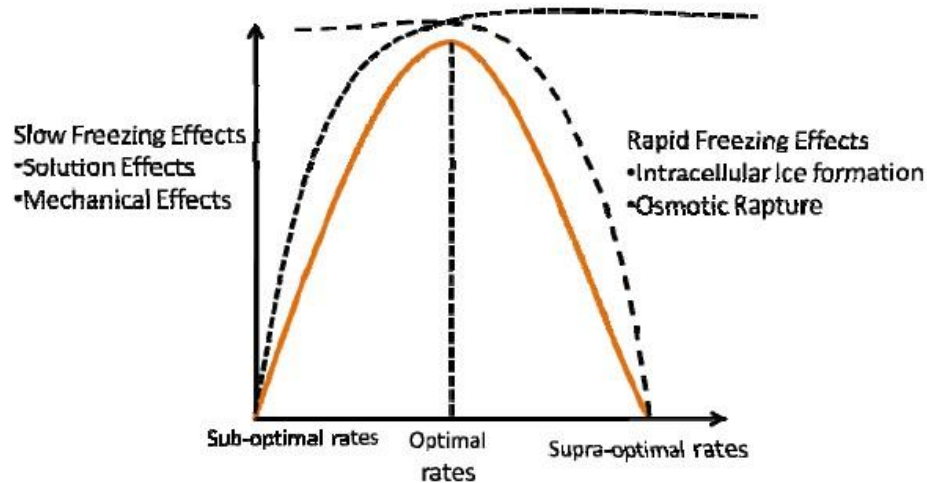


Figure 2.3 A survival-cooling rate profile of a hypothetical cell demonstrated the onset of cell death at exceedingly low and exceedingly high cooling rate. At very low cooling rate, high intracellular salt concentrations and mechanical deformation from in growing ice crystals will lead to cellular damage. At very high cooling rate, osmotic flux and intracellular ice formation will lead to low fraction of surviving cells. Image redrawn from previously published work [12].

2.3.5. Thawing and CPA Removing Process

The thawing should be carried out immediately after the samples are removed from the cryogenic environment, i.e. the freezer and the liquid nitrogen tank, to enable controlled thawing. Normally, the samples are directly thawed in a 37°C water bath till nearly fully thawed with little ice crystal within the cryovessel. In order to allow the cryopreserved cells recover to isotonic solutions and elevated temperatures, the recovery medium should be introduced to cell suspension gradually, sometimes with gently shaking of the samples, to dilute the CPA. With such implementation, the osmotic shock can be minimized. Eventually, the CPA was removed entirely by replacing with fresh medium.

2.4. Constituents of the Cryoprotective Agents

In order to obtain a greater understanding of the cryopreservation process, it is necessary to investigate the cryoprotective agents used. Proper choice of cryoprotective agents is crucial because there are a few factors that might cause cell damage. Ice crystal might grow inside or outside the cells, and there might be water permeating through cell membranes, causing mechanical damage, toxic concentrations of solutes across the cell membrane. Cryoprotectants, lyoprotectants and nucleators can be applied to minimize the damages mentioned above, and this section will focus on the working mechanisms of these cryoprotective agents, giving some basic rules of how to choose them and maximize their protecting functions in the cryopreservation process of cells.

2.4.1. Cell Membrane Permeable Cryoprotectants

The basic principle of cryopreservation is to inhibit intracellular and extracellular crystalline ice forming, by using cryoprotectants [52, 53]. Cell membrane permeating cryoprotective agents (CPAs) are organic solvents which can permeate into cells and function as cryoprotectants by inhibiting the ice crystal forming. Another feature of this type of CPA is that they usually have outstanding cell membrane permeation capacities. One of the most widely used cryoprotectants, dimethyl sulfoxide (DMSO) is one example of this types of CPAs. Other representatives of CPAs include glycerol, hexylene glycol and propylene glycol. The crystal inhibition basis of CPAs relies on their special chemical structures. They usually have hydrophilic and hydrophobic centres. The hydrophilic areas can act as hydrogen bond acceptors, while the hydrophobic centres which do not interact

with water molecules will disrupt the formation of an ice crystal lattice during freezing. These CPAs benefit from their ability to penetrate cell membrane freely, which can inhibit the formation of intracellular and extracellular ice crystals during freezing process. They have the dual effect of encouraging vitrification instead of ice crystallisation as well as depressing the freezing point of intracellular water. In addition, these CPAs also reduce “solute effects” injury by encouraging the formation of a gel type glass within the cell, thus reducing the efflux of intracellular water. This translates to less shrinkage and thus less stresses in the cell membrane. [52, 54]. Similarly, the viscosity of the freezing medium was increased by CPAs, and this effect can interfere with water molecules movement [12, 55]. As a result, the initial water molecule required for ice crystals growth will be exhausted [55], and hydrated proteins provided they are excluded from the hydrated protein structure will be stabilized [12, 56, 57]. The stabilization of protein structure is temperature-dependent. The lower the temperatures are, the stabilization effect of these CPAs will be better. However, as temperatures increase, proteins will be gradually denatured.

The acting mechanisms of dimethyl sulfoxide (DMSO) of protecting cells in cryopreservation is closely linked with its ability to permeate the plasma membrane faster, but the cytotoxicity of DMSO is a major disadvantage of its application. As Figure 2.4 illustrates, DMSO is toxic to cells with a concentration higher than 12%. This is the reason why some experiments adopt 1M as optimum DMSO concentration during cryopreservation process. Moreover, cells that are exposed to DMSO for 90 minutes or longer are considered as to have been affected by the toxicity of DMSO. It is worthwhile pointing out here that the CPA toxicity is cell specific.

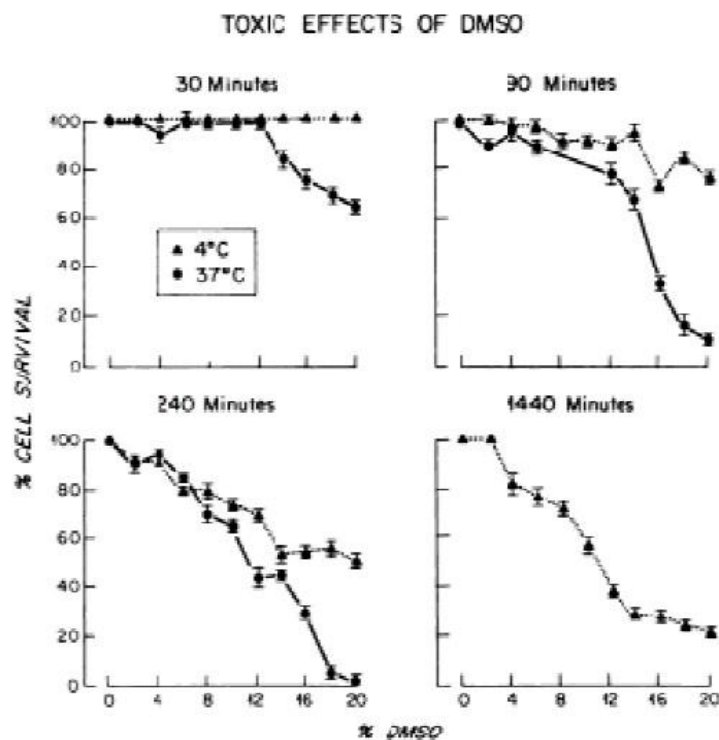


Figure 2.4 Toxicity of DMSO in terms of concentration and exposure time. The Cell type that used to demonstrate these results are bovine articular chondrocytes[58]. Image redrawn from Dr. Sammy Sambu's work, Institute of Biomedical Engineering, University of Oxford

Another cell-protecting mechanism of DMSO is that its molecular polarity allows it to interact with charges on the cell membrane, resulting in integral stability of the cell structure [12]. Apart from glycerol and DMSO, who are classic permeating cryoprotectants, other permeating cryoprotectants include Trimethylamine acetate (TMA), whose molecular structure allows a fast-penetrating and excellent space-filling of the molecules. However, the disadvantages of TMA include its unpleasant smell, and evidences have shown that it is not widely suitable for a large variety of cells and tissues [15]. One essential limitation of the above ice crystal inhibition hypotheses is, however, there is still no direct proof demonstrating these mechanisms, because there are no proper molecular tags on subject molecule at a molecular and atomic scale currently available [59]. Other types of CPAs

include non-permeable and semi-permeable CPAs. As the name indicated, these CPAs are not freely entering the cells. They are polymers and/or proteins and usually used together with cell membrane permeable CPAs.

2.4.2. Synthetic High Molecular Weight Polymers

By encouraging the exosmosis of water to discourage intracellular ice formation, polymers, such as PEO (poly ethylene oxide), PEG (poly ethylene glycol), can potentially inhibit ice crystal formation, and protect cells in cryopreservation. Compared with simple solutes, like most binary inorganic salts, these high molecular polymers have much smaller contribution to the osmolality of the extracellular solution. Nevertheless, their ability to inhibit ice crystal formation has been confirmed by some research [60]. They can only minimize extracellular crystalline ice formation, since they cannot permeate the plasma membrane.

2.4.3. Proteins

Research has shown that some proteins can protect cells in cryopreservation [61]. One explanation of this effect is that proteins, interacting with membrane-associated proteins and lipids during freezing, can stabilize the cell structure [62]. In the cryopreservation protocols, proteins have mild effects, under the assumption that the extracellular solution does not exceed osmotic limits in the presence of protein. Historically, serum proteins have always been part of cryopreservation solutions, but the poor clarification of their components has limited their clinical applications [63]. As an alternative, purified serum

proteins such as albumin [64] or sericin [63] can be used in replace of serum, to improve cell survival rates in the process of cryopreservation.

2.4.4. Lyoprotectants

Apart from osmotic shock, the possibility of cell dehydration is another fatal challenge which must be considered and minimized. In freezing process, large quantities of ice will form surrounding the cells, and the remarkable efflux of water may lead to an extremely high intracellular solute concentration, which may denaturize proteins and membrane phospholipids in the cells [61]. There are two aspects in which CPAs can play the protecting roles for cells in the above situation – first, they can provide the replacement of the hydrogen bonds, which were previously supplied by water to intracellular proteins, thus stabilize the structure of those proteins. Second, they can suppress the extracellular ice formation, which may prevent further dehydration [65]. The possibility of preventing cell death via the mechanism of dehydration led researchers to look for inspiration from anhydrobiotic cells, by the method of biomimetic engineering. The amazing feature of these cells is that by nature they can produce sugar complexes to resist the death threat in waterlessness. These important sugars include trehalose and sucrose. The cell protecting mechanisms of these complex sugars include to avoid sudden changes between lipid phase and ensuing phase, to minimize the effects of the appearance of a lipid phase transition temperature [66]. Moreover, these sugars may act as a part of hydrated protein complexes, providing replacing hydrogen bonds which previously provided by water molecules [67]. Another suggested mechanism is that these sugars may lower the possibilities of intracellular ice crystal growth by influencing the formation of extracellular ice [68]. The

investigations on trehalose, as well as mixtures of complex sugars such as sucrose and raffinose, pointed out that their anhydrobiotic cell protection capabilities are due to their high glass transition temperature and low potential to crystallize [69]. However, in the formulation for mammalian cells, complex sugars only act as non-permeating solutes [12]. However, incubation of trehalose solutions witnessed some success of a solute accumulation in the cells [61]. After all, since no direct quantified assays of this passive method is available, the use of complex sugars in cryopreservation processes is still considered as mainly based on their extracellular cryoprotective effects.

The discovery of porins and the development of genetic engineering provide alternatives to the above cryoprotection mechanism - to allow extracellular trehalose enter into the cytoplasm by engineered switchable gateways on cell membranes. Two well established and widely used techniques are electroporation and sonoporation. The pores on the cell membrane surface are induced by high energy (high voltage electric pulses or ultrasound) which provides temporary and auto-repairable passages for extracellular molecules, such as trehalose, to pass through the cell membrane. The trehalose-containing cells can be stored at -196°C for several months, implying that trehalose can be a valuable addition to cryopreservation formulation [70]. In addition, previous studies have shown the loading trehalose to routine DMSO treatment can increase cell viability [61]. In summary, the addition of complex sugars to CPA formulations may contribute to a rise of cell survival-freezing rate, and increase the overall cell surviving viability in many current cryopreservation procedures.

2.4.5. Nucleators-Seeding

Although evidences have shown the ice inhibiting effects of cryoprotectants, it is still possible that ice crystals nucleation formed in extracellular medium can cause the crystals growth randomly in other areas of the sample [12]. To decrease this risk, efforts have been applied to initiate the crystalline ice in a more controllable way, with its location and rate of growth more predictable. Not depending on specific chemical or reagent, a particular procedure called nucleators seeding can provide solution to the above issue. Nucleators seeding refers to the deliberate induction of extracellular ice crystals in samples at the temperature slightly below the freezing point of the solution. The principle of this technique is to prevent spontaneous nucleation of ice occurring as a random event at a high subcooling temperature in the medium and the samples.

An example is a chilled needle to be used manually and the growth of extracellular ice at the “seeding temperature” can be initiated. A further cooling stage may be applied. The seeding method mentioned above will significantly lower the possibility of ice formation within the cells, eliminate the randomness of the intracellular and extracellular ice formation and the rates of cell survival can be enhanced consistently [12]. Alternatively, research has pointed out the cooling rate could be optimized in concern of specific cell type, and this optimization could be an efficient way of extracellular ice growth control, saving the need for extracellular seeding. For instance, the freezing protocol adopted by the cryopreservation procedure of human hepatocytes in this study contains a step with a -40°C/min as the cooling rate. Such a high cooling rate or local ‘cold shock’ could act as the seeding process.

2.5. Three-Dimensional (3D) Cryopreservation

Early technical breakthroughs in cryopreservation were concerned with blood, namely red blood cells (RBCs). RBCs exist in suspension and are extremely active when in suspension and suspension cryopreservation has since been deemed as suitable in this case. In recent studies, cryopreservation in the reproductive field focused on oocytes and spermatozoa, both are also attachment-independent. A point to consider, though, is that for a large number of cells separate from somatic tissues, suspension, it has been found that cryopreservation is not the most yielding procedure. It has its severe disadvantages such as significantly reduced yields of cell survival, [71] and alterations in phenotype which were not expected [13]. Most cells attach to a surface (in culture) or to an extracellular matrix (in body). It is worth exploring the cryopreservation of attached cells or encapsulated cells.

Many challenges in the development of an accepted cryopreservation procedure may well be solved by utilising modern techniques in cell immobilization. For example, microencapsulation creates a platform technology, capable of fulfilling some requirements for both a merchandisable and a calculable cryopreservation system. In addition, it is able to provide an environment where cell survival is very encouraging with the use of regulating biotic/biochemical fluxes spanning the 3D matrix. Microencapsulation will certainly aid in the feeder-free culture of stem cells [72, 73]. Also, microencapsulation controls heat and mass fluxes [72], providing a protection from microbial contamination [72] as well as protection from uncontrolled extreme conditions whilst processing such as shearing [72, 74]. Also, a 3D matrix has been studied and proven to create a barrier against ice crystal formation [75], hypothesized to support the cell stabilization membrane from

undue stresses in cryopreservation [71] and finally help to update water permeability and cell nucleation parameters through the cytoskeletal rearrangement and modifications in cell morphology [12, 71].

All the advantages of 3D cell cryopreservation may well be further improved by encouraging cells to bind to the 3D scaffold. Attached cells have a higher survival rate when compared to cells trypsinized in suspension [71]. Water permeability parameters being higher and the water permeability activation/energy coefficients, means that attached cells exhibit increased water permeability coefficients, they also equilibrate at a quicker rate due to the ever changing state of the extracellular medium whilst freezing [71]. It therefore translates itself to the published general operation ranges relating to both freezing rates and loading periods [71]. Attachment also makes sure that that cells gain anti-apoptotic indications from the substratum, hence lowering the rates of apoptosis whilst the loading process is performed, after thawing and re-suspension.

Methods adopted, up to this point, cell encapsulation can be achieved by any of the following: manual, electrostatic ejection [76, 77], centrifugation whilst the process of cross-linkage is carried out [78], the piezoelectric droplet generation [79], aerodynamically aided bio-jets (AABJ) [80], finally the use of cutting implements [81, 82]. Since rapid advancements in technology concerning encapsulation have allowed for creation of constructs within the micrometer scale, any cell-material composite size for cryopreservation is after limited at approximately 300 microns for practical reasons [83]. At the higher end, requirements to limit exposure to CPAs because of their cytotoxicity and also to limit temperature differentials whilst cooling results in the fact that construct size is

set at a limited diameter of less than 2 mm, due to cell volume transients differing for cells at differing areas in the construct [84].

Expressed potential problems concerning oxidative stress, hydrodynamic stress, creation of ice crystal nucleation and growth/freeze concentration have been generally met with concentration on cell survival following the process of cryopreservation. On the other hand, when stem cells are cryopreserved, any stemness and potency must also be conserved during cryopreservation. The CPA solution must hence offer a correct chemical environment in order to avoid the induction of signals concerning terminal differentiation aimed at the given specimen. The non-differentiating chemical environment, certainly varied, is dependent upon the cell. For instance, samples of mouse embryonic stem cells (ESCs) may keep their pluripotency whilst being cryopreserved in suspension [85] whereas human ESCs, a proven fact, can maintain their pluripotency once cryopreserved in an attached condition (see Matrigel) [61, 73]. The mechanism used in the employment of ECM signalling in the process of cryopreservation is deemed as a factor in anti-apoptotic activity.

Alginate is commonly used for cell encapsulation. It is a family of polyanionic copolymers derived from brown sea algae. In the molecular terms, the monomers of the alginate are arranged in a pattern of blocks along the chain (Figure 2.5). G-block is the most important structure feature for alginate gel formation because divalent cations, for instance Ca^{2+} and Sr^{2+} , are prefer to bind to G-blocks in a highly cooperative manner [27].

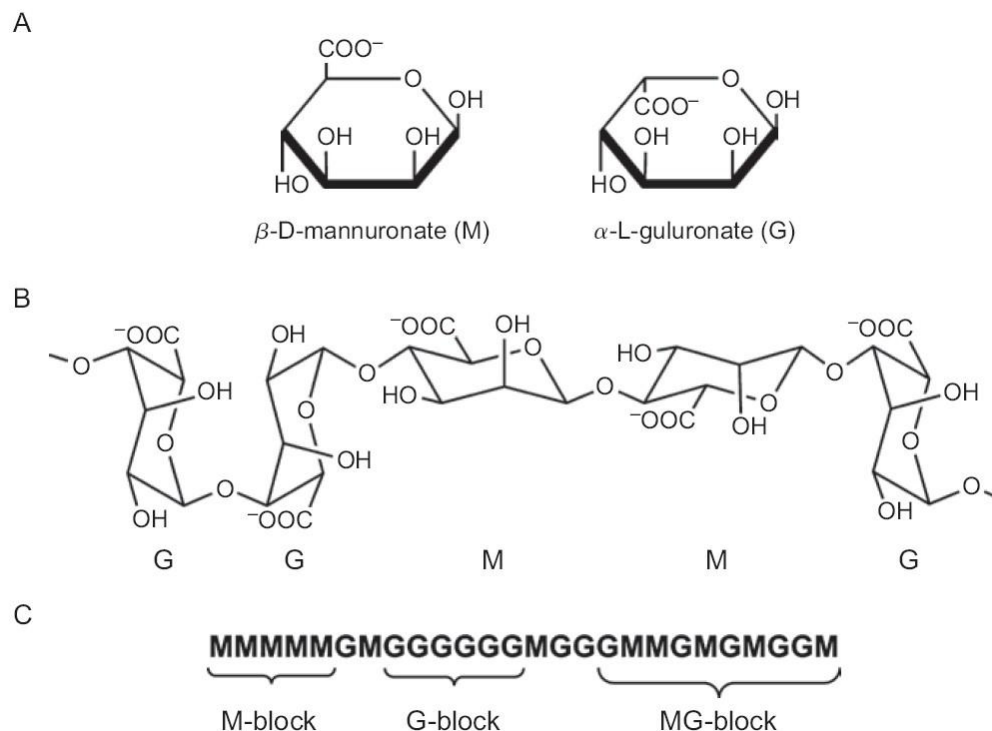


Figure 2.5 Structure of alginate: (A) alginate monomers, (B) chain formation, and (C) block distribution [86, 87].

Image quote from Selimogly and Elibol et al.[88]

Amongst many varied techniques involving 3D cell culture, alginate has become the most widely used method by immobilizing living cells [89, 90]. Alginate gel beads were generally considered to be the most versatile and promising method for cell immobilization and culture methods for both cell lines and small organs [91, 92]. The cells encapsulated in alginate can be easily recovered. The wide application of alginate was because of the “single-step process” and the ambient environment provided [27, 93]. Thus these characteristics resulted in alginate gel being compatible with most cell lines [94-96].

The concentration of Na^+ -alginate and CaCl_2 will have an effect upon mechanical porosity and strength of alginate gel. Higher alginate concentration results in increased stable and convenient bead structure for a long-term cultivation [27]. However, lower concentration of alginate leads to increased diffusional characteristics but at the same time also results in a reduced stable time due to the destabilization of the beads. In the view of to Stabler et al. [27], in order to satisfy sufficient bead stability and maximum mass transfer, the optimal concentration of alginate should certainly be in the range of 1% to 1.5% (w/v) [27].

2.6. Targeted Cell Types

Cryopreservation outcome depends on cells types and often the protocols are cell specific. This research targets important cells, so that the outcome and results can make a significant impact. For this purpose, two types of cells are targeted for cryopreservation study – human embryonic stem cells for regenerative medicine application and human primary hepatocytes due to their importance in drug testing applications.

2.6.1. Human Embryonic Stem Cells (hESCs)

The successful derivation of human embryonic stem cells traces back to the year of 1998, carried out by scientific studies in the US led by James Thomson and John Gearhart [97]. Since then, the applications of hESC have been pursued by many researchers. The most valuable property of human embryonic cells is that it can be “culture in vitro”, expanded in number indefinitely in the undifferentiated state characteristic. This means that hESCs

cultured in vitro still possesses the ability of developing into any cell type in the human body. With such incredible properties, the hESC has been used for a variety of purposes, such as novel approaches to the study of human embryonic development and disorders, gene expression for biological data mining, tissue regeneration and repair, creating disease models in vitro to assist basic research and drug discovery and toxicology [98].

With the growing demands of hESCs, banking of large quantity of hESC, where straw vitrification becomes impractical, is becoming increasingly important. Cryopreservation of human embryonic stem cells is problematic due to low cell survival rate, poor cell health, a high rate of apoptosis and spontaneous differentiation following recovery. An optimized cryopreservation protocol in order to overcome the above negative issues is urgently required. Given the great importance of hESC, new method of hESC cryopreservation or optimisation of the existing protocol, which can lead to better post-thaw cell quality, is in great need of investigation.

2.6.2. Human Primary Hepatocytes

Human primary hepatocytes are generally considered as a valuable experimental resource both in academia as well as for commercial purpose. The important roles played in both the drug toxicity study and new medicine development areas are self-evident. For instance, human hepatocytes are used in every stage of emerging drug development processes, ranging from early screening of the most effective new chemicals, to the determination of important properties of drugs for new medicine applications [99]. In addition, with recent advances in surgical techniques, human hepatocytes can be used for cell transplantation as a

method of treatment for many end-stage liver disease and liver-based metabolic disorders [100, 101]. This application of human hepatocytes benefits from being inexpensive and less invasive [101].

Whether the requirement is a large population of cells for drug development or the immediate availability in cell transplantation surgery, it is required that human hepatocytes are well preserved. Thus, the development of the optimum cryopreservation protocol for this particular cell type is vital. Due to the high occurrence of cell apoptosis and fragile cell condition, the cryopreservation of human primary hepatocytes has been always challenging and is a problem unsolved.

Actually the isolation and purification of human primary hepatocytes from fresh liver tissue samples is the first challenge. The liver tissue samples are usually obtained from liver or cancer surgery. The condition of the tissue sample may well be varied. The samples are either different in size or from different segments of the liver. Some of the liver samples are from severe fatty liver. All types of liver samples listed above could cause problems in the isolation procedure, which is impossible to predict. In addition, the quality of freshly isolated human hepatocytes is highly dependent upon the pre-exciting condition of the liver tissue. For instance, liver tissue from patients who have undergone pre-surgery radiation therapy or chemotherapy normally lead to poor cell function; also, liver tissue from fatty liver always leads to difficulties in separating normal hepatocytes from fat cells. Consequently, different liver tissue samples require different treatment with proper isolation protocols in order to maximum the isolation efficiency and more importantly the hepatocytes functions.

2.6.3 Human Colorectal Cancer Cells (DLD1)

The two targeted cell types are important, but expensive and difficult to obtain in large quantity. To test developed technologies and protocols, an inexpensive easy-to-get cell type is needed, and the human colorectal cancer cells line (DLD1) is used for this specific purpose. The human colorectal cancer cell line is commonly used in cancer drug testing and in vitro tumour model development. Furthermore, the difficulty of cryopreservation concerning this cell line is relatively low. However, its cultivation has dual advantages of low requirement of maintenance and high cell growth rate. A large population of high quality cells can be obtained in just a number of days. Given large number of cell samples, it has the great advantage to be used as an experimental model for studying novel cryopreservation techniques, such as electroporation and sonoporation.

2.7 Sonoporation

Sonoporation refers to the technique of utilizing high frequency sound or ultrasound in order to make cell membranes temporarily permeable to chemicals or particles to which they are naturally impermeable, such as genes, drugs, etc. [102]. Though the diagnostic applications of ultrasound are well established and widely used, its therapeutic applications are more recent but have managed to revolutionize a number of medical and biological methods: ranging from therapeutic treatment (e.g. High Intensity focused ultrasound which is used for cancer ablation), drug delivery, sonophoresis and also numerous industrial applications. Due to the development of this technique, there have clear evidences that the

application of ultrasound to cells enhances the uptake of large molecules, e.g. DNA, that is used in gene therapy [103]. In general terms, enhancing the permeability of cell membranes is achieved by “punching holes” on the cell walls. The pores that are created by the application of ultrasound are transient and recover automatically after a short period of time. The consequences of applying ultrasound to these cells are variable and some are neither acceptable nor tolerable. Moreover, some of the consequences are harmful to the cell and affect their viability and re-culturing. Cavitation induced by ultrasound is considered as the major cause of cell death and dysfunction, a concept which is generally accepted by most researchers [104].

2.7.1. Cavitation

Cavitation is termed as vapour bubbles formed under the influence of fast flowing liquid where the pressure it creates within itself is lower than the surrounding medium or liquid [105]. This phenomenon can be found in water and a cavitating propeller model could be a good explanation of this phenomenon. Although the physical process of cavitation is similar to boiling, the vapour bubbles formed in cavitation have vital differences compared with the vapour formed during the boiling process. In boiling, water suffers from high temperature heating and vapour forms on the surface of the bottom of the water container. This extremely high energy forces water to change its phase from liquid to gas and rise to the surface. The pressure of the vapour formed at the onset of the boiling process is higher than its local ambient pressure. In contrast, the pressure of vapour in cavitation is lower than its local ambient pressure. Hence, the thermodynamic paths that precede the formation of the vapour are essential.

Cavitation can be further divided into two parts - inertial cavitation, (transient cavitation) and non-inertial cavitation, (stable cavitation).

In the 19th century, the inertial cavitation effect was initially studied by Lord Rayleigh [106]. He defined that the formation of the cavity was due to the rupturing of liquid whilst exposed to a sufficiently low pressure. Following this, more recent researchers discovered that there are alternative methods that can generate cavitation voids such as intense focused laser pulse (optic cavitation) or electrical discharge through a spark or in the presence of an acoustic field [105]. Consequently, the ultrasound is adopted in this project to generate an acoustic field with high intensity. In inertial cavitation, the cavity is not in a complete vacuum. By increasing the pressure of the surrounding liquid, the vapour gas bubbles will inevitably collapse. It always requires an acoustic field with a high intensity in order to create inertial cavitation [107]. When the intensity of the acoustic field is relatively low, the cavitation phenomenon is non-inertial than inertial. Non-inertial (stable) cavitation activation generally just requires a relatively low level of ultrasound intensity to activate a pre-existing gas body, which may be stabilized by solid structures and may also be, approximately, of resonant size before the onset of ultrasound exposure [108, 109]. Compared with inertial cavitation, non-inertial cavitation is considered as a safe process since it results in significantly less erosion and it is adopted when delicate materials are required to be cleaned, such as silicon wafers.

2.7.2 Mechanisms of Ultrasound-induced Bubbles

The bioeffects, regardless of whether they are positive or negative, are products of the ultrasound-induced bubbles' "form-collapse" process. In the presence of ultrasonic field, the bubble will initially grow in size and then rapidly collapse. As a detailed explanation, the bubble will implode violently rather than simply collapse. Differing intensities of ultrasound result in varying bubble behaviour. This is also the key feature to distinguish the inertial and stable cavitation.

Figure 2.6 perfectly explains the behaviours of the bubbles. At the onset of the cavitation effect, the bubbles will grow at every negative portion of the ultrasonic wave and shrink slightly at the positive portions. This is due to the surface area for the dissolved gas to enter the bubble during the expansion phase being greater than the surface area available for gas to diffuse out of the bubble during the compression phase, a process known as rectified diffusion[110]. The bubbles commence oscillation under the low-intensity ultrasonic field. The diameter of pre-existing bubbles will steadily increase until it reaches a resonant diameter, accompanied, at the same time, by the accomplishment of stable oscillation. With high-intensity ultrasonic field, the bubbles will form and grow rapidly during the first several wave cycles. After a period of time, the inertial energy, which exists in the surrounding medium of the bubbles during the compression wave cycle (positive cycle), become so great that they cannot reverse direction when the next expansion cycle (negative cycle) has an effect [111]. The bubble will implode violently by the inertial energy, which continues to invade the bubble. This implosion is accompanied by local heat, shock wave

and free radical productions. Quantitatively, the temperature could be several thousand Kelvin and the pressure could be several hundred atmospheres[111].

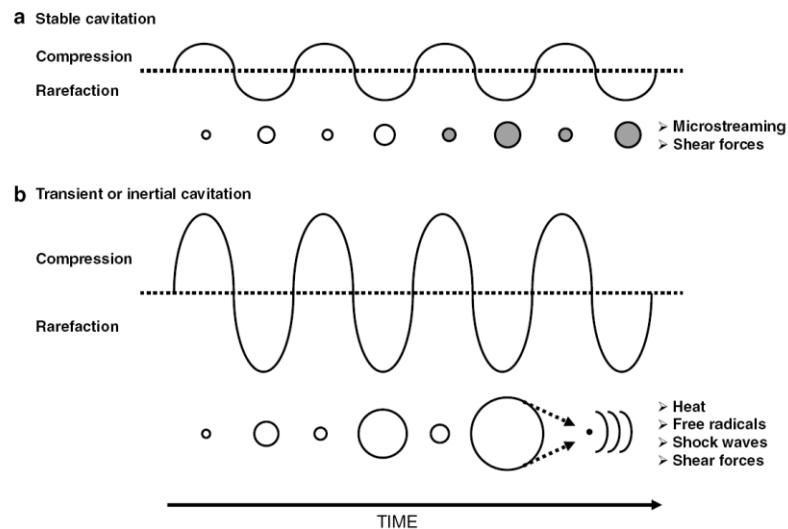


Figure 2.6 Behaviours of ultrasound-induced micro bubbles. (a) The stable cavitation under relatively low ultrasound intensity. (b) The inertial cavitation under high ultrasound intensity [111]

It would not be surprising to learn that the inertial cavitation releases much more energy than stable cavitation. Simultaneously, the inertial cavitation causes more bioeffects when ultrasound is applied to cell suspension rather than stable cavitations. Although some of the bioeffects are undesirable and potentially harmful, the cell poration (reversible permeabilization of cell membrane) is the fundamental point of departure for this project. Thus, the object of sonoporation is to achieve reversible permeabilization of cell membrane whilst minimizing the remaining undesirable bioeffects to keep the cell in a high viability state [104].

2.7.3. Exposure System

The exposure system is the practical implementation method to apply ultrasonic field to cell suspension in order to achieve bioeffects on cells. Careful consideration is required when the researcher designs this exposure system. A number of systems for exposing cells *in vitro* are proposed and adopted by many pioneers in this area which provides dependable and accurate references. MW Miller, DL Miller and AA Brayman [112] reviewed a number of exposure systems utilized in the past decades. All the systems are illustrated in Figure 2.7 below.

The container of the cell suspension itself also influences the character of the ultrasound exposure. Typically, vessels and Petri dishes are used to carry out these experiments. For plastic material tubes, due to the existence of acoustic impedance differential between the tube wall and the contents, these tubes normally result in a standing wave field within the sample under continuous or semi continuous-wave sound field. [113] A standing wave is considered as a cause of the segregation of cells from lytically effective bubbles [114]. Moreover, a standing wave can cause problems in ultrasound dosimetry [115]. A substitution could be initiated in order to avoid the standing wave and sonolucent material containers are ideal alternative choices. Differing researchers have confirmed the same phenomenon, which is that the vessel walls could cause cavitation nuclei and subsequently affect cells [116] [117] [118, 119].

The air/liquid surface could create the perfect reflection of the ultrasonic wave. As illustrated in no. 6-8 schemes, Petri dishes are placed directly on or under the transducer. With the medium in the Petri dish, a perfect stationary wave can be obtained.

The rotating system is popular among researchers. This system benefits from enhancing the bioeffects induced by ultrasound by avoiding cell accumulation at microbubbles-free areas [119]. This circumstance frequently happens in stationary systems. In addition, rotating can help to increase fluid nucleation [117, 118, 120].

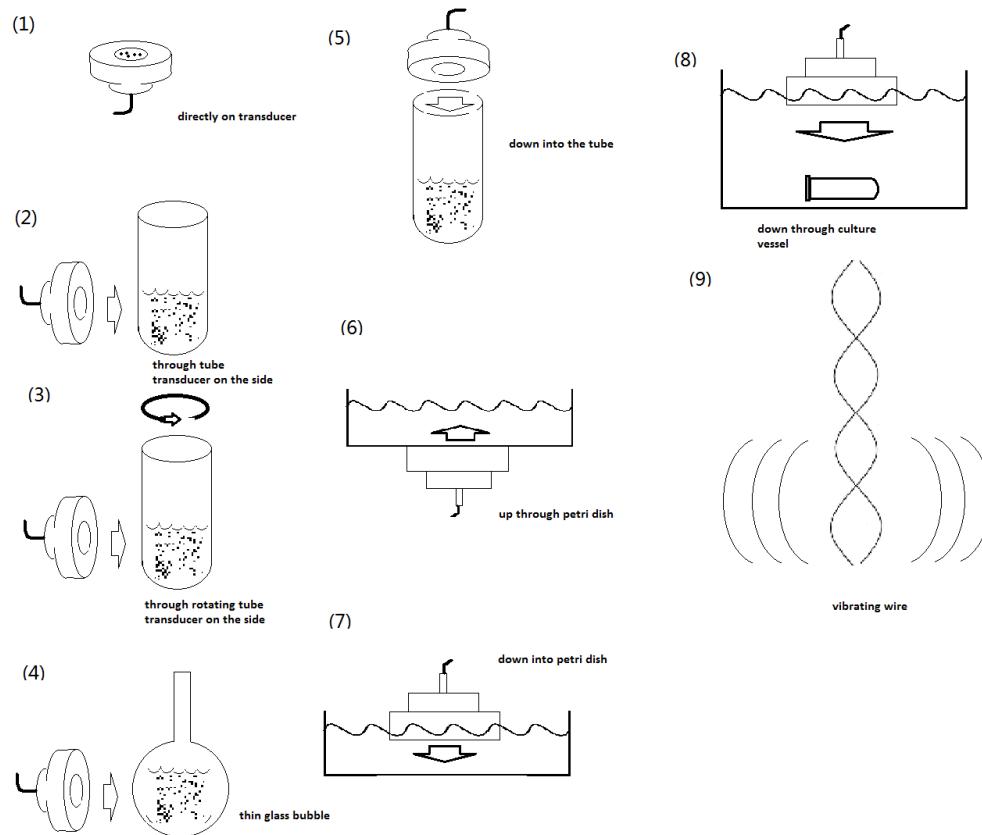


Figure 2.7 Typical ultrasound exposure systems. (1) [121], (2) [122], (3) [115], (4) [123], (5) [124], (6) [125], (7) [126], (8) [127], (9) [128]

2.7.4. Ultrasound-induced Biological Effects *in vitro*

In general, the biological effects of ultrasound can be categorized as thermal and nonthermal [129]. As the behaviours are explained above, one of the ultrasound-induced products is heating which is the dominating effect of thermal bioeffects. In theory, if the temperature increases by more than a few degrees above normal cell suspension due to heating and micro bubbles collapse, this would perturb biological systems and also affect cell viability to some degree. In fact, thermal mechanisms only play a minor role in cell permeabilization. A simple water bath could perfectly control the local temperature of the cell suspension. Any other bioeffects without significant temperature increase (i.e., less than about 1 degree), are considered as nonthermal mechanisms. The nonthermal mechanism can be further divided into the cell lysis effect, structure/function effects or effects on DNA. Typically, cell lysis refers to cell membrane which is ruptured. This is the reason why cell membrane permeability increases, but also why it can lead to cell death due to the fact that the poration of cell membranes is permanent. Cell lysis can easily occur in the presence of ultrasound-induced micro bubbles causing collapsing and inertial cavitation. An increasing number of researchers have confirmed that cell lysis in suspension is the primary effect of cavitation. Many researchers pay increased attention on the structure/function effects and the effects upon DNA, but these effects are beyond the scope of this project. Only a brief introduction of these effects is listed. For example, morphology alterations, chromosome breakage, sister chromatid aberrations, cell transformation, calcium uptake and mutation have been reported to be a consequence of ultrasound exposure of cell suspensions.

According to several researchers' findings, there is not only purely mechanical processes of bioeffects that have occurred during the experiment, but also a secondary effect, chemically mediated processes. The sonochemical hydrogen peroxide is readily detectable after modest *in vitro* exposures producing inertial cavitation, and this chemical can damage DNA in viable cells [130, 131].

2.8. Electroporation

Electroporation is a membrane phenomenon first indicated in the 1970s. Transient pores are created in the cell plasma membrane when an external electrical pulse is applied (Figure 2.4), which allows the delivery of DNA and other biologically active molecules into the host cells [132, 133]. Given the weak interactions between the hydrophobic interior and hydrophilic exterior and also the bilayer's ability to spontaneously reassemble after disturbance, the electroporation technique uses a controlled, voltage shock for a millisecond to temporarily disrupt the phospholipid bilayer, followed by a dramatic uptake of specific molecules, all without jeopardizing the integrity of the cell (Purves, et. al., 2001).

Electroporation is effective with the vast majority of cell and species types [134], and the number of biologically active molecules required is small compared to alternative methods (e.g. micro precipitates, microinjection, and biological vectors) [135]. Due to its high versatility and efficiency, this procedure has been widely utilized in many areas of molecular biology research for the transformation of cells with plasma and the delivery of drugs and genes [136]. More recently, *in vivo* electroporation was successfully performed

by Saito and colleagues, who transferred a DNA construct with a fluorescent reporter gene into intact embryonic mouse brain tissues at various stages [137].

2.8.1. Mechanism and Applications of Electroporation

Electroporation is conducted in electroporators. The cell suspension is pipetted into a cuvette with two aluminium electrodes on both sides, before the suspended solution containing the biological material is added into the cuvette. As shown in Figure 2.8, the application of a rapid electrical shock leads to the formation of temporary aqueous pores in the cell membrane. Typically, 500-100,000 V/cm (varying with cell size) in a pulse lasting from a few microseconds to a millisecond is required for electroporation. During the time that these pores exist, a significant amount of previously injected biomolecules is taken into and trapped in the host cell for further functional study. After a short period of time, the phospholipid bilayer discharges and the pores reseal, leaving the membrane unharmed [133].

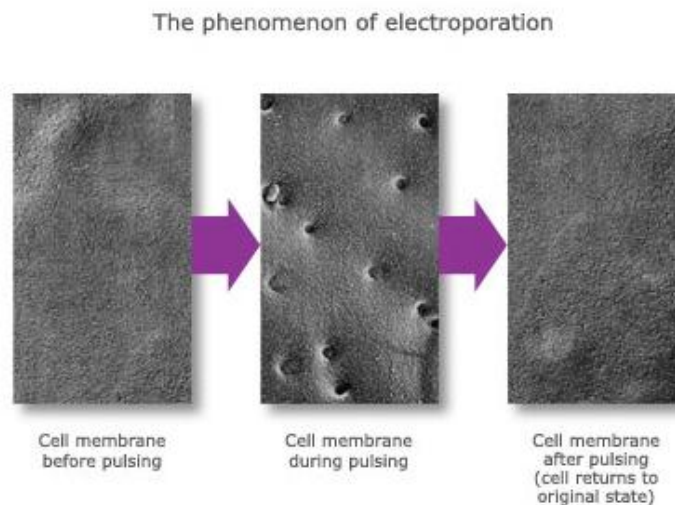


Figure 2.8 The phenomenon of electroporation. Controlled, quick electrical pulses induce temporary pores in the cell plasma membrane, which then reseals and is left undamaged (Source: Inovio Pharmaceuticals, 2010)

As mentioned previously, electroporation is now commonly used in molecular biological and medical studies. Some typical applications of electroporation include DNA transfection, transformation of plasmids between cells, drug delivery, electro chemotherapy and gene therapy. More specifically, the electroporation-based DNA delivery technology has been applied to introduce DNA vaccines into host cells in order to examine the structure and function of specific genes and proteins [134]. In addition, electroporation has been found to be beneficial in enhancing the effectiveness of chemotherapy. As in electroporation for DNA transfection, the electrical pulse disrupts the membrane of the tumour cell and increases the quantity of drugs delivered to the targeted site. For instance, Maeda et al. [138] reported increased tumour reduction when applying electroporation to cancerous cells in rodents. In addition, the technique has been used in gene therapies such as DNA-based immunotherapies and vaccines.

2.8.2. Key Parameters

A variety of parameters might well influence the electroporation procedure, including field strength, pulse length, the number of field pulses, temperature, cell density and the choice of electroporation buffer.

The field strength (V/cm) of the electrical pulse could influence the survival rate and the transfection rate of the cells. When the field strength of the pulse exceeds a certain threshold, reversible permeation occurs in the cell membrane. The determination of a minimum permeation voltage is associated with the temperature, the gap width of the cuvettes, as well as the size of the cells used. For example, the necessary field strength of the pulse for electroporation at 4 °C is approximately twice as high as the strength required for room temperature [139], and cells with smaller diameters need higher field strengths in order to achieve membrane permeation. Moreover, an optimal transfection rate is usually achieved when a higher pulse voltage is applied, but this may well lead to an increased cell mortality rate.

The pulse length and the number of field pulses are further two important factors for a successful transfection. The pulse length is mainly dependent on the diameter of the cell – larger cells usually require longer pulses for permeation of the membrane, and it has been demonstrated that the ideal pulse lengths for electroporation is 40–100 µs at room temperature and 15–40 µs at 4 °C. As for the number of field pulses, while electroporation is usually carried out with a single pulse, multiple pulses are used when one pulse is

insufficient, with a 60-second interval between pulses to allow the regeneration of the membrane and prevent excessive injury.

In addition to the effect on the permeation voltage, the temperature at which electroporation takes place also has a direct effect on the cell membrane regeneration following electroporation. Evidence shows that the resealing of membrane pores at low temperatures is much slower than the same process at room temperature [133], and this slower healing process could result in an increased amount of transfection material absorbed. However, cells in a cold environment are more likely to suffer from irreversible damage and therefore higher mortality rate. In addition, it has been suggested that a cell density of 1×10^6 cells/ml should be used for electroporation, since the electrical field is effective on the cells in this range, whereas homogeneous field conditions can no longer be guaranteed in higher cell densities.

Chapter 3 Three-dimensional Cryopreservation of Human Embryonic Stem Cell

3.1. Introduction

Human embryonic stem cells (hESC) are a potential source of cells for cell therapy and regenerative medicine [97]. However, hESC storage via cryopreservation has a low recovery and a slow growth rate [140]. Moreover, these disadvantages also coupled with a vital problem which is a high incidence of short-term spontaneous differentiation after thawing. In order to overcome the apoptosis challenge, current approaches of hESC cryopreservation involve the presence of inhibitors such as Rho-associated coiled coil forming protein serine/threonine kinase inhibitor (ROCK inhibitor, Y-27632) to prevent apoptosis post-thaw [141]. Although it has been confirmed by many researchers that the use of ROCK inhibitor can improve the outcome of hESC culture and cryopreservation, other challenges of hESC cryopreservation still stay as not-entirely solved. The stem cell storage is fated to result in diminishing stocks unless better and more efficient cryopreservation methods are adopted.

Standard suspension cryopreservation using DMSO and slow-cooling has yielded low recoveries [142]. Stem cell markers such as Oct-4 have expression levels as low as 10% of the recovered cell population [140]. Post-thawing growth rates can be so slow and unpredictable that recovery of original stocks takes week to months [140]. Some of the

problems with suspension cryopreservation include the lack of a substratum for cells to attach; a sharp, sudden exposure to high cryoprotective agent (CPA) concentrations and the use of expensive ROCK inhibitor supplementation. These problems can be minimized through cryopreservation of cells using materials that support cell attachment as a transitory anti-apoptotic mechanism. The attachment mechanism may also steady expression of stem cell markers such as Oct-4 and osteopontin that are intimately linked to integrin-ligand activity [143].

Several researches demonstrated that attachment can inhibit post-thawing apoptosis [141]. Mechanistically, attached cells function better than free-floating cells and are less likely to apoptose [144]. Cell survival, differentiation, migration and proliferation [145, 146] are hinged on cell attachment [147]. When attached, cellular biophysical properties such as hydraulic conductivity and solute permeability increase compared to free-floating cells [71]. This higher permeability has been exploited plasmid delivery [148] and cryopreservation [61]. For attachment, the arginine-glycine-aspartic acid-serine (RGDS) sequence is an essential recognition site for cells. Many cell-adhesive proteins e.g. fibronectin, vitronectin, osteopontin, collagens, thrombospondin, fibrinogen and von Willebrand factor present in extracellular matrix and in blood contain RGDS. RGDS domains on these extracellular matrix molecules bind to transmembrane domains such as $\alpha V\beta 3$ integrins which play significant roles on cell survival during embryonic development [149]. On a macro-scale, integrin-RGDS interactions also affect cell permeability [149]. Such interactions, when active during cryoprotective agent (CPA) loading, may help improve cell response to changes in CPA concentration.

Microencapsulation is one of the broadly adopted methods to provide cells with three dimensional extracellular matrix (ECM) and has been demonstrated in mammalian cell culture and storage [150-152]. Encapsulation may be accomplished by chemical, physical or mechanical means [152] resulting in a core-shell [152] or a solid capsule. In encapsulation with solid capsules, each cell is surrounded by a highly swollen polymeric 3D network preferably reversibly cross-linked (e.g. calcium alginate). Although this technique has been applied to many areas in both research and clinical, it has few application in cryopreservation area especially involve human embryonic stem cell. During cryopreservation, encapsulation may help increase post-thaw cell viability, inhibit spontaneous differentiation and inhibit the growth of ice crystal during freezing process [72, 153]. With these potential advantages that can improve the cryopreservation outcome, applying microencapsulation to cryopreservation of human embryonic stem cell is worth exploring.

This chapter aims at using RGDS-functionalized alginates to improve human embryonic stem cell survival rate and cell health after thawing while maintaining their stemness using a simple constant-rate cooling protocol. This objective is followed by investigation of differences between lab-made and commercial available RGDS-alginate. In addition, due to ROCK inhibitor's expensive cost and complexity of handling during hESC maintenance and expanding, the alternatives that preserve hESC without the presence of ROCK inhibitor are being sought. This work is based on a feasibility study done by Dr. S. Sambu and his inconclusive results. Consequently, a detailed study of 3D cryopreservation of hESC without ROCK inhibitor was carried out and the results are also presented in this chapter.

3.2. Materials and Methods

3.2.1. Culture of Human Embryonic Stem Cells

In this chapter, all experimental data is based on human embryonic stem cell line HUES2 (Howard Hughes Medical Institute, Department of Molecular and Mellular Biology, Harvard University, USA) which is approved by the UK Stem Cell Bank Steering Committee.

3.2.1.1. Feeder-independent hESC Culture

The human embryonic stem cells were cultured in matrigel pre-coated culture flasks, in culture medium MTeSRTM (Stem Cell Technologies, France) which supplemented with 1% penicillin (Invitrogen, UK) and 1% Rock inhibitor Y-27632 (100X) (Sigma, UK), at 37°C, under 5% CO₂ and 21% O₂. The culture medium was completely changed every 24 hours. With the high risk of contamination, all medium involved with the hESC culture were pre-filtered through 0.22 µm filter (MILLEX, UK). Cell passaging should be carried out when hESC forms a perfect mono-layer. Cells should be observed daily to prevent cell overlap hence detachment.

For passaging, hESC colonies were washed with PBS first and then detached by StemPro Accutase (Invitrogen GIBCO, UK) at 37°C for 3-5 minutes. The flask was gently flushed with pipette in order to assist cell detachment. Then, the culture flask was washed by adding another 1.5 mL culture medium. Cell suspension was removed and transferred to new matrigel-coated culture flasks which were prepared in advance. Normally, the split

ratio is 1:3. The Rock inhibitor was only added to the day 1 culture and is not required after passaging.

3.2.1.2. Matrigel Coating

Since matrigel coating is indispensable and of significant importance to hESC culture, the pre-coated culture flask should be carefully prepared. Most importantly, the matrigel should always stay cold to preserve its functions and capabilities. Therefore, tubes and pipette tips which are used during the making of matrigel should be pre-cooled in a -20°C freezer and only removed when used.

At the beginning of the procedure, prepare a large polystyrene box and pack it with ice. Push the cold 50mL tube and cryotube containing frozen matrigel (VWR International, UK) into the ice and leave them for 5 minutes. The matrigel tube should not be put into the water bath since it will turn to a thick gel. Thus, the matrigel should be thawed only by mixing with cold DMEM solution. Quickly load a frozen tip onto the pipette, load and dispense the tip 3 times into the cold DMEM medium (PAA, UK), then begin mixing. Mix 200 µL of matrigel with 35mL DMEM medium and then store them inside a 4°C freezer for later use. The entire procedure should operate on ice if possible.

3.2.2. Cell Function Assays

3.2.2.1. Viability Assay

To determine cell viability, cells were assayed for cell membrane integrity. Trypan blue exclusion method was adopted as the method of cell viability assay for cells in both 2D and

3D culture. Cells that were encapsulated by alginate were removed from the alginate beads and then applied viability assay.

3.2.2.2. Cell Apoptosis Assay

Cell apoptosis rate was quantified using flow cytometry. Cells were thawed and recovered from RGDS-alginate beads and cultured in medium with/without rock inhibitor supplementation for a fixed duration of 2, 4 or 24 hours; controls were suspension cryopreserved cells with/without rock inhibitor supplementation. At the appointed times, cells were fixed. All sample conditions were stained using FITC-conjugated Annexin V and propidium iodide from the kit as supplied (Trevigen, UK), incubated at room temperature in the dark for 15 minutes, resuspended in a binding buffer and analysed by flow cytometry (model: BD FACSCalibur[®] BD Biosciences, USA) at a minimum of 10000 events per condition. The results were processed using WinMDI software (Scripps Institute, USA).

3.2.3 RGDS Modification of Alginate

Medium viscosity alginate (2000cP at 2 wt.% polymer at 25°C, sigma, UK), derived from brown algae, was dissolved over a 12 hour period in a buffer made from 50mM 2-(N-morpholino) ethanesulfonic acid (MES) (sigma, UK) in 0.15 NaCl (pH 6.5) with concentration of 1g alginate per 100mL buffer. The solution needed to be mixed and dissolved completely. RGDS was dissolved in the solution and bonded onto the alginate via carbodiimide chemistry using highly water-soluble N-hydroxysulfosuccinimide (sulfo-NHS) (Sigma, UK) to ensure stabilization against hydrolysis. The ratio of sulfo-NHS added to 1-ethyl-(dimethylaminopropyl) carbodiimide (EDC) (Sigma, UK) was 1:2 whilst the ratio for

the peptide was 1:4. The sulfo-NHS, EDC and peptide (in that order) were added in quick succession after dissolution of each reagent to alginate solution at room temperature with constant stirring. Hydroxylamine hydrochloride (HA-HCL) (Sigma, UK) was added to the reaction after a period of 20 hours at a ratio of 18:1000 (HA-HCL to alginate weight ratio). The solution was left for another 2 hours to allow the solution to completely dissolved, it was then transferred to a dialysis tubing (MWCO 3500, Fisher, UK). The final step was dialysis. The dialysis tubing containing RGDS modified alginate was immersed in NaCl solution. The NaCl solution was changed every 8 hours over a period of 3 days, with decreasing concentrations of 15g, 12.5g, 10g, 7.5g, 5g, 2.5g, 0g, 0g, and 0g NaCl in 2L Milli-q water. Then the RGDS-alginate was freeze-dried. Successful bonding of the RGDS peptide to the alginate was confirmed by Transmission-FTIR and combustion analysis.

3.2.4 RGDS-alginate Analysis

3.2.4.1 FTIR Analysis

After RGDS-alginate was freeze-dried, the successful coupling of the RGDS peptide was analysed by FTIR. The modified alginate in solid form was loaded to Tensor 37 FTIR spectrophotometer (Bruker, Ettlingen, Germany) and analysed using a Miracle ATR sampling unit. All FTIR spectra were baseline corrected for lab atmospheric conditions (CO₂ and water) and using unmodified pure alginate as reference. The results was analysed and exported using OPUS 6.0 software (Bruker, Ettlingen, Germany).

3.2.4.2 Combustion Analysis

CH&N analysis were carried out by combustion analysis to quantify the percentage composition of the elements of Carbon, Hydrogen and Nitrogen. Since the RGDS peptide is the only source for nitrogen in RGDS-alginate, the percentage of nitrogen could indicate how much peptide were successfully bonded onto the alginate.

3.2.5. Cell Encapsulation

Cells were centrifuged down then supernatant was removed. Cell pellets were resuspended in 1.2 wt. % alginate solution in 0.9 wt. %NaCl at a cell density of 1 million cells/mL. Due to the viscous nature of alginate, some cells are lost in the “dead volume” of left-over alginate in the pipette tips. Therefore, it is important to reconcile the cell numbers after recovery. Following this, the cell suspension was loaded into a syringe (BD, UK) with a 30 gauge needle (BD, UK). The syringe was firmly fitted onto a portable infusion pump. The pump was placed vertically with the needle pointing down and the infusion rate was set to 3 mL/min. The cell suspension was ejected through the needle into a 50 mL tube of 102mM CaCl_2 solution from a 3 cm height, giving an average alginate beads radius of 0.6 mm (standard error of 0.1mm). While ejecting, the tube was gently shaken by hand to avoid alginate beads forming a larger cloud. The osmolarity of CaCl_2 solution used in this experiment, 310 mOsm/L, is a vital point to consider since it balances the osmotic pressure of the hESC membrane. The encapsulation procedure is demonstrated below in Figure 3.1.

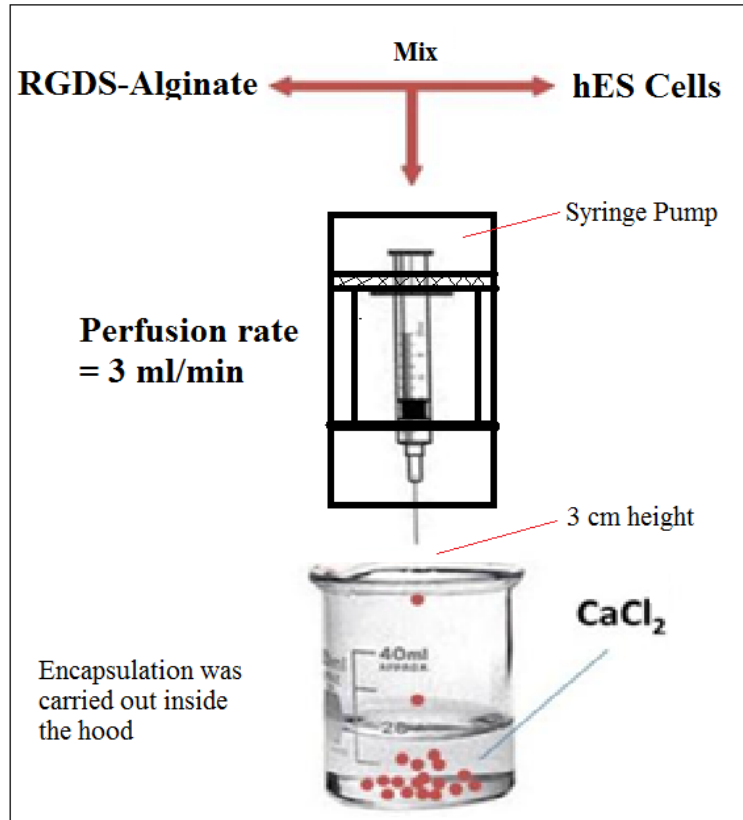


Figure 3.1 Diagram of cell encapsulation procedure.

After alginate beads were formed, they were left within the solution for 10 minutes for cross-linking. Then the CaCl₂ was removed by pouring the solution that containing alginate beads through a 40 μ m Nylon mesh (Fisher, UK). The alginate beads were washed by PBS containing 10% 102mM CaCl₂ solution. Finally the alginate beads were resuspended in the culture medium.

3.2.6. Cell Recovery from Alginate Beads

Before analysis for post-thaw cell survival rate, cells in alginate beads were resuspended in 12 mL of 0.055M sodium citrate (Sigma, UK) in 50mM NaCl solution and buffered with

10mM morpholinoethanesulphonic acid (MOPS) (Sigma, UK) to a pH of 7.4 to dissolve the alginate for 15 minutes on a roller mixer. The osmolarity of the dissolving agent is 340mOsm, this reading is very similar to the osmolarity of the CaCl₂ solution and the hESC membrane. The alginate should not be left on a roller mixer for longer than 15 minutes otherwise the viability of the cells may be affected. Also, the roller mixer should be set to low spin in order to avoid exerting shear stress on cells. The completion of dissolution can be easily monitored by visual observation when samples are held against dim light. Cells were then centrifuged at 148g for 5 minutes then resuspended in culture medium. Supernatant was checked for the presence of cells before discarding. Cell density after resuspension was reconciled to 1 million cells/mL.

3.2.7. Cryopreservation Procedures

3.2.7.1. Cryoprotectant

Dimethyl sulfoxide (DMSO) was used as cryoprotectant for all cryopreservation purposes. Cryoprotective efficiencies of different DMSO concentrations were investigated in terms of cell survival rate after thawing and the results were compared in groups.

3.2.7.2. Freezing Protocols

Samples are loaded with different concentrations of DMSO and left at room temperature for a period of 20 minutes, 30 minutes or 45 minutes; this is known as the loading time. The impacts of different loading times to post-thaw cell survival rates were analysed and compared.

Afterwards, the samples were transferred to freezing devices for the freezing procedure. Two freezing rates, 1 °C/min and 5°C/min, were adopted and the efficiencies were analysed and compared.

The freezing rate of 1 °C/min was achieved by placing the samples inside a Cryo 1 °C freezing container (NALGENETM, cat. No. 5100-0001) and leaving the container in -80 °C freezer. The container has a buffer layer filled with anhydrous 2-propanol, which can provide a constant freezing rate of 1 °C/min.

The freezing rate of 5 °C/min was achieved by using programmed freezer (Kryo 560 Planer, UK). The programmed freezer can monitor the real-time temperature of the samples in the chamber and provide a precise freezing rate of 5 °C/min, commencing from room temperature to -80°C.

After freezing, the samples chosen be tested overnight would remain in -80°C freezer; samples which were going to be tested in the future, 7 days or longer, would be transferred to liquid nitrogen tank for long-term storage.

3.2.7.3 Thawing Protocols

For recovery from cryogenic storage, cryopreserved cells were immediately warmed by immersion into a 37°C water bath. Thawing should take less than 2 minutes in total with frequent swirling by hand. Cells should be removed from the water bath when cells are nearly fully thawed with a little ice left at the top. Further thawing could be carried out by adding fresh, pre-warmed culture medium. Then, mTeSR® culture medium were added to

the cell suspension gradually in order to reduce the CPA concentration to less than 1% and the samples were left at room temperature for 10 minute for cells to adapt to the recovery environment.

For cells cultured in 3D form, cells should be recovered from alginate beads followed by alginate dissolving protocol and then prepared for later function tests.

3.2.8. Imaging Analysis

LIVE/DEAD[®] Viability/Cytotoxicity Kit (Life Technologies, Invitrogen[™], UK) was used for cell imaging. This kit consists of two types of fluorescence dye – Calcein-AM which colours the live cells green and EthD-1 which stains the dead cells red. Both bright field images and fluorescence images of cell adhesion were taken by using fluorescence microscopy (Nikon eclipse Ti-E, Nikon, UK). In order to perform fluorescence imaging of the hESC in both 2D and 3D conditions, the culture plate containing hESC was removed from the incubator and the culture medium was removed from the well. Then, 1 μ M calcein-AM in PBS and 5 μ M EthD-1 in PBS were added to each well until the cell was fully covered. The culture plate was left at room temperature for 30-34 minutes in the dark. Finally, the sample was place under a fluorescence microscope for imaging.

Live cells are clearly identified by the presence of ubiquitous intracellular esterase activity, illustrated by the enzymatic conversion of the nonfluorescent cell-permeant calcein-AM to the much stronger fluorescent calcein which has an excitation/emission wavelength of 495/515 nm respectively. The polyanionic dye calcein is well preserved within the live cells, which produces a strong constant green fluorescence in live cells. EthD-1 enters the

cells with clearly damaged membranes and goes through a high enhancement of fluorescent (X40) upon interacting with nucleic acid, thus producing clear red fluorescence in dead cells (ex/em ~495nm/~635nm). Since EthD-1 is excluded by the plasma membrane, live cells will remain the unchanged.

3.2.9. Statistical Analysis

Each experiment was performed in triplicate and repeated three times. Analysis of variance (ANOVA) was adopted as the method to determine the statistical significance between groups. P-values were also calculated using T.TEST function with a two-tailed distribution in Excel. A p-value of less than 0.05 was considered as statistically significant. All data presented are in the format of mean $\pm$ standard deviation.

3.3. Results and Discussion

3.3.1. RGDS Modification of Alginate

Microencapsulation is one of the broadly adopted methods to provide cells with three dimensional matrix. This technique has been applied to cell cryopreservation and proven to be both useful and efficient. The advantages of this technique include increasing post-thaw cell viability, inhibiting spontaneous differentiation and inhibiting ice crystal growth during the freezing process. The most popular material to create encapsulation is alginate. Cell attachment can be greatly facilitated by the 3D matrix provided by alginate when the cells

are encapsulated by the alginate beads. As previously stated, the attached cells are of a superior quality compared to free-floating cells in terms of cell survival, differentiation, migration and proliferation.

The word “modification” in this case, refers to the successful bonding of the RGDS to the backbone of alginate.

The ECM molecules are found to have a variety of short amino acid sequences that can promote cell attachment. Similar to amino acids, the RGDS (arginine-glycine-aspartic acid-serine) sequences have the property of containing integrin receptors that allow reactions with corresponding receptor integrin of cell transmembrane, such as $\alpha_5\beta_1$ integrins. Thus, the RGDS sequence is the vital recognition site during cell attachment. Previously, it has been discovered that many cell-adhesive proteins within ECM and blood contain RGDS. Examples of such proteins include osteopontin, thrombospondin, collagens, fibrinogen, fibronectin and the von Willebrand factor. After the successful bonding of RGDS to alginate, the ECM provided by RGDS modified alginate can promote the cell attachment in a superior way to alginate [149]. Such an effect upon RGDS can greatly improve the cell response to changes in DMSO concentration during the freezing process. Hence, the successful production of RGDS modified alginate is crucial to the cryopreservation of human embryonic stem cells (hESCs) in 3D.

However, the free-form EDC and HA-HCL in alginate solution is lethal (according to the hazard classification data published by sigma-aldrich, both products are classified as GSH07, which means acute toxicity) when encountered with cells during the encapsulation process. Thus, the dialysis process is vital. It should be noted that the dialysis requires

regular timetabled changing of the dialysis medium (every 8 hours). The entire process takes 3 days and the sample can be left in the medium for longer if necessary in order to guarantee that no free-form EDC and HA-HCL exists in the modified alginate. A freeze-drying process is followed in order to perform the analysis of RGDS-bonding efficiency. Since the freeze-drying may accelerate the reactions of the bonding when the concentrations of chemicals increase due to loss of water within the alginate solution, the process was not expected to affect the quality of the RGDS-alginate. The bonding efficiency was examined by FTIR by comparing spectra of differently modified alginates. Moreover, FTIR can also determine if the dialysis or freeze-drying eliminates any valuable properties of the alginate by comparing the FTIR results of normal alginate and the RGDS modified alginate. Since the modification is achieved by bonding the RGDS peptide onto the backbone of the normal alginate, new peaks are expected on the absorption spectra of modified alginate but the shape of the spectra should still match the one obtained from normal alginate.

To obtain the findings above, the absorption spectrum of the RGDS modified alginate after dialysis and freeze-drying was compared to that of pure alginate. As Figure 3.2 illustrated, the result did not show any shift in peaks. This means that the major properties of the alginate were well preserved. It can also be noted that the RGDS modified alginate has three distinct peaks at 1300, 1262 and 973 cm^{-1} . These peaks cannot be found in unmodified-alginate. These distinct peaks may have been introduced by the bonding process of the RGDS peptide. As the ninhydrin-UV method confirmed [154, 155], the FTIR absorption spectra of amino acid group on alginate-amino acid derivatives was shown at 1734, 1245 and 1037 cm^{-1} [156]. By referencing the Typical Infrared Absorption

Frequencies table (Appendix 1), the absorption frequencies of three important chemical bonds, which are C=O, N-H and C-N, can be identified. These chemical bonds are indicators of a successful carbodiimides chemistry process. It should be noted here that the C=O bonds exist in alginate before and after carbodiimides chemistry and the N-H bonds only exist in the modified alginate after carbodiimides chemistry. The C=O bond (amide I bond) has its frequency range of 1630-1695 cm^{-1} and the N-H bond has its frequency range of 1590-1650 cm^{-1} . Since the latter is slightly smaller than the former one, the peak around 1600 cm^{-1} of RGDS modified alginate slightly shifts to the right compare to the one in the spectra of normal alginate (Figure 3.2). The C-N bond (amide II bond) is the outcome of the carbodiimides chemistry, which has its frequency range of 1000-1300 cm^{-1} . Hence, the observed peaks in the region from 1000 cm^{-1} to 1300 cm^{-1} of the modified alginate in Figure 3.2 are contributions by RGDS. In addition, the peak nearing the 979 cm^{-1} indicates the successful bonding of RGDS to the alginate backbone [157]. All the observed peaks are sufficiently distinct from the peaks expected in alginate. By summarizing the evidences cited above, it can be concluded that the observation of peaks at 1300, 1262 and 973 cm^{-1} are attributable to the successful coupling of RGDS onto the alginate.

The FTIR was also performed on commercially available RGDS-alginate purchased from NovaMatrix. The same peaks were observed from the ATR-FTIR absorption spectrum, confirming our success on RGDS modification (Figure 3.3).

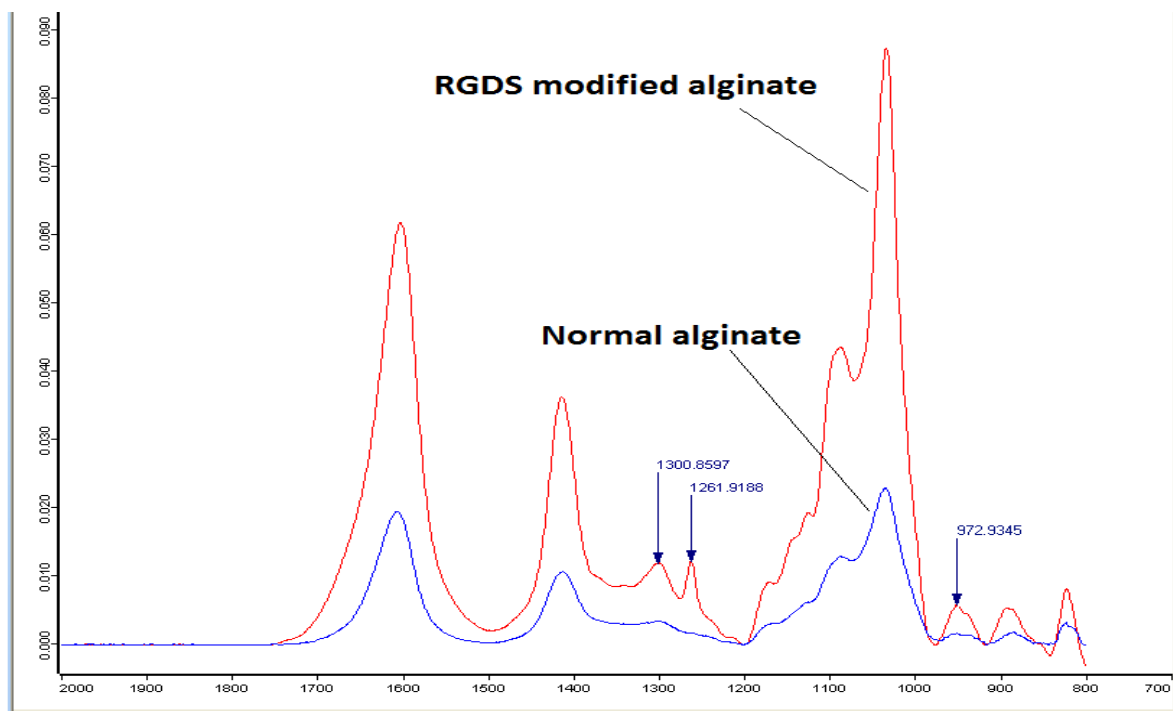


Figure 3.2 Comparison of ATR-FTIR absorption spectra for RGDS modified and unmodified alginate. An unmodified alginate spectrum is plotted in blue; the RGDS-modified alginate spectrum is plotted in red.

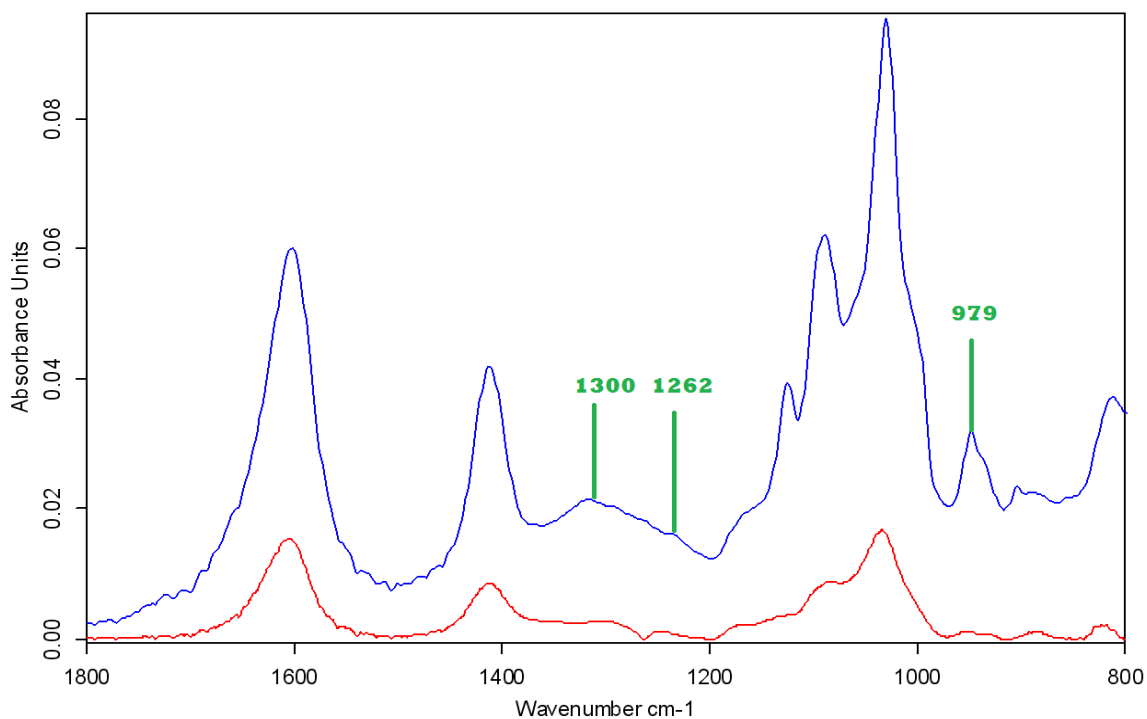


Figure 3.3 Comparison of ATR-FTIR absorption spectra for commercially available RGDS modified alginate from NovaMatrix and unmodified alginate from Sigma. An unmodified alginate spectrum is plotted in red; the RGDS-modified alginate spectrum is plotted in blue.

The combustion analysis was performed in order to quantify that how much RGDS peptide has bonded onto the alginate by analysing the percentage composition of the elements of Carbon, Hydrogen and Nitrogen. Since the RGDS peptide is the only source for nitrogen in RGDS-alginate, the percentage of nitrogen could well indicate the amount of the peptides that successfully bonded onto the alginate. This combustion analysis was carried out by MEDAC LTD and the results are shown below in table 3.1.

Table 3.1 Analytical report of combustion analysis

MEDAC LTD			
Analytical and chemical consultancy services			
ELEMENT	C	H	N
% Alginate (Sigma 71238)	28.50	4.28	<0.10
% Alginate (NovaMatrix LVG)	31.31	5.09	<0.10
% RGDS-Alginate (NovaMatrix MVG-RGDS)	33.48	4.94	0.80
% RGDS-Alginate (Our batch)	31.33	5.09	0.48

According to the data in table 3.2, the RGDS-alginate created by us has 0.48% of RGDS peptide bonded onto the alginate scaffold. Since the weight of other remaining chemical, such as EDC, sulfo-NHS and etc., and water is unable to calculate, the RGDS to alginate ratio is roughly 1:100. Thus, the RGDS bonding efficiency is around 51%. This result matches the result (bonding efficiency is between 50% and 60%) published by Rowley et al. [158]. Although the bonding efficiency is slightly lower than the commercially available RGDS-alginate from NovaMatrix, it still represents significant success in making RGDS modification of alginate in the lab. The data also indicates the accuracy of the modification protocol. The technique of RGDS medication has been mastered.

During the modification process, the suitable amount of attached RGDS peptide remains to be agreed upon. The common usage is 12.5 mg RGDS peptide per gram alginate. In this

study, different uses of the RGDS peptide were analysed by FTIR and the resulting data was compared.

The bonding efficiencies using 1, 5, 10, 12.5 mg per gram alginate were analysed. In FTIR, the integration of the spectrum indicates the content of the chemical. From Figure 3.4, the efficiency of RGDS modification nearly reached the maximum. Given the high cost of the RGDS peptide, 10 or 12.5 mg peptide per gram alginate is considered as the optimum quantity of RGDS.

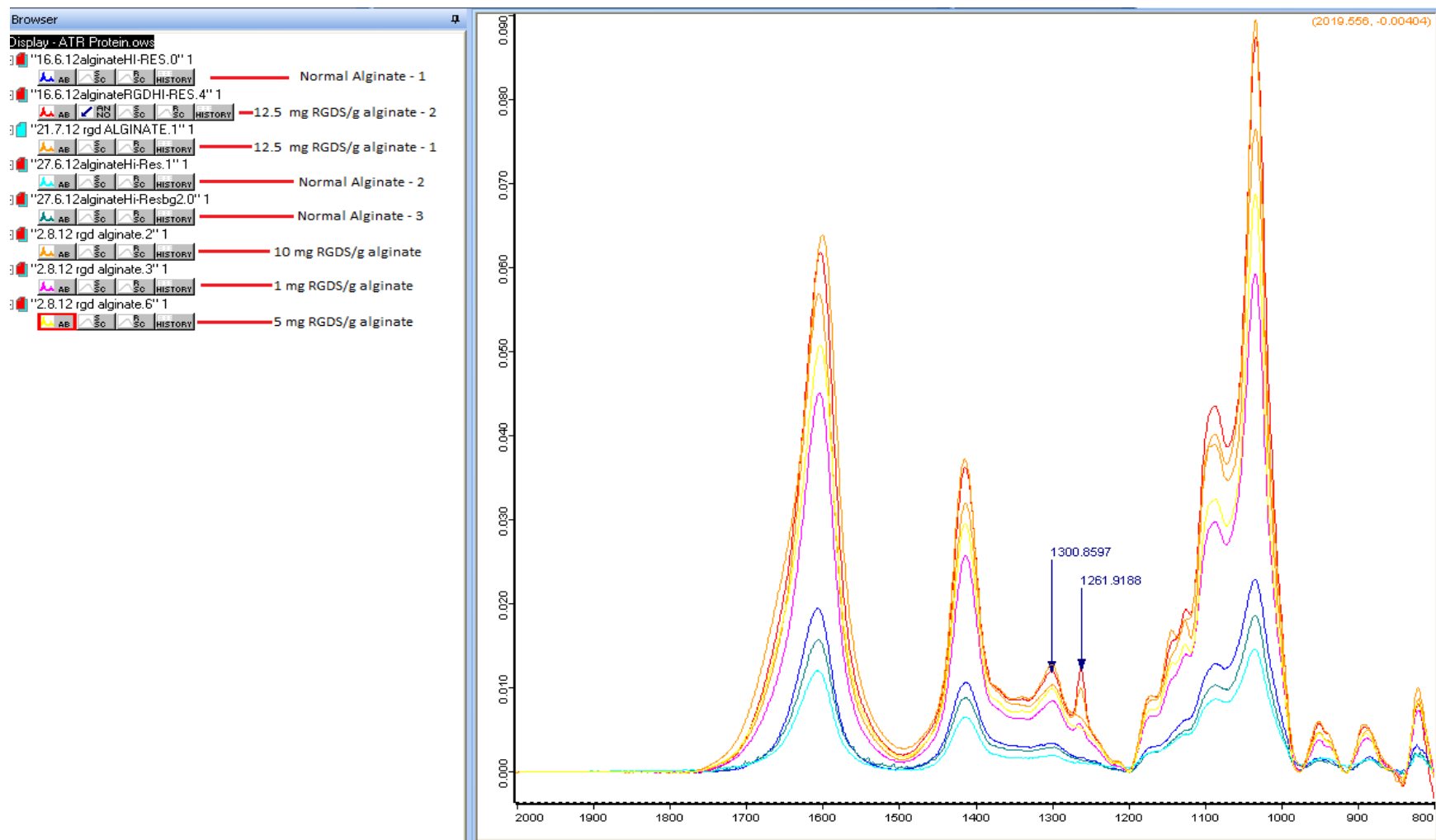


Figure 3.4 Comparison of ATR-FTIR absorption spectra with different use of RGDS peptide during the modification process.

3.3.2. Optimum DMSO Concentration for hESC Cryopreserved in Suspension

Post-thaw viability of hESCs was analysed as the CPA concentration was altered. The results are shown in Figure 3.5. Cells preserved with 10% DMSO supplementation demonstrated the highest post-thaw viability. This concentration of DMSO was adopted as a standard CPA concentration in cryopreservation of multiple cell lines including mouse embryonic stem cell [159], but has not been widely used in cryopreservation of human embryonic stem cell [160]. Many groups reported extremely low survival rate after thawing, which is only around 10% [61, 160-164]. However, Li et al. proposed that such problem can be solved with the presence of ROCK inhibitor (RI), and 10% DMSO is the optimal CPA concentration for cryopreservation of hESCs with RI [160]. The results in this study are equivalent with the observations obtained by Li et al. [160].

Having identified the optimum DMSO concentration, the evaluations of 3D cryopreservation efficiencies were carried out using 10% DMSO. After this stage, the possibility of reducing DMSO concentration by encapsulating hESCs in RGDS modified alginate beads was explored.

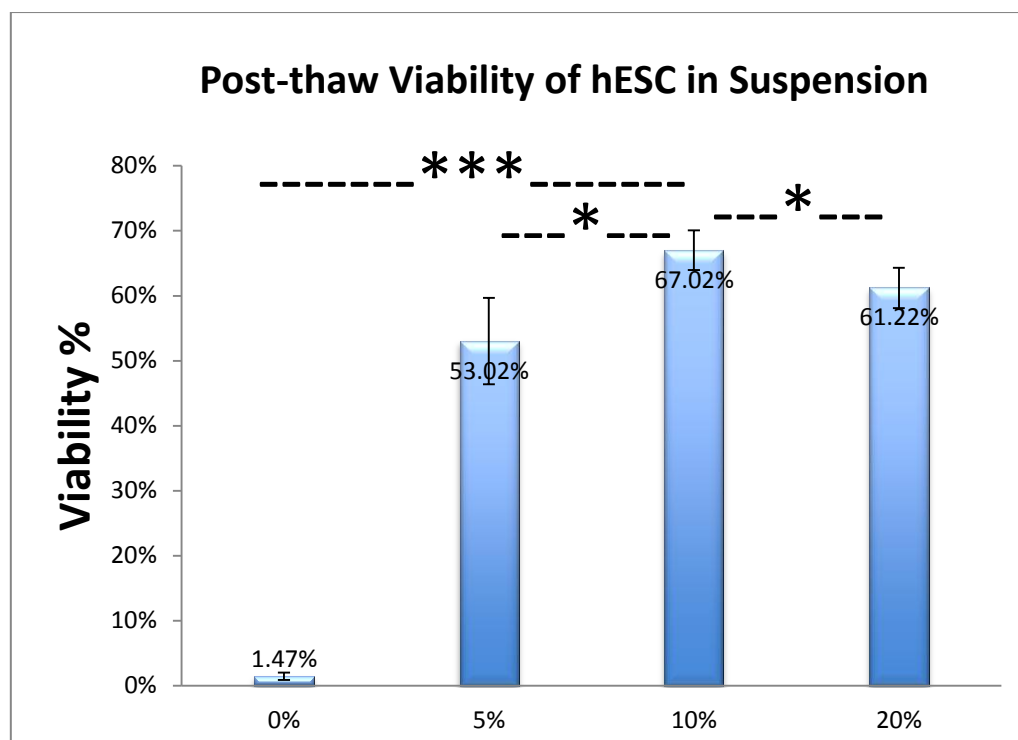


Figure 3.5 Relationship between DMSO concentration and post-thaw viability for hESCs cryopreserved in suspension. The loading time was 30 minutes and the cell densities of all tested samples were 1 million cells per ml. The result obtained from 10% DMSO concentration was identified as significantly higher (* $p < 0.05$, *** $p < 0.001$) than the other doses based on a single factor ANOVA analysis. Data is given as mean $\pm$ standard deviation, obtained from at least three independent trials. {Experiment conditions: loading temperature: 20 °C, 1 °C/min cooling rate, freezing medium: mTeSR culture medium supplemented with DMSO, FBS, 1% 100X ROCK inhibitor and DMEM}

3.3.3. Loading Time

Duo to the toxic property of DMSO, the loading time can have an effect upon the outcomes of cryopreservation. The investigation concerning the loading time for 3D cryopreservation is of great importance. As data in Figure 3.6 illustrates, 3D cryopreservation of hESC had an optimum loading time of 30 minutes. The outcome of 3D cryopreservation was consistent with the results obtained using the suspension model (data not shown). It can

also be concluded from this result that the change of diffusion rate of RGDS-alginate can be ignored.

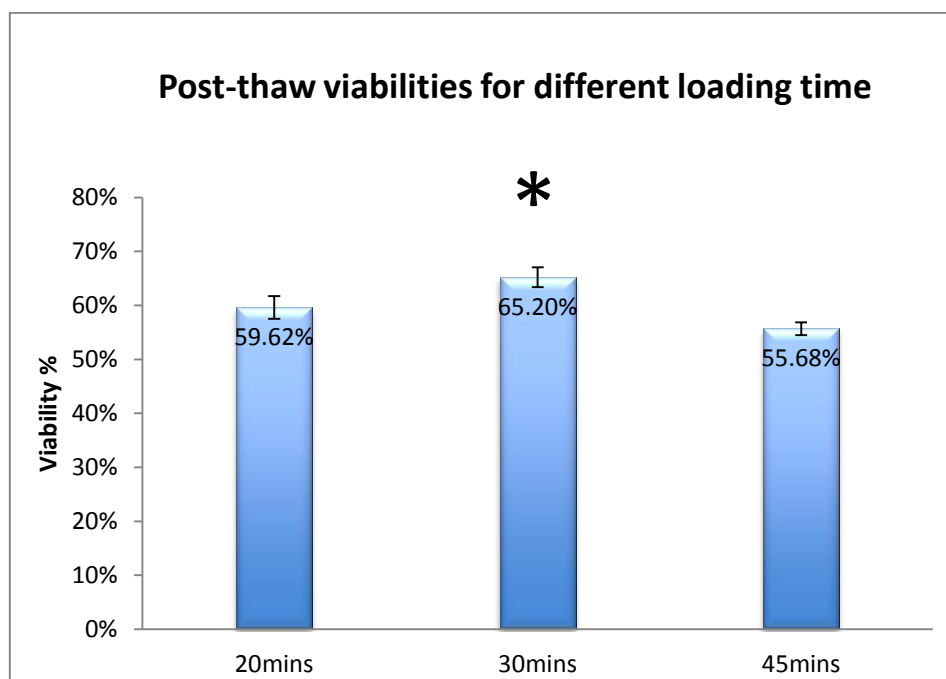


Figure 3.6 Relationship between loading time and post-thaw viability for hESCs cryopreserved in RGDS-alginate beads. The result obtained from loading time of 30 minutes was identified as significantly higher (* $p < 0.05$) than the other doses based on a single factor ANOVA analysis. Data is given as mean $\pm$ standard deviation, obtained from at least three independent trials. {Experiment conditions: loading temperature: 20 °C, -1 °C/minute cooling rate, 1×10^6 cells/ml, cells encapsulated by 1.2 wt% RGDS-alginate beads with mean diameter of 0.5 mm, freezing medium: mTeSR culture medium supplemented with DMSO, FBS, 1% 100X ROCK inhibitor and DMEM}

3.3.4. Cooling Rate

As an important parameter of cryopreservation, altering the cooling rate for 3D cryopreservation was attempted. A cooling rate of 1 °C/min resulted in a higher post-thaw

viability when compared to a higher cooling rate of 5°C/min (Figure 3.7). This result is consistent with other researchers' findings. Hunt and Timmons report that one of the most critical factors that affects the cell survival is the cooling rate between -1°C/min and -1.8°C/min [165]. Additionally, Ware et al. performed further experiments that compared different cooling rates of -0.1, -0.3, -1, -1.8, -2.7 °C/min and -1°C/min showed best cell survival rate [164]. However, in the presence of ROCK inhibitor, the cooling rate could be higher to approximately -5°C/min without affecting the survival rate [166]. Such finding is obtained by using modified CPAs and controlled-rate cooling device. Thus, -1 °C/min is preferred in this study since it is easier to achieve in the lab and gives slightly better results than -5°C/min.

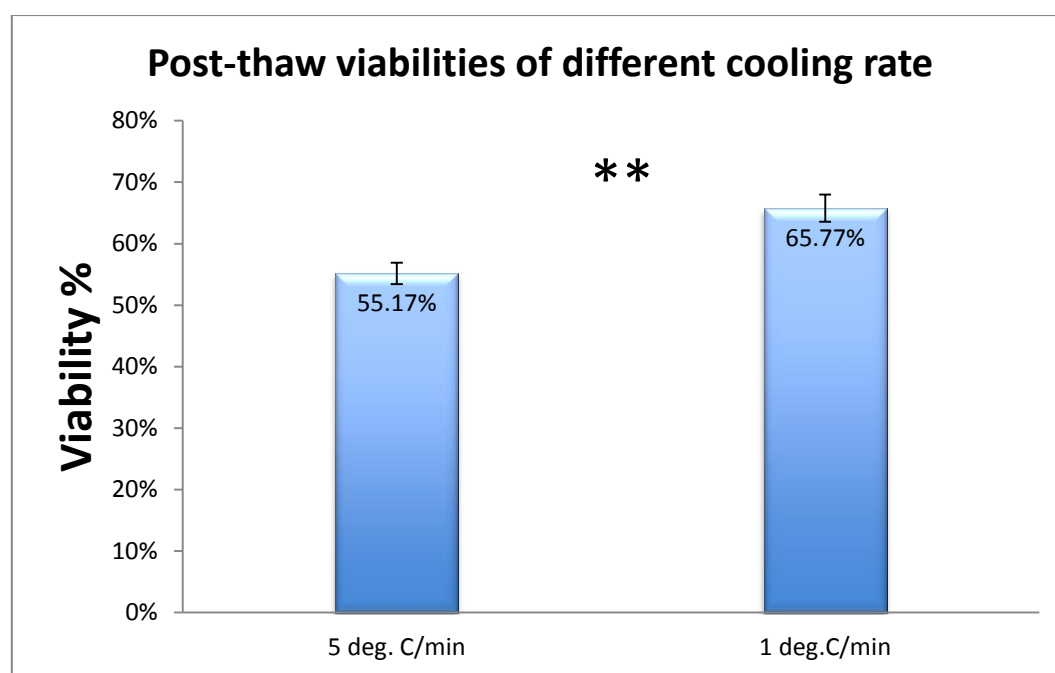


Figure 3.7 Effect of cooling rate on post-thaw cell viability. The viability obtained from 1 °C/min is significantly higher than the other data according to the result of two tailed distribution T-Test (**p<0.01). Data is given as mean±standard deviation, obtained from at least three independent trials. {Experiment conditions: loading temperature: 20°C, 1×10⁶ cells/ml, cells encapsulated by 1.2 wt% RGDS-alginate beads

with mean diameter of 0.5 mm, freezing medium: mTeSR culture medium supplemented with DMSO, FBS, 1% 100X ROCK inhibitor and DMEM}

Having identified the optimum DMSO concentration, loading time and cooling rate, the following experiments were then carried out following this plan.

3.3.5. 3D Encapsulation Effect on hESC Cryopreservation

3.3.5.1 Post-thaw Viability

The cryopreserved hESCs were recovered by quickly immersing the cryo-vessel in a 37°C water bath with frequent swirling by hand. The samples were removed from the water bath and then the cell suspension was transferred into tubes. The culture medium was added into the tube drop by drop with constant shaking of the tube in order to minimize the osmotic pressure induced by the fresh medium. The viability assay was carried out by the trypan blue method once the hESCs was recovered from the alginate beads and the results are shown in Figure 3.8.

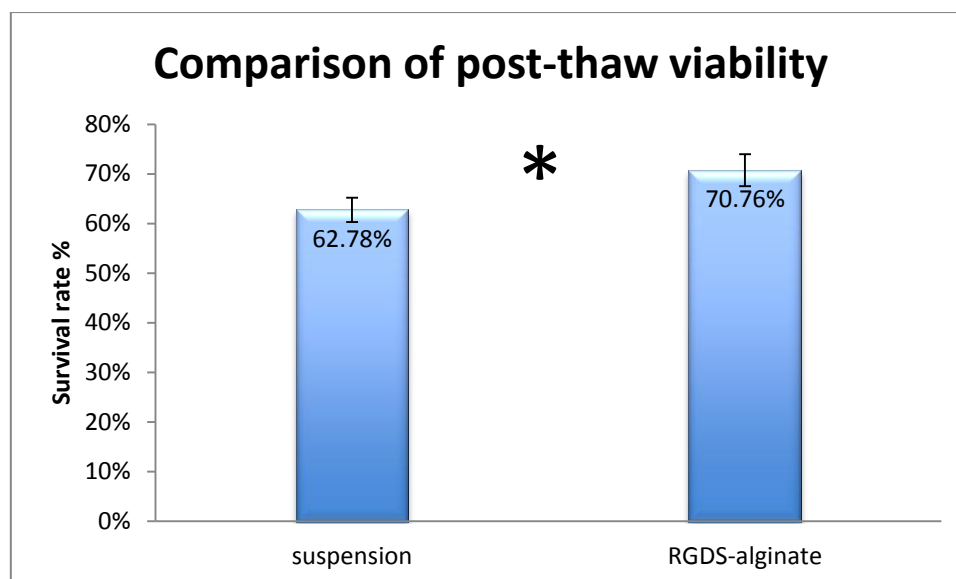


Figure 3.8 Comparison of cell survival rate for hESCs cryopreserved in suspension and RGDS-alginate beads. (* $p < 0.05$, according to two-tailed distribution T-Test). Data is given as mean $\pm$ standard deviation, obtained from at least three independent trials. {Experiment conditions: loading temperature: 20 °C, -1 °C /minute cooling rate, 1×10^6 cells/ml, cells encapsulated by 1.2 wt% RGDS-alginate beads with mean diameter of 0.5 mm, freezing medium: mTeSR culture medium supplemented with 10% DMSO, 20% FBS, 1% 100X ROCK inhibitor and DMEM}

The hESCs preserved in RGDS-alginate beads had a higher survival rate compared with cells preserved in the suspension. Given the same composition of the CPA, the RGDS-alginate can provide increased cell protection during cryopreservation. Related researches have rarely been done in the area of 3D cryopreservation of hESCs, because the cell encapsulation using normal alginate does not provide more benefits to the outcome of the cryopreservation of hESCs. However, the RGDS-alginate does. In this study, the normal alginate was further modified with RGDS peptide and the results showed great improvement on the outcome of the cryopreservation of hESCs.

Before the thawed hESCs were recovered from the alginate beads, cells were stained by calcein-AM (Green) and EthD-1 (Red) to assess both the post-thaw cell morphology and viability.

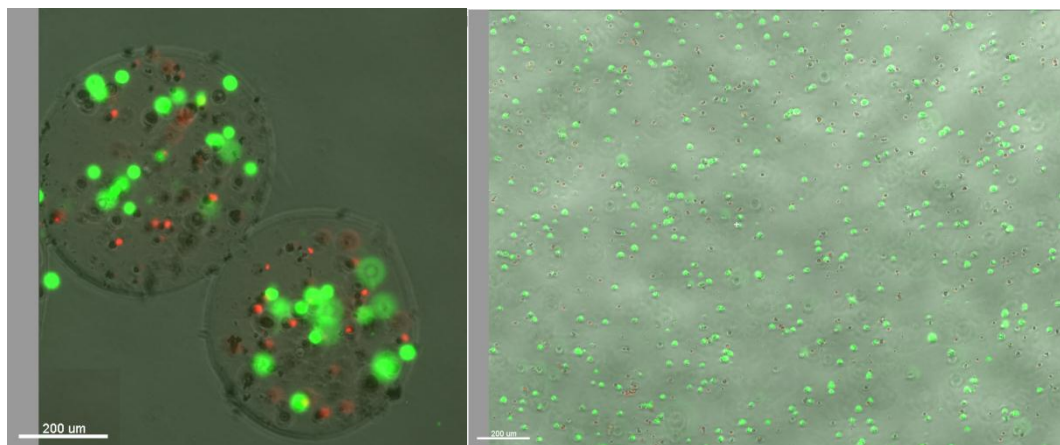


Figure 3.9 Images of thawed hESCs cryopreserved in RGDS-alginate and suspension. Image on the left is RGDS-alginate encapsulated hESC whilst the hESCs in suspension is on the right. {Experiment conditions: -1°C /minute cooling rate, 1×10^6 cells/ml, cells encapsulated by 1.2 wt% RGDS-alginate beads with mean diameter of 0.5 mm, freezing medium: mTeSR culture medium supplemented with 10% DMSO, 20% FBS, 1% 100X ROCK inhibitor and DMEM}

The hESCs were stained immediately after thawing. The viability observed in the figure 3.9 are consistent with the results obtained from the trypan blue assay.

3.3.5.2. Analysis of Post-thaw Cell Apoptosis Rate

In addition to investigating the cell metabolic rate, the apoptosis rates of the cells in RGDS-alginate encapsulation and suspension were quantified using AnnexinV-FITC and propidium iodide 2 hours after the thawing. The results are shown below in Figure 3.10.

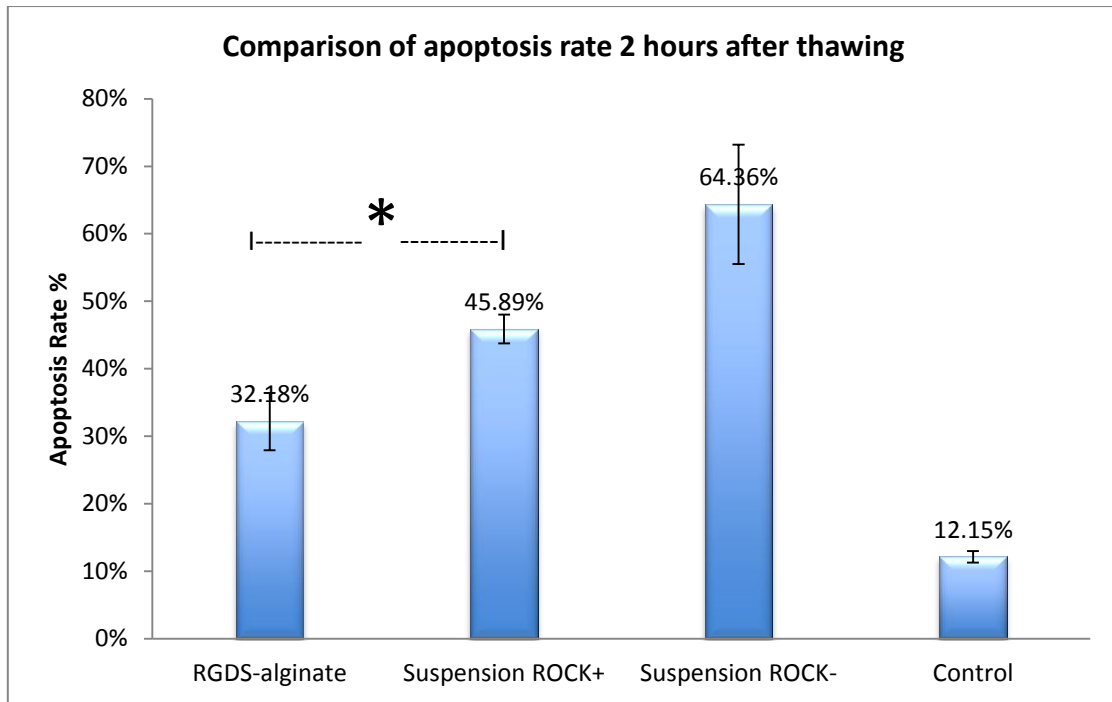


Figure 3.10 Analysis of apoptosis rates of cells cryopreserved in RGD-alginate with ROCK inhibitor, suspension with ROCK inhibitor, suspension without ROCK inhibitor. The control group is hESCs trypsinized from culture. {Experiment conditions: -1 °C /minute cooling rate, 1×10⁶ cells/ml, cells encapsulated by 1.2 wt% RGDS-alginate beads with mean diameter of 0.5 mm, freezing medium: mTeSR culture medium supplemented with 10% DMSO, 20% FBS, and DMEM}

The RGDS-alginate encapsulated hESCs have a lower apoptosis rate compared with cells in suspension as per Figure 3.10. The encapsulated cells have the benefit of attachment with engages anti-apoptotic pathways known to encourage cell survival and growth such as the cellular (caspase-8) FLICE-like inhibitory (c-FLIP), focal adhesion kinase (FAK) pathway and etc.. It has been previously reported that the ligation of the Fas receptor can lead to irreversible cell damage [144]. The encapsulation also blocks the apoptotic pathway. The Bcl-2 pathway is also activated which is known to stop the apoptotic cascade by regulating mitochondrial membrane permeability [167, 168]. Conversely, the hESCs, preserved in suspension supplemented with ROCK inhibitor, do not have such advantages.

Thus, the lower apoptosis rate of encapsulated hESCs matches this expectation. As the negative control group, the hESCs preserved in suspension without ROCK inhibitor supplementation, shows a consistently higher apoptosis rate than all the tested groups. The hESCs trypsinized from culture, as the positive control group, has the lowest apoptosis rate consistent with the absence of the negative effects of cryopreservation.

To sum up, the application of 3D encapsulation to hESC cryopreservation can greatly increase the qualities of the cryopreservation outcomes. Cryopreservation of hESC in calcium RGDS-alginate capsules with ROCK inhibitor supplementation diminishes cryoinjury and apoptosis in comparison to cryopreservation of hESC in suspension.

3.5.6. Viability Recovery of Subsequent Culture

3.5.6.1. Apoptosis Rate during Recovery

The cell apoptosis rate was monitored at the indicated times, namely 2, 4 and 24 hours after thawing. The comparative results are illustrated in Figure 3.11. As previously stated, the positive control group is hESCs in culture whilst the hESCs cryopreserved without ROCK inhibitor were used as the negative control group.

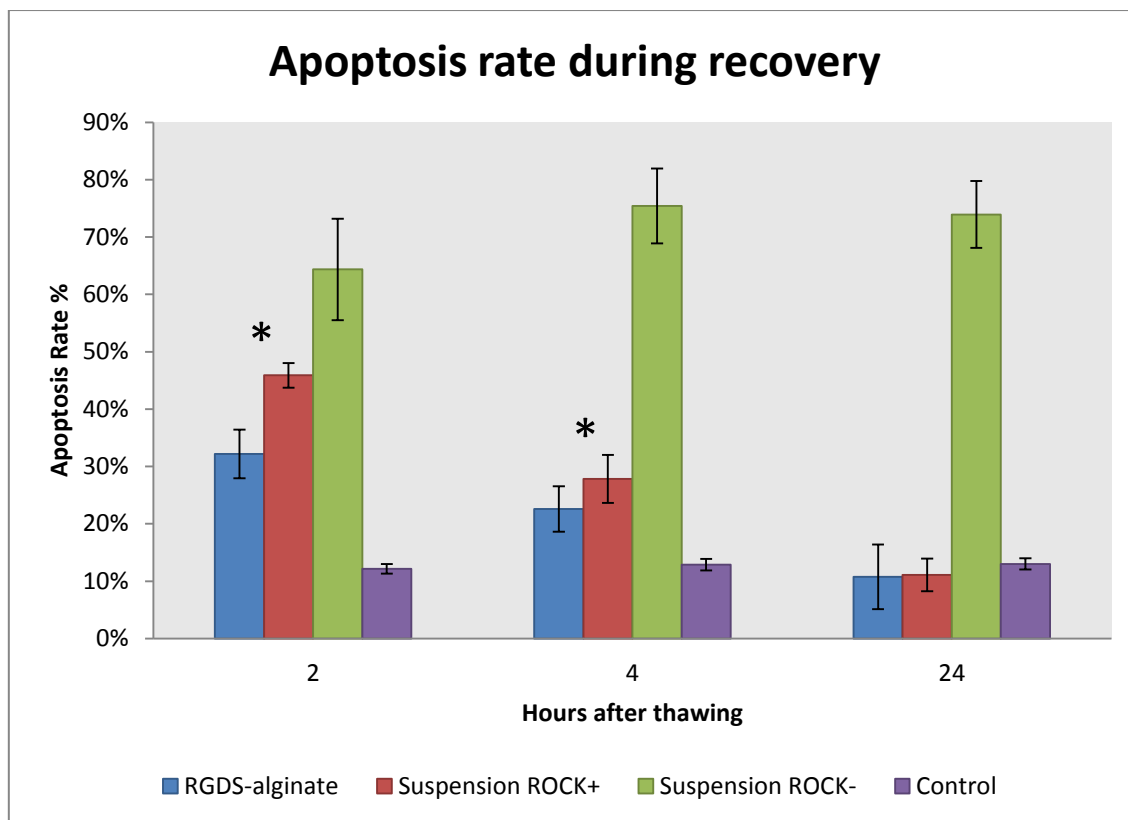


Figure 3.11 Analysis of 24 hours apoptosis rates in cells cryopreserved in RGD-alginate beads with ROCK inhibitor, cells cryopreserved in suspension with ROCK inhibitor supplementation post-thaw, following suspension cryopreservation without ROCK inhibitor supplementation (negative control), after trypsinization from culture (positive control). {Experiment conditions: $-1^{\circ}\text{C}/\text{minute}$ cooling rate, 1×10^6 cells/ml, cells encapsulated by 1.2 wt% RGDS-alginate beads with mean diameter of 0.5 mm, freezing medium: mTeSR culture medium supplemented with 10% DMSO, 20% FBS, and DMEM}

The same observation was obtained at 4-hour. Following the discussion of apoptosis rate observed at 2 hours post-thaw, the same explanation is applicable to the result obtained at 4-hour. There is no significant difference between the two cell populations in the 24-hour post-thaw where the benefits of 3D cryopreservation appear to be overwhelmed by cell doubling and acclimation to the post-thaw incubation condition. This can be gleaned from the similarity in the apoptotic rate observed in the control group and in the suspension ROCK+ group. The negative control group shows a consistently higher apoptosis rate of all

the groups tested. The decline in apoptosis rates observed in cells supplemented with ROCK inhibitor shows that apoptosis is reversible – this has previously been demonstrated [169].

3.5.6.1. Cell Attachment

After quantifying the cryopreservation outcomes following thawing, the hESCs in suspension and recovered from RGDS-alginate beads were seeded and grown at a 0.5×10^5 cells/cm² cell density on a 10 cm² well plate with ROCK inhibitor supplementation. The cell attachments were monitored after 24 hours incubation.

As the images in Figure 3.12 illustrate, the 3D encapsulated and in suspension cells exhibit a good rate of recovery after thawing and steady growth 24 hours after thawing. The result from Calcein-AM and EthD-1 staining is confirmed visually using images of cells cultured on 2D surfaces. The cells in image A and B (Images D and E are corresponding fluorescence images) are attached with limited numbers of floating cells, whilst the cells without ROCK inhibitor supplementation are mostly rounded, unattached and have large numbers of dead floating cells and cell debris.

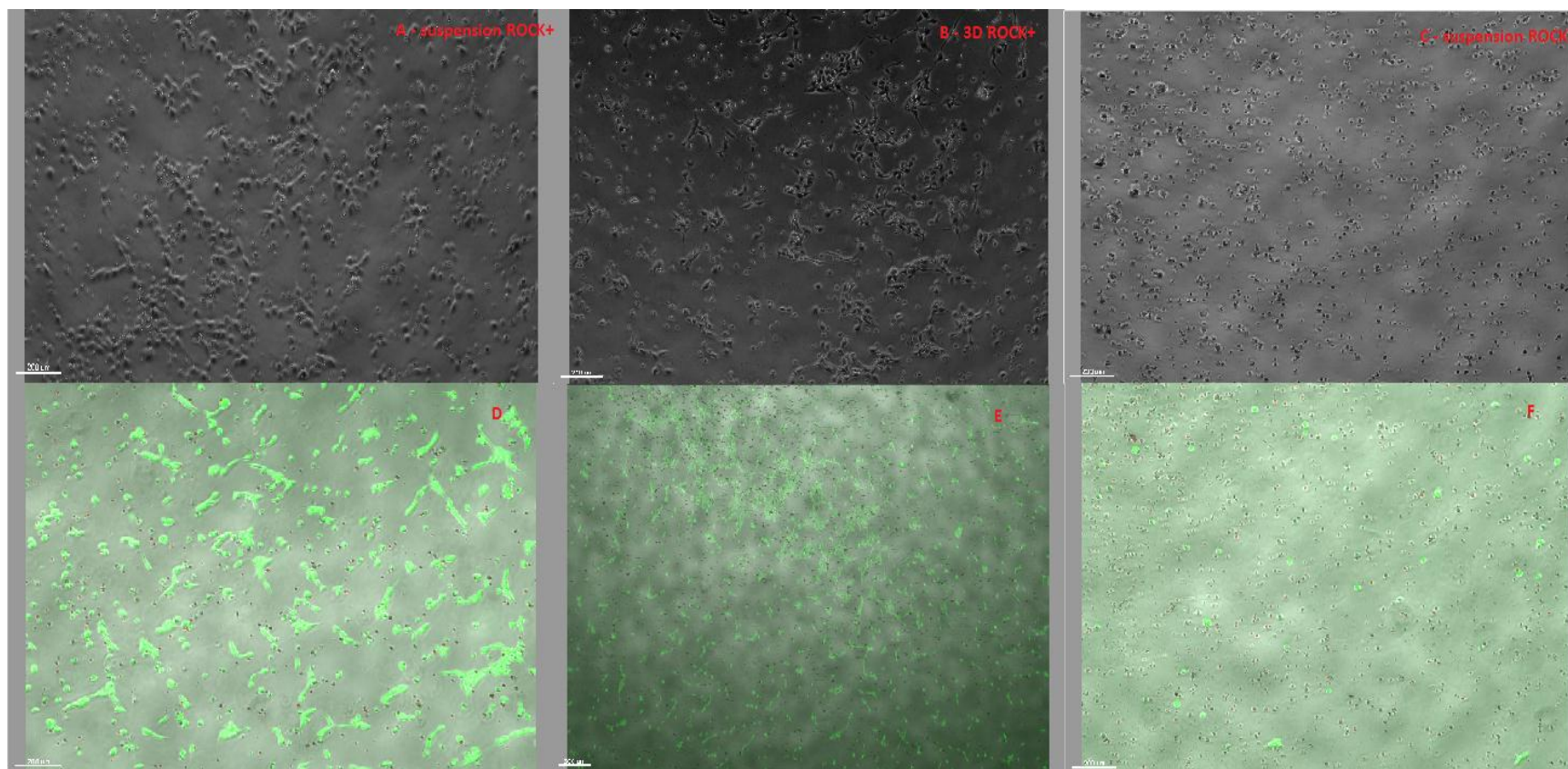


Figure 3.12 Cell growth 24 hours after cryopreservation compared with cells cryopreserved in 3D RGDS-alginate beads with ROCK (A), cells cryopreserved in suspension with ROCK (B) and cells cryopreservation in suspension without ROCK (C). Images of D, E and F are corresponding fluorescence images of A, B and C respectively. {Experiment conditions: $-1^{\circ}\text{C}/\text{minute}$ cooling rate, 1×10^6 cells/ml, cells encapsulated by 1.2 wt% RGDS-alginate beads with mean diameter of 0.5 mm, freezing medium: mTeSR culture medium supplemented with 10% DMSO, 20% FBS, 1% ROCK inhibitor and DMEM}

3.5.6.2. Subsequent Culture of hESC within the RGDS-alginate Beads after Thawing

After thawing, the hESCs encapsulated in RGDS-alginate beads were not recovered from the 3D scaffold. The beads containing hESCs were directly cultured in mTeSR culture medium supplemented with 1% ROCK inhibitor and 1% penicillin, at 37°C, 5% CO₂ and 21% O₂. The cell growth was monitored after 24 hours and the cell viability assay followed.

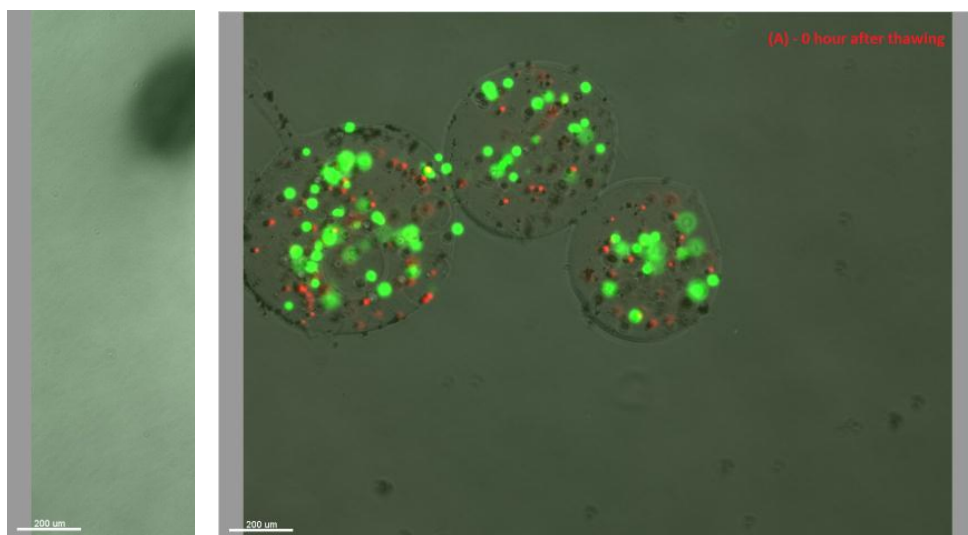


Figure 3.13 Cell growth within RGDS-alginate beads after thawing. Image A was captured 0 hours after thawing while image B was captured after 24 hours cultivation. {Experiment conditions: cells encapsulated by 1.2 wt% RGDS-alginate beads with mean diameter of 0.5 mm, 1×10^6 cells/ml, culture medium: mTeSR culture medium supplemented with 1% ROCK inhibitor and 1% penicillin}

The decrease of the cell survival rate was observed from Figure 3.13 after 24 hours culturing. This decrease was confirmed by cell viability assay after hESCs recovery from RGDS-alginate beads. The results are shown in Figure 3.14.

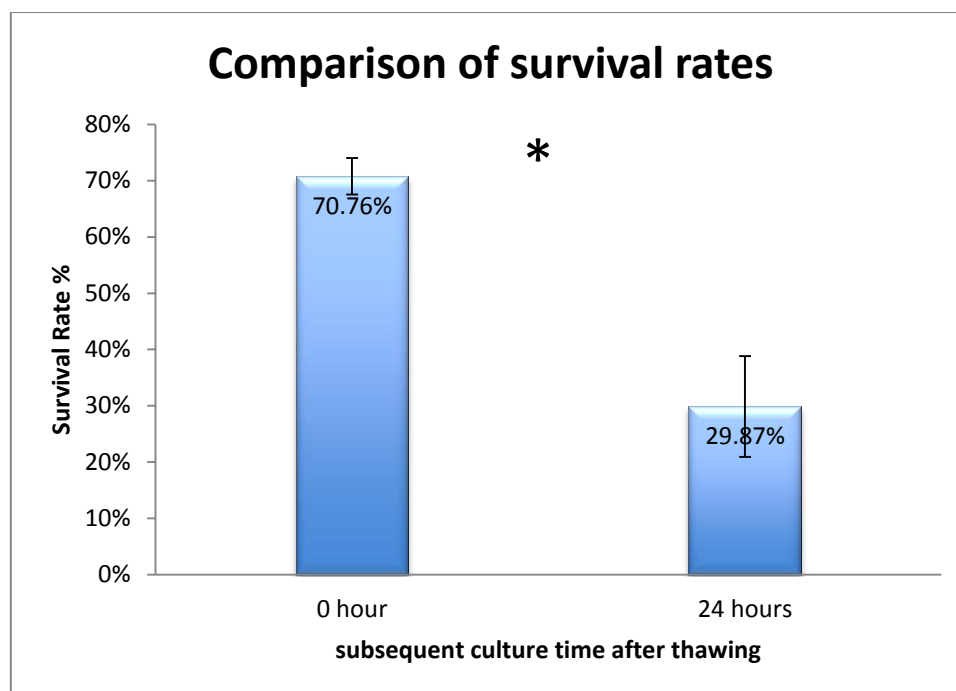


Figure 3.14 Comparison of cell survival rates between 0 hour and 24 hours subsequent culture. (* $p < 0.05$, according to two-tailed distribution T-Test). Data is given as mean $\pm$ standard deviation, obtained from at least three independent trials. {Experiment conditions: cells encapsulated by 1.2 wt% RGDS-alginate beads with mean diameter of 0.5 mm, 1×10^6 cells/ml, culture medium: mTeSR culture medium supplemented with 1% ROCK inhibitor and 1% penicillin}

The hESCs, recovered from encapsulation after 24 hours subsequent culture, show a significantly lower survival rate than the cells recovered immediately after thawing. It can be concluded from this observation that the environment of RGDS-alginate is far from perfect for hESC cultivation. The cell growth and the formation of cell colony require good cell-to-cell interactions. The loss of communication between neighbouring cells can lead to unstable cell growth [170]. Before the encapsulation process, cells were mixed thoroughly with alginate solution. After encapsulation, cells were separated from each other due to the viscous property of alginate. Thus, the cell-to-cell contacts were completely cut, which may well lead to cell apoptosis. Moreover, in order to maintain the spherical structure of the

beads and prevent adhesion of neighbouring alginate beads, a limited amount of calcium solution was added to culture medium. The induced calcium ions can prevent dissolving and re-formation of the alginate beads. As Maeno et al. stated, the high concentration of calcium ($>10\text{mM}$) is cytotoxic and leads to cell death [171]. Consequently, the hESCs cryopreserved in alginate encapsulation should be immediately recovered from the alginate beads after thawing in order to obtain an optimum preservation outcome. Since superior cell adhesion relies upon stable cell-cell interactions, the 2D culture for long-term hESC cultivation is irreplaceable.

3.5.7. Comparison of RGDS Modified Alginate to Unmodified Alginate

The major objective of 3D cryopreservation is to preserve the cells in their attached-state in order to increase the desired preservation outcome. Although the 3D ECM provided by unmodified alginate encapsulation can promote cell attachment, it does not have the advantages of the RGDS sequence, such as the ability to facilitate reactions between RGDS integrin receptor and corresponding receptor integrin of cell transmembrane. Thus, the cells encapsulated in RGDS-alginate have a higher attachment rate, therefore they have a superior cryopreservation quality. This conclusion is consistent with the results obtained from Figures 3.15 and 3.16.

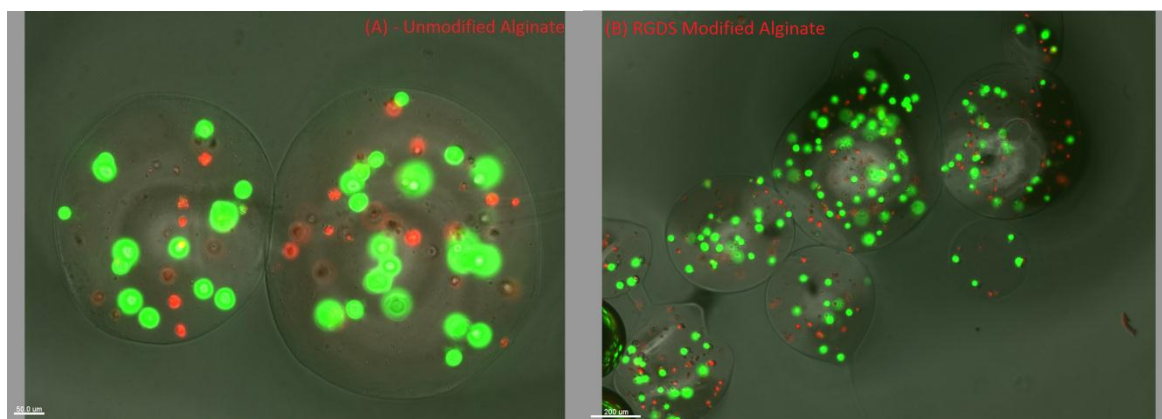


Figure 3.15 Comparison of cell survival rate between different encapsulation materials. (A) hESCs was cryopreserved in unmodified-alginate encapsulation. Scale bar was set to 50 micrometres (B) hESCs was cryopreserved in RGDS-alginate encapsulation. Scale bar was set to 200 micrometres. The cell survival rate observed from image B is higher than the one of image A. {Experiment conditions: $-1^{\circ}\text{C}/\text{minute}$ cooling rate, 1×10^6 cells/ml, cells encapsulated by 1.2 wt% RGDS-alginate/unmodified alginate beads with mean diameter of 0.5 mm, freezing medium: mTeSR culture medium supplemented with 10% DMSO, 20% FBS, 1% ROCK inhibitor and DMEM}

In order to quantify the exact cell survival rate, hESCs were recovered from the alginate beads and viability assay was applied. The post-thaw cell survival rate of hESCs cryopreserved in RGDS-alginate is significantly higher than the cells in unmodified alginate beads. The results obtained from Figure 3.16 are consistent with the observations in the fluorescence images as per Figure 3.15.

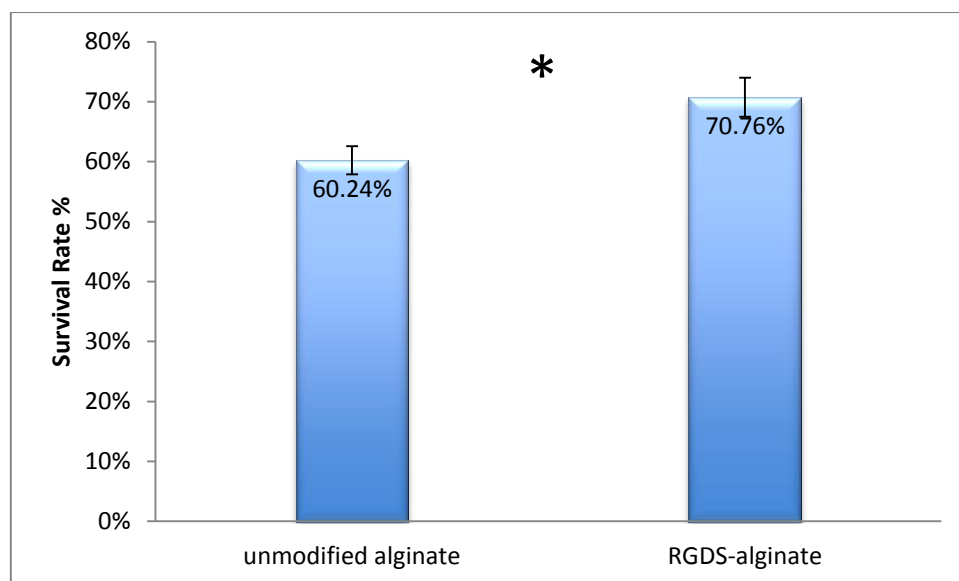


Figure 3.16 Comparison of post-thaw survival rate of hESCs recovered from unmodified and RGDS-modified alginate encapsulation. (* $p < 0.05$, according to two-tailed distribution T-Test). Data is given as mean $\pm$ standard deviation, obtained from at least three independent trials.

It can be concluded from the above data that the modification of alginate is necessary to achieve an efficient 3D cryopreservation process and also can lead to a more satisfactory outcome than unmodified alginate and suspension.

3.5.8. Possibility of Reducing CPA Concentration in 3D Cryopreservation

Given the toxicity of DMSO, the method of cryopreserving hESC in lower concentration of DMSO is constant area of exploration. The possibility of cryopreserving hESC in RGDS-alginate bead with 5% DMSO, as a low-toxicity cryopreservation method, was previously investigated. Any success was determined by the post-thaw cell survival and apoptosis rate.

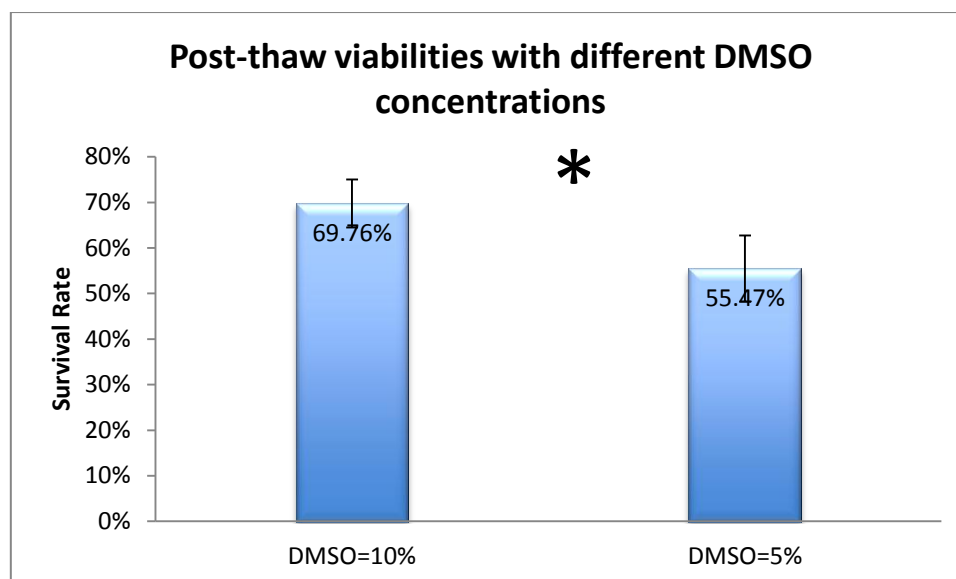


Figure 3.17 Comparison of cell survival rate for encapsulated hESCs cryopreserved in 5% and 10% DMSO. (* $p < 0.05$, according to two-tailed distribution T-Test). Data is given as mean $\pm$ standard deviation, obtained from at least three independent trials. {Experiment conditions: loading temperature: 20 °C, -1 °C/minute cooling rate, 1×10^6 cells/ml, cells encapsulated by 1.2 wt% RGDS-alginate beads with mean diameter of 0.5 mm, freezing medium: mTeSR culture medium supplemented with DMSO, 20% FBS, 1% 100X ROCK inhibitor and DMEM}

From Figure 3.17, 10 % DMSO supplementation still resulted in a higher survival rate when compared to 5% DMSO. This result is consistent with the 2D cryopreservation (the finding covered in 3.3.2). Thus, 10% is also considered as the optimum DMSO concentration for 3D cryopreservation.

After quantifying the post-thaw cell survival rate, the samples were seeded in 6-well plate to assess the cell recovery. Analysing the images in Figure 3.18, both hESCs cryopreserved in 5% and 10% DMSO exhibit a good rate of recovery after 24 hours incubation. Given the lower cell survival rate of 5% DMSO group, fewer cells were attached to the matrigel-coated surface when compared to the 10% DMSO group.

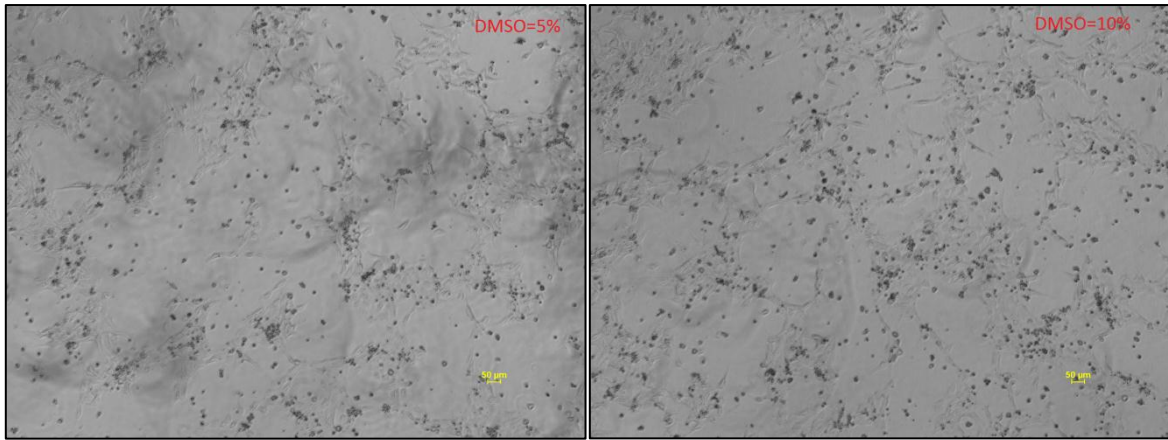


Figure 3.18 Comparison of cell attachment of hESCs preserved in 5% and 10% DMSO. Images were captured after 24 hours incubation. {Experiment conditions: cells recovered from RGDS-alginate beads after thawing and cultured for 24 hours, culture medium: mTeSR culture medium supplemented with 1% ROCK inhibitor and 1% penicillin, cells cultured on matrigel-coated flask}

3.5.9. ROCK Inhibitor

Due to ROCK inhibitor's expense cost and the complexity of handling during hESC maintenance and expanding, the experimental protocol was specifically designed to test the efficiency of 3D cryopreservation of hESCs without the presence of ROCK inhibitor.

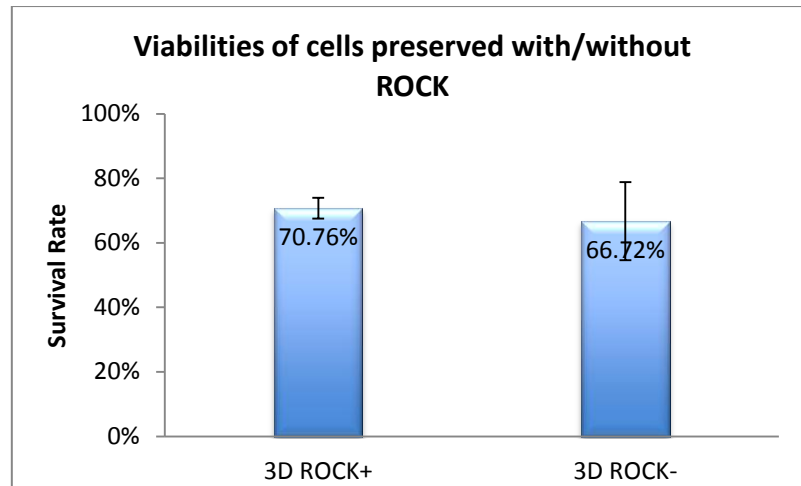


Figure 3.19 Comparison of post-thaw cell survival rate of 3D cryopreservation of hESC with and without ROCK inhibitor. Data is given as mean $\pm$ standard deviation, obtained from at least three independent trials. {Experiment conditions: loading temperature: 20 °C , -1 °C /minute cooling rate, 1 $\times$ 10⁶ cells/ml, cells encapsulated by 1.2 wt% RGDS-alginate beads with mean diameter of 0.5 mm, freezing medium: mTeSR culture medium supplemented with 10% DMSO, 20% FBS, and DMEM}

As Figure 3.19 illustrates, the addition of ROCK inhibitor does not affect the degree of cryoinjury since there is no significant difference between the two groups. In order to further analyse the effect of ROCK inhibitor on cell cryopreservation, the cell apoptosis rate was examined.

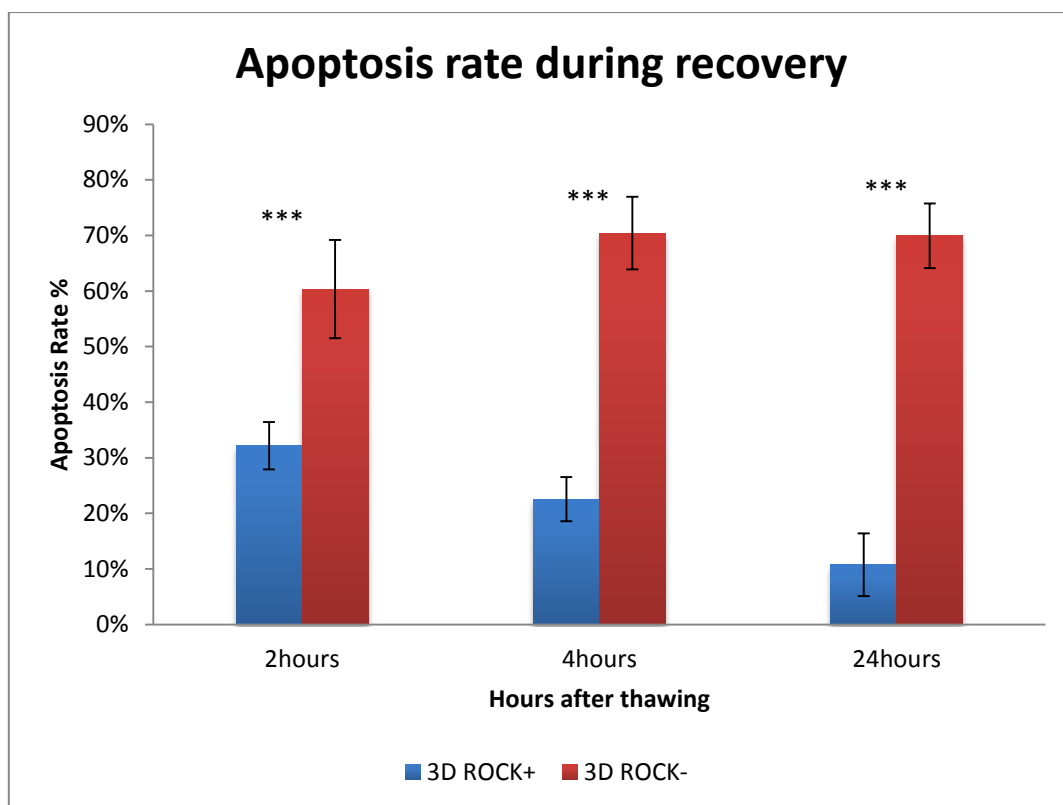


Figure 3.20 Analysis of apoptosis rates in cells cryopreserved in RGD-alginate beads with and without ROCK inhibitor. The data obtained at the time points of 2, 4 and 24 hours after thawing. (***p<0.001, according to two-tailed distribution T-Test). Data is given as mean±standard deviation, obtained from at least three independent trials.

It can be concluded from Figure 3.20 that the 3D cryopreservation without ROCK inhibitor shows a consistently high apoptosis rate. The significant higher apoptosis rate than ROCK+ group indicates that the hESCs cryopreserved without ROCK inhibitor would die successively after thawing. At the conclusion, less than 5% viable cells were observed after 24 hours. Therefore, the effects and abilities of inhibiting post-thaw cell apoptosis of ROCK inhibitor was strongly supported by the results demonstrated above.

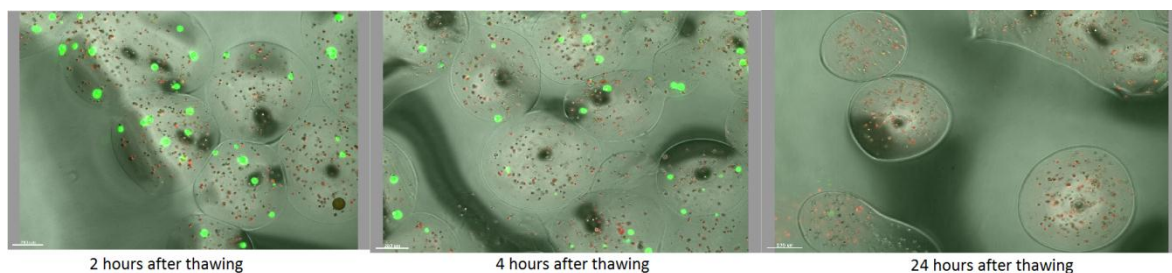


Figure 3.21 Subsequent culture of hESCs within the RGDS-alginate beads after thawing. All the samples were cryopreserved in RGDS-alginate beads without ROCK inhibitor supplementation. Images were captured after 2, 4 and 24 hours cultivation after thawing. {Experiment conditions: loading temperature: 20°C, -1°C /minute cooling rate, 1×10^6 cells/ml, cells encapsulated by 1.2 wt% RGDS-alginate beads with mean diameter of 0.5 mm, freezing medium: mTeSR culture medium supplemented with DMSO, 20% FBS, and DMEM, culture medium: mTeSR culture medium supplemented with 1% ROCK inhibitor and 1% penicillin }

The hESCs were subsequently cultured within alginate beads after thawing. From Figure 3.21, the decrease of cell viability was observed. The reason for this is supported by the fact that the decrease of cell viability is triggered by post-thaw apoptosis, due to the fact that the hESCs were not preserved with ROCK inhibitor [160]. A further reason concerns the imperfect 3D ECM provided by RGDS-alginate for hESC cultivation, as previously stated in 3.5.6.2. The end result, because of these two factors, was that the cells were all dead after 24 hours.

To sum up, the efficiency of hESC cryopreservation is low without the presence of ROCK inhibitor. Although the post-thaw viabilities of hESCs with and without ROCK were similar, the apoptosis rate of cells preserved without ROCK inhibitor was consistently higher after thawing. In regard to inhibiting post-thaw cell apoptosis, the 3D encapsulation does not superseded the presence of ROCK inhibitor in the CPA. Thus, the addition of ROCK inhibitor during 3D cryopreservation process is necessary and vital to storing and repopulating hESC stocks.

3.4. Summary

The aim of this chapter is to explore the feasibility of 3D cryopreservation of human embryonic stem cell using RGDS modified alginate, which is the one of the most important original points of this study. The technique of microencapsulation has been widely used as a powerful tool in genetic engineering, antibody-based genetic or cell therapy, anti-angiogenic cancer therapy and cell transplantation [172-176]. In cell cryopreservation area, microencapsulation using normal alginate has achieved successful preservation of certain types of cell line, such as rat hepatocytes and pancreatic islets [177]. However, there are not much researches regarding the application of microencapsulation using RGDS-alginate to human embryonic stem cell cryopreservation. This study provides a good foundation for the research in 3D cryopreservation of hESCs

In this work, a protocol of attaching RGDS peptide chemically to alginate backbone was developed. The presence of RGDS on alginate was demonstrated using FTIR and combustion analysis and 0.48% wt. RGDS attachment was identified. Comparing to commercially available RGDS-alginate production from NovaMatrix (0.8% wt.), the bonding efficiency was significant.

Human embryonic stem cells were cultured and expanded to a quality for the cryopreservation study. The effects of concentration of DMSO, loading time, cooling rate and thawing protocol were studied and suitable parameters were selected. An optimum procedure to encapsulate hESCs in RGDS-alginate was determined, so the 3D cryopreservation, thawing and cell recovery.

The viability of cryopreserved cells was determined under different conditions using various protocols. The following best viabilities were achieved, $62.78\% \pm 2.45\%$ in suspension and $70.76\% \pm 3.25\%$ in 3D encapsulation with RGDS modified alginate, which is higher than that with unmodified alginate. However, the post-thaw cell viability in suspension is not as good as the one achieved by other researchers (higher than 80%) [160, 178]. This is due to the high passage number of the cell in our lab. Dr. S. Sambu obtained higher than 80% post-thaw viability using hESCs with low passage number. As long as the data obtained using the cells from the same bench, the conclusion can be drawn that the 3D encapsulation using RGDS-alginate indeed improves the hESC cryopreservation outcome

Apoptosis was identified following thawing. ROCK inhibitor was applied and proved to be effective to increase cell attachment and long-term viability in the subsequent culture. Although the 3D encapsulation with RGDS-alginate also improved the cryopreservation outcomes regarding the post-thaw survival rate and apoptosis, its effect did not supersede the presence of ROCK inhibitor during freezing and thawing. Thus, the ROCK inhibitor is still considered necessary and vital to cell cryopreservation.

Chapter 4 Isolation and Characterisation of Human Primary Hepatocytes

4.1. Introduction

High quality hepatocytes are treasured products for biomedical research, pharmaceutical industry needs and therapeutic purposes [179]. Isolated primary hepatocytes and developed hepatocyte cell lines have been used widely to study xenobiotic metabolism for more than 30 years [180]. But it is well established and widely accepted that the majority of hepatocyte cell lines lack of primary key aspects of healthy primary hepatocytes functions. Therefore, they are likely to give less predictive results in research and are ultimately unsafe for therapeutically use [181]. Human primary hepatocytes are thus becoming highly demanded in all the areas above. In addition, due to the rapidly increasing needs of liver transplantation for treatment of many end-stage liver diseases and lack of organ donors, human primary hepatocytes caught scientists and surgeons' eyes and potential imagination for usage. In recent years, human primary hepatocytes have been used for cell transplantation to treat liver diseases due to its advantages of being less expensive and less invasive than orthotopic liver transplantation [182].

Although human primary hepatocytes are highly rated in both research and therapeutic areas, the quality and functional activity of the cells are variable due to different liver tissue conditions, isolation methods and culture protocols [183]. Since donor liver tissue's health

condition is beyond lab control, high hepatocyte yield isolation method and reliable cell culture protocol are in the process of being developed and need to be optimised.

Most importantly, the freshly isolated human hepatocytes can greatly affect the results of the hepatocytes cryopreservation. The freshly lab-isolated human hepatocytes in this study have the advantages of traceable tissue source, uniformity between benches and maintaining the original state of the cells before cryopreservation. However, commercially available human hepatocytes sample is also an option for the experiments. But it is not suitable for the studies of hepatocytes cryopreservation. The commercially available human hepatocytes do not possess some valuable properties of the cells, hence, lead to unreliable experimental results. Firstly, the sources of the commercially available hepatocytes are untraceable, which means the original condition of the liver tissue is unknown and may lead to lack of discussion of the results of hepatocytes cryopreservation. Secondly, commercially available human hepatocytes sample is a mixture of cells that isolated from multiple liver tissues. These liver tissues may come from different tissue donors. Such mixture makes the results of the cryopreservation are incomparable between different benches [184]. Last but not least, most of the commercially available human hepatocytes are cryopreserved cells because of the requirements of the storage and transportation. The post-thaw cells are nature-selected cells, which are totally unsuitable for the studies of hepatocytes cryopreservation. Thus, establishing a laboratory applicable and efficient isolation protocol of human hepatocytes and using freshly isolated human hepatocytes from same tissue source are desired and importation to the study of the hepatocytes cryopreservation.

In this chapter, two different human primary hepatocytes isolation methods are developed and presented in order to match different liver tissue conditions. Experiments were performed on various cohorts of non-diseased and diseased liver tissues, which are small piece of liver tissue (less than 10g with one or multiple cut surfaces), large liver tissue (weight between 50g to 150g with one cut surface) and whole but unsuitable for transplantation liver (severe fatty liver). The results of isolation efficiency are presented in terms of cell viability, cell adhesion rate, phase I and II enzyme activities and albumin secretion. At last, the freshly isolated hepatocytes were going to use as samples to study the cryopreservation of hepatocytes. The results are presented in next chapter.

4.2. Ethical Statement

The liver tissue samples were obtained from Tianjin Medical University Cancer Institute and Hospital. The experiments of using liver samples were approved by the Ethics Committee of the hospital following the procedure set up by the national ethic guidelines, with full consents of the patients.

4.3. Patient Information

The patients were screened from pancreatoduodenectomy (whipple procedure), liver cancer, kidney cancer and receiver of liver transplantation. Informed consents were obtained from the patients for the use of liver tissue for research purposes. Basic patient information

relevant to the study e.g. sex, age, ethnicity, medication and serological data were recorded. Virological status for HIV1 and HIV2, Hepatitis B (HBV) and Hepatitis C (HCV) was carried out on the patient by serological screening using approved diagnostic kits.

4.4. Materials and Methods

4.4.1. Liver Tissue Samples

There are 3 major type of liver tissue that are obtained and used in this experiment, (A) small piece of liver tissue (less than 10g with one or multiple cut surfaces), (B) large liver wedge (weight between 50g to 150g with one cut surface) and (C) whole liver but unsuitable for transplantation liver (severe fatty liver) (Figure 4.1).



Figure 4.1 Liver tissue samples. (A) Small piece of liver tissue, around 10 gram with 1 cut surface. Obtain from end-stage liver cancer. (B) Large liver wedge, around 80 gram with one cut surface. Two 20 gauge cannulae were sutured in to 2 selected vessels and clamped by hemostats. (C) Large liver wedge, around 200 gram with one cur surface. Cut From whole severe fatty liver which is unsuitable for transplantation.

As shown in Figure 4.1 above, the first type (Type A) of tissue samples were obtained from liver tumour removal surgery. Traditionally, the surgeon would treat the patient by cutting into his/her body and removing the located tumour along with some surrounding healthy tissue to make sure that entire cancer is cleared. After cancer tissue is removed, the surgeon will help to separate the healthy tissue apart from the tumour tissue. In this case, the liver tissue would not be large and normally less than 20 grams. Despite the small pieces, this type of tissue contains healthy human primary hepatocytes. However, it is hard to control the size and shape of the liver tissues, as it is beyond our control, which leads to the uncertainty whether there is any blood vessel inside the tissue. This is the disadvantage that cannot be ignored during isolation process, as the blood vessels are necessary if perfusion is used in isolation procedure.

The second type of liver tissue samples (Type B) are the ideal sample for isolation experiment and human primary hepatocytes study, as it has a blood vessel network and only has one cut surface. Normally this kind of liver tissue samples are obtained from pancreatoduodenectomy and kidney cancer. Since 200 gram of liver tissue is the minimum requirement for children liver transplantation, which has to be the priority for use of the liver tissue, the sample weight will not exceed 180 gram. In our case, as illuminated in Figure 4.1(B), the sample weight is 94 gram with only one cut surface. Its wedge shape indicates that it is from liver segment II and III, which are the ideal segments for hepatocytes isolation. As Figure 4.3 shows, segment II has 3 main blood vessels. This gives us huge advantage on perfusion based hepatocytes isolation experiment.

Isolation of human primary hepatocytes from the third type of liver tissue samples could benefit from the liver integrity and large isolated hepatocyte number (Figure 4.1(C)). However, the drawback cannot be ignored. Since the sample is severe fatty liver, the isolated cells are considerable fragile along with significant numbers of adipose cells. Thus, the difficulty of separating them apart could leads to unstable cell function or unsuccessful culture.

4.4.2. Materials

The following chemicals and biochemical were used in the experiments: Cannulae (Vasculon 14-20 gauge, vein size dependent, Becton Dickinson), Hank's balanced salt solution (HBSS, 14175-053, Invitrogen), Trypan blue (0.4 % T8154, Sigma), Isotonic Percoll (L6143, SEROMED), Na₂HPO₄ (Sigma), KCl (Sigma), NaCl (Sigma), CaCl₂ (Sigma C7902), Ethyleneglycoltetraacetic acid (EGTA, Sigma), Collagenase (Gibco powdered collagenase for hepatocyte isolation, Invitrogen), Williams E medium (A1217601, Invitrogen), GlutaMAX-I Supplement (100x, 35050-079, Invitrogen), Penicillin/Streptomycin (15140122, Invitrogen), Bovine insulin (I5500, Sigma), Fetal calf serum (FCS, 10270-098, Invitrogen), Collagen-coated 24-well plate (Becton Dickinson), HEPES (1 M, sigma), Dexamethasone (sigma), Insulin-Transferrin-Selenium (ITS, GIBCO), BSA (Sigma), Linoleic acid (sigma), 7-Ethoxyresorufin (E3763, Sigma), β -naphthoflavone (N-3633, Sigma), α -naphthoflavone (N5757, Sigma), Resorufin sodium salt (fluorescent labelled, TRC-R146500, LGCstandards, www.lgcstandards.com), P450-GloCYP3A4 Assay with Luciferin-IPA (V9001/V9002, Promega), Beetle Luciferin

Potassium salt (E1601, Promega), hepatocyte growth medium (HGM), 7-hydroxycoumarin (7-HC, TRC-H924875).

4.4.3. Buffer Preparation

Liver tissue wash buffer: 0.8 mM Na_2HPO_4 , 0.5 mM EGTA, 2.7 mM KCl and 137 mM NaCl, pH7.4.

Digestion buffer: 0.8 mM Na_2HPO_4 , 2.7 mM KCl and 137 mM NaCl, 5mM calcium chloride, 5mM magnesium chloride, 0.05% w/v collagenase (adding collagenase just before perfusion digestion start).

- Hepatocyte wash buffer: 6.2 mM KCl and 120 mM NaCl, 0.9 mM CaCl_2 , 10 mM HEPES, 0.2% Albumin.
- Percoll solution: To make 6 ml of Percoll solution by mixing 4.32 ml of 1X HBSS (72%) with 1.68 ml of Isotonic Percoll (28%) for purifying $6-8 \times 10^6$ hepatocytes.
- Hepatocyte seeding medium: 1X William's E medium supplemented with 1% 100X GlutaMAX-1, 1% Penicillin and Streptomycin, 4 $\mu\text{g/ml}$ bovine insulin, 15mM HEPES(1 M), 1 μmol Dexamethasone and 5% FCS.
- Hepatocytes sandwich culture medium: 1X William's E mediums supplemented with 0.5% Penicillin and Streptomycin, 15mM HEPES (1 M), 6.25 $\mu\text{g/mL}$ ITS, 1.25 mg/mL BSA and 5.35 $\mu\text{g/mL}$ Linoleic acid.

4.4.4. Liver Tissue Transport

4.4.4.1. Small Piece of Liver Tissue

Small piece of liver tissue is obtained from on-site liver cancer removal operation. Following the cancer operation, surgeon or assistants separate the surrounding normal tissue apart from the removed cancer tissue and place the normal tissue pieces into pre-cooled 50 mL tube filled with protective medium. This tube will then be placed inside 4 °C box and shipped to lab for isolation procedures.

4.4.4.2. Whole Liver

Once the liver was removed from the surgery, a step of pre-perfusion is required in order to remove any remaining blood and other fluid within the liver. This procedure is normally carried out by experienced surgeon with special tools. The perfusion tool is an infusion liked circulating system with one input cannulae sutured into main hepatic vein and an output one sutured into portal vein. Then the sterilized bag contained 2 litres of UW solution (static preservation solution, Belzer) will be raised to 1.5 meters higher than liver tissue. With the force of gravity, this system could achieve minimum 400 mL/min perfusion rate. Following this, the liver tissue will be immersed into sterilized bag filled with UW solution and then sealed. This bag will be placed inside 4 °C box and shipped to lab for isolation procedures.

4.4.5. Human Primary Hepatocytes Isolation Procedures

As motioned above, two different isolation protocols were developed for different type of liver tissue. One of the methods is called direct-digestion which is a reasonable isolation method for small piece of liver tissue where it is difficult to identify blood vessels. Alternatively, with whole liver or liver wedges obtained from either segments II/III or segments V/VI, isolations could be carried out using a modified “two-stage” collagenase perfusion procedure which originally developed by Berry and Friend [185]. The original perfusion method has been well established and widely adopted by many laboratories [183, 186, 187].

4.4.5.1. Direct-digestion Method

As its name suggested, the direct-digestion method is to digest small piece liver by enzyme. On arrival of the liver tissue at the laboratory, the liver tissue was gently removed from the tube and placed on a sterilized stainless steel plate. Then the tissue was dissociated by a mechanic force, for example scissors or scalpels (Figure 4.2). The stainless steel plate was filled with digestion buffer until the dissociated liver tissue was mostly immersed and it was left for around 2 minutes for the digestion process. The suspension was gently stirred with a scalpel and the suspension became increasingly cloudy. The suspension was poured out onto sterile mesh filter and consecutively filtered in the sequence of filter pore size of 850, 425 and 106 μm . In addition, the filter was shaken slightly and gently squeezed down the mixture above the filter during the filtering process in order to let it through the filter quickly and efficiently. After collecting the filtered suspension, the cell pallet was centrifuged at 50g for 2 minutes at 4 °C, and wash three times with HBSS. Then the

supernatant was removed, cell pellet was resuspended with cold seeding medium. The tube that contained the cell suspension was placed on ice for viability assay.



Figure 4.2 Direct-digestion method. Cutting the liver tissue by scalpel to smaller piece.

4.4.5.2. Two-stage Perfusion Method

This two-stage perfusion method of hepatocytes isolation procedure was originally designed by Berry and Friend [185], which was widely used for obtaining viable hepatocytes from rat liver. Afterwards, this method has been modified by many researchers [186-188] and applied to isolation of human primary hepatocytes. Although the protocols adopted by different researchers are similar in general, it is worth pointing out that minor modification of a single step could lead to different isolation results. Thus, the protocol should be modified on the actual needs of the experiments and the equipment of the laboratory.

Unlike direct-digestion method, the perfusion method requires the integrity of liver wedge and visualized blood veins. On arrival at laboratory, the liver was removed from the transportation bag, placed in ice-cold liver tissue washing buffer to remove any remaining blood and other liquid from within the liver wedge and to identify two/four suitable vessels depending on the size of the liver tissue that could be used for cannulation and subsequent perfusion of buffers. Two important criteria should be matched when choosing the right vessels for cannulation. First of all, a buffer wash without enzyme could apparently illustrate how large the area that each vessel can reach and perfuse. The greater the liver area that the vessel can reach gives a more favourable chance that this vessel will be chosen. Secondly, the different selected vessels should reach different areas of the liver wedge in order to achieve maximum efficiency. Subsequently, two/four 20 gauge cannulae were sutured into the selected vessels and then haemostat was used to secure the connection in order to ensure minimal loss of perfusion fluids (Figure 4.1(B)). The final stage of liver tissue preparation was to oversee any suspicious small vessels for the purpose of maintaining pressure within the liver tissue throughout the perfusion procedure and then place the liver wedge on an iron plate.

The perfusion system was set up based on a well-established protocol. One end of the perfusion tube was connected to the sutured cannulae, the middle segment of the tube was inside the peristaltic pump (model no. minipuls3, GILSON, France) and another end of the tube was fed into the perfusion buffer [184]. All perfusion buffers used in the procedure were pre-warmed in water bath to 37°C. A washing stage was initiated by using chelating wash buffer in a non-recirculation pattern at perfusion rate of 20 mL/min/cannula for 10 min. the purpose of this step was to disrupt cell adhesion to the underlying matrix. Since

the enzymes used to digest the liver tissue highly rely on calcium and magnesium for activation, a further perfusion without EGTA (non-recirculation, 1 minute) was carried out to remove any remaining EGTA from the tissue. After the washing stage, the digestion stage was initiated by recirculation of the digestion buffer for 20-30 minutes at a flow rate of 20mL/min/cannula. The decision to stop perfusion was prompted when the liver tissue was soft and fragile. After digestion stage, cell suspension was obtained by mechanic dissociation using scissors and passed through a sterile mesh filter in the orders of filter pore size of 850, 425 and 106 μm . The cell pellet was centrifuged at 50g for 2 minutes at 4°C and washed three times with HBSS. Then the supernatant was removed and resuspended the cell pellet with cold seeding medium and placed on ice for viability assay.

4.4.6. Cell Yield and Viability

Cell yield and viability was analysed by the trypan blue method and considered acceptable if greater than >50 % at this stage. If viability is lower than 50% but total cell yield is high, Percoll density gradient centrifugation of cell suspensions would be carried out in order to improve the yield of viable cells.

4.4.7. Purification with Percoll.

If necessary, human hepatocytes can be purified using Percoll solution. Briefly, slowly place 3 ml of cell suspension above 6 ml of Percoll solution (incline the tube at 30 °with the end of the pipette being in contact with the inferior inner side of the tube). Be careful not to mix cell suspension with the Percoll solution. Centrifuge at room temperature for 3 min at

180g. Remove the supernatant, and re-suspend the cell pellet in 3 ml of cold seeding medium then count the cell. Put the tube with the cell suspension on ice or at +4 °C until using of the cells.

4.4.8. Sandwich Culture of Human Primary Hepatocytes

The difficulty of culturing human primary hepatocytes is widely acknowledged due to the cells' sensitivity to changing environments [189]. A well-established culture protocol named collagen sandwich culture is adopted in this project. Human hepatocytes were sandwiched between two layers, which are a collagen basal surface and a matrigel apical surface. Such a design like this could provide a stable culture environment that more closely resembles the in-vivo polar geometry for hepatocytes.

Firstly, the cell pellet was resuspended with hepatocytes seeding medium and 2.5×10^5 cells were seeded into each well of collagen pre-coated 24-well plate along with 500 μ L to 1 mL seeding medium. Then the 24-well plate was placed into incubator for 24 hours for cell adhesion. After cell attachment, the supernatant was collected and the cell adhesion rate was calculated. Secondly, add 500 μ L hepatocytes sandwich culture medium with 5% matrigel to each well and place into incubator for another 12 hours. Finally, the matrigel solution was replaced with same volume of sandwich culture medium and then leave the plate in the incubator for function test. The culture medium should be changed every two days.

4.4.9. Cell Adhesion Assay

Hepatocytes adhesion assay was carried out after cell attachment. Unlike cell viability assay, the cell adhesion rate was obtained by counting the dead cell number in the supernatant and then calculating the attached cell rate.

4.4.10. Function Assays of Human Primary Hepatocytes

Functions of Isolated human primary hepatocytes are analysed by measuring CYP1A2 and CYP3A4 activities and Hepatocyte Phase II Enzymes Assay, as both would be the key features of human hepatocytes and important for drug metabolism.

4.4.10.1. CYP1A2 Assay

CYP1A2 activity is measured using ethoxyresorufin as a substrate, β -naphthoflavone as an inducer, and α -naphthoflavone as an inhibitor. The assays are performed in 24-well plate with serum-free medium. CYP1A2 metabolic activity is expressed by pmole resorufin production/million cell/minute. Fold increase/decrease on CYP1A2 activity after induction and/or inhibition is analysed by dividing control which is baseline CYP1A2 activity.

The protocol is as follow:

- Resorufin standard prepare stock solutions:

1 mM ethoxyresorufin: dissolve 0.001 g ethoxyresorufin in 416 μ l sterile DMSO.

30 mM β -naphthoflavone: dissolve 0.0082 g β -naphthoflavone in 1 ml sterile DMSO.

30 mM α - naphthoflavone: dissolve 0.0082 g α - naphthoflavone in 1 ml sterile DMSO.

- At a testing point, samples are grouped to four, named as control, induction, inhibition and induction + inhibition.
- Discard the culture medium for each sample, and add 500 μ l of serum-free medium to each sample well.
- Add 0.5 μ l inducer β -naphthoflavone into each well of sample group named induction and induction + inhibition only (1:1000 dilution, final concentration is 30 μ M). Incubate all sample groups in a 37°C 5% CO₂ incubator for 72 hours.
- Add 0.5 μ l α - naphthoflavone (1:1000 dilution, final concentration is 5 μ M) in each well of sample group named inhibition and induction + inhibition, and incubate all sample groups at 37°C for 1 hour before addition of 7-Ethoxyresorufin.
- Add 2.5 μ l of 1 mM ethoxyresorufin (1:200 dilution, final concentration is 5 μ M) in each well of all sample groups, mixed well. Immediately take 100 μ l samples from each well of all sample groups into a black 96-well plate. Put a lid on the plate, marked as T0 and keep it in the dark. Incubate rest volume of samples at 37°C for 1 hour.
- After incubation, transfer 100 μ l of samples to the same black 96-well plate which contains T0 samples, and marked as T60. Read samples on a FLX800 fluorescence microplate reader from top at 530 nm excitation, 590 nm emission. Resorufin standard (starting 2.5 μ M) diluted in buffer as a standard.

- All T60 data will be substrate T0 before calculating resorufin production based on resorufin standard curve and/or fold increase/decrease of resorufin production normalised to control.

4.4.10.2. CYP3A4 Assay

Human hepatocytes CYP3A4 activity is measured using the P450-GloTM substrate from Promega. In the assay a luminogenic substrate is added to the culture medium and incubated with cells for an hour. Intracellular CYP enzymes convert the substrate to D-luciferin, which passes out of cells into the medium, where it can be detected. The light output of the luciferase reaction is proportional to CYP activity using a standard curve.

The protocol is as follow:

- Stock solutions

Luciferin Detection Reagent: transfer the entire contents of the bottle of the appropriate reconstitution buffer to the amber bottle containing Luciferin Detection Reagent. Mix by swirling or inverting several times, and equilibrate to room temperature before use. Store the unused portion at -20 °C.

20 mM stock D-luciferin standard: dissolve 50 mg of Beele Luciferin potassium salt in 7.85 ml of water. Aliquot and store at -80 °C in dark.

- Treat cells with inducers for 24-72 hours. Optimal treatment time should be determined empirically; however, 48 hours is a common starting point. Omit this step if only detect basal enzyme activity.
- After an experimental treatment, remove spent medium and wash cells with working luminogenic substrate medium (1:1000 dilution in complete culture medium). Add 0.5 ml per well for sandwich culture plate.
- If inhibitors of basal or induced CYP enzyme activity are being tested, add them at this point with the luminogenic substrate. Omit this step if only detect basal or induced enzyme activity.
- D-luciferin standard curve: dilute 20mM stock standard solution in complete culture medium to generate 2 μ M working standard solution (1:10000). Prepare duplicate standard points by serially diluting working standard solution to produce concentration of 1 μ M (taking 500 μ l 2 μ M standard solution into 500 μ l complete culture medium), 0.4 μ M (taking 200 μ l 1 μ M standard solution into 800 μ l complete culture medium), 0.08 μ M (taking 200 μ l 0.4 μ M standard solution into 800 μ l complete culture medium), 0.016 μ M (taking 200 μ l 0.08 μ M standard solution into 800 μ l complete culture medium). Complete culture medium serves as the zero standard (0 μ M). Transfer 50 μ l of the serial dilution standard solution into wells of a 96-well opaque white luminometer plate. 50 μ l of luminogenic substrate medium was as blank.
- Incubate plates containing samples and standards and blank at 37 °C for 60 min in the dark.

- After incubation, transfer 50 µl of sample medium from each sample well to the 96-well opaque white luminometer plate containing standards. Add 50 µl of Luciferin Detection Reagent to each well to initiate a luminescent reaction.
- Incubate the plate at room temperature for 20 min in the dark, and then read luminescence using a luminometer.
- Calculate net signals by subtracting blank luminescence values (no-cell control) from sample values.
- Calculate CYP enzymatic activity using a standard curve or fold changes by dividing net induction/inhibition values by net basal values.

4.4.10.3. Hepatocyte Phase II Enzymes Assay

Glucuronidation is a major phase II metabolic pathway for inactivation and excretion of many drugs and the assay is performed by UGT. This activity is measured by monitoring the reduction in 7-HC fluorescence, the substrate of UGT, with glucuronic acid and sulphate group which mediated by Phase II enzymes (UPD-Glucuronyl-transferase and Sulfo-transferase).

The protocol is as follow:

- Making working substrate solution: dilute stock 50 mM 7-HC into culture medium (1:1000 dilution) to make 50 µM 7-HC substrate solution. Reserve 0.5 ml of this substrate solution in room temperature in the dark as Standard solution.
- For Sandwich culture: remove culture medium, add 500 µl working substrate solution in each sample well and one cell-free well as blank 1.

- Take 0.5 ml of working substrate solution into a 1.5 ml glass Chromacol vial as blank 2 (input).
- Incubate both samples and blanks in the dark at 37 °C for 1 h.
- After incubation, transfer 100 µl of samples, blank 1 and blank 2 to the black 96-well plate. Reserved substrate solution 7-HC (starting 50 µM) diluted in culture medium as a standard. Read samples and standards on a FLX800 fluorescence microplate reader from top at 410 nm excitation, 510 nm emission.
- All data will subtract to blank 1 before calculating Phase II activity based on standard curve. Phase II activity is expressed as pmole/million cells/min, or pmole/mg total cell protein/min.

4.4.10.4. Albumin Secretion Assay

Albumin secretion is determined by a sandwich enzyme-linked immunosorbent assay (ELISA), using a human albumin ELISA kit. Human albumin is used for establishing the standard curve. Albumin secretion rate is expressed as ug/million cells/24h.

- Centrifuge spent cell culture media at 3000 ×g for 10 min to remove debris. Collect supernatants and store at -20°C. Thaw samples before assay.
- Prepare all reagents, working standards and samples as instructed. Bring all reagents to room temperature (RT) before use. The assay is performed at RT.
- Add 50 µL of standard or sample per well of microplate strips. Cover wells with a sealing tape and incubate for one hour.

- Wash five times with 200 μ L of Wash buffer. Invert the plate and decant the contents, and hit it on absorbent paper towel to completely remove liquid at each step.
- Add 50 μ L of Biotinylated Human Albumin Antibody to each well and incubate for 30 minutes.
- Wash five times with 200 μ L of wash buffer.
- Add 50 μ L of streptavidin-peroxidase conjugate to each well and incubate for 20 minutes.
- Wash five times with 200 μ L of wash buffer.
- Add 50 μ L of Chromogen Substrate per well and incubate for about 12 minutes or till the optimal blue colour density develops. Gently tap plate to ensure thorough mixing and break the bubbles in the well with pipette tip.
- Add 50 μ L of stop solution to each well. The colour will change from blue to yellow. Read the absorbance on a microplate reader at a wavelength of 450 nm immediately.

4.4.11 Cell Morphology and Viability

LIVE/DEAD[®] Viability/Cytotoxicity Kit (Life Technologies, Invitrogen[™], UK) was used for hepatocytes imaging in order to examine the cell viability and also morphology. This kit consists of two types of fluorescence dye – Calcein-AM which colours the live cells green and EthD-1 which stains the dead cells red. Both bright field images and fluorescence images of cell adhesion were taken by using fluorescence microscopy (Nikon eclipse Ti-E, Nikon, UK). In order to perform fluorescence imaging of the attached hepatocytes, the

culture plate containing human hepatocytes was removed from the incubator after cell attachment and the culture medium was removed from the well. Then, 1 μ M calcein-AM in PBS and 5 μ M EthD-1 in PBS were added to each well until the cell was fully covered. The culture plate was left for incubation at room temperature for 30-34 minutes in dark. Finally, the sample was place under a fluorescence microscope for imaging.

4.5. Results and Discussion

4.5.1. Sample Categorization

The selected samples are initially labelled in the order of operating date and then further divided into 4 different groups in terms of size and isolation protocols. Samples classified as Group A were treated by using the direct-digestion method and they are smaller than 10g. The sizes of the samples in Group B are also smaller than 10 g but the perfusion method is used to isolate cells. Compared to the previous two groups, samples in Group C are treated with the same method as Group B and their sizes are between of 10g and 200g. Group D differs from the other three groups since it is a mixed group from the same fatty liver. Sample 11 is slightly smaller than 10g and isolated by using direct-digestion method while sample 10 is larger than 100g and isolated by using perfusion method.

The patients' information and results are all listed below in table 1 and table 2.

Table 4.1 Patients information and Comparison of isolated hepatocytes viability and cell yield rate. * Viabilities were based on the average reading of three independent cell counting of the same sample.

	Sample	Weight	Gender	Age	Disease	Cell No.	Viability before Percoll*	Cell No. after Percoll	Viability after Percoll*	Cell yield/g (million cell/g)
Group A	1	2.54g	M	69	liver cancer	1.5×10^5	64.59% $\pm$ 2.36%			0.06
	2	7.95g	M	71	liver cancer	1×10^6	73.78% $\pm$ 3.01%			0.13
	4	4.6g	F	68	liver cancer	1.6×10^6	57.48% $\pm$ 2.79%			0.35
	5	0.75g	M	59	pancreatic cancer	5.2×10^5	64.89% $\pm$ 4.03%			0.69
Group B	7	5.3g	M	60	liver cancer	1.7×10^7	82.79% $\pm$ 3.98%			3.21
	8	4.75g	M	73	Cholangiocarcinoma	5.49×10^6	40.00% $\pm$ 4.21%	4.29×10^6	77.90% $\pm$ 0.96%	1.16
	12	6g	F	62	gastric cancer	1.78×10^7	51.00% $\pm$ 2.87%	9.6×10^6	87.12% $\pm$ 1.36%	2.97
	13	3.4g	M	54	liver cancer	5×10^6	23.00% $\pm$ 1.36%			1.47
Group C	3	10.48g	M	72	Cholangiocarcinoma	3.49×10^7	76.00% $\pm$ 5.03%	2.77×10^7	96.80% $\pm$ 1.00%	3.33
	6	15.25g	F	63	Cholangiocarcinoma	1.22×10^8	87.69% $\pm$ 4.25%			8.00
	9	16.5g	M	65	renal cancer	2.32×10^7	41.70% $\pm$ 1.85%	1.4×10^7	80.80% $\pm$ 1.56%	1.41
	14	94g	F	66	pancreatic cancer	4×10^8	67.32% $\pm$ 2.84%			4.26
	15	43g	F	50	renal cancer	2.9×10^8	73.00% $\pm$ 4.36%			6.74
Group D	10	140	M	45	fatty liver	2.89×10^7	35.00% $\pm$ 1.25%	1×10^7	87.30% $\pm$ 1.36%	0.21
	11	10	M	45	fatty liver	1.25×10^6	46.00% $\pm$ 1.59%	3.2×10^6	80.00% $\pm$ 1.09%	0.13

Table 4.2 Comparison of average viability and average cell yield rate between different groups

	Sample Size	Isolation Method	Average Viability	Average Cell Yield/g (million cell/g)
Group A	sample<10g	direct-digestion	65.19%	0.31
Group B	sample<10g	perfusion	49.20%	2.20
Group C	10g<sample<200g	perfusion	69.14%	4.75
Group D	10g<sample<200g	mixed methods	40.50%	0.17

4.5.2 Viability and Cell Yield Rate after Isolation

4.5.2.1. Effect of Different Isolation Methods on Hepatocytes Viability and Isolation Efficiencies

The cell viabilities and cell yield rates are listed in Table 4.2 in order to compare the differences that occur between all the groups as a whole. It is apparent from the Table 2 that the viability of group A is higher than group B and the only difference between these two groups is the isolation method. However, it is obvious from table 1 that the viability of sample 7 is significantly higher than any sample in group A. A further observation is that the average viability of group C is higher than group A. Comparatively, the argument over which method is better is not a conclusive one. Consequently, there is no direct relationship between viability of isolated hepatocyte and the isolation method. This is hardly surprising as one major factor, the liver tissue status, is beyond control, and the location, size and shape of the samples.

Although the average viability of group A is relatively high, the cell yield rate is extremely low compare to group B and group C. To illustrate this, a comparison between sample 2 and sample 12 can better demonstrate this issue. Even though these two samples have similar in size and adopt same isolation method, the cell yield rate of sample 12 is twenty-two times greater than sample 2. Such low cell yield rates of group A indicate that direct-digestion method lacks efficiency and wastes tissue sample and experiment materials. Thus, for the hepatocytes isolation the perfusion method is much preferred because of its higher cell yield rate.

It is also worth comparing our results with other published results. Baccararani et al. published their results based on 2-year experience and hundreds of cases in 2003. They achieved a cell yield rate of 7 million hepatocytes/g of tissue and a post-isolation cell viability of $73\% \pm 14\%$ [190]. Another group, R.R.Mitry et al., obtained similar results in the hospital lab in 2002, which is 70% post-isolation cell viability for healthy liver tissue and 35% for transplantation-rejected liver tissue [191]. The results of group C, who has the ideal samples for hepatocytes isolation, are not as good as theirs but very close. The main reason is that they have the equipment that can achieved 350mL/min perfusion rate, which is normally the one used for preparation before liver transplantation surgery in hospitals. Higher perfusion rate provide higher pressure within the liver tissue that allow the digestion buffer to reach larger area of the tissue, which give great advantages in isolation efficiency [190, 191]. Unfortunately, our peristaltic pump can only provide 20 mL/min/cannula perfusion rate in the lab. But it should be noted that it is not always possible to apply fast perfusion into small pieces of liver tissue.

Comparing to others' results which based on similar protocol to ours, great improvement was observed in this study. Bhogal et al. published their result based on over a hundred cases in 2011, including normal liver tissue, alcoholic liver disease, biliary cirrhosis and primary sclerosing cholangitis, and other end-stage liver diseases, and achieved 40% overall post-isolation cell viability and 55% on normal liver tissues [184]. Moreover, Alexandrova et al. achieved a mean post-isolation viability of 64% and a mean yield rate of 2.6 million cell/g liver tissue [192]. The post-isolation cell viability and cell yield rate achieved in this study, which are 69.14% before purification for viability and 4.75 million cell/g liver tissue for cell yield rate, demonstrate that the adopted protocol is accurate, efficient and more adaptable to laboratory use.

4.5.2.2. Effect of Different Sample Size on Hepatocytes Viability and Isolation Efficiencies

The data presented in Table 4.1 and Table 4.2 suggest that group C shows much higher viabilities as well as cell yield rate when compared with group B. The results match the expectation. Large liver tissue has three distinct advantages. First of all, most of large liver tissues have a single cut surface. Since the liver tissues were cut by electronic scalpel during the operation in order to minimize the blood loss of the patient, the more times that the live tissue has been cut, the more tissue would have been damaged. Compare to small pieces of liver tissue with multiple cut surfaces, large liver tissue can maintain the integrity of the tissue surface. This could well lead to high cell survival rate [184, 193]. Secondly, large liver tissues are normally obtained from liver segment II/III. As Figure 4.1 shows, liver tissue of segment II/III consists of countless blood vessels. The complexity of the blood vessel system gives the hepatocytes isolation procedure great advantages by allowing

the digestion buffer to perfuse to more areas of the liver tissue hence increasing the efficiency of the isolation procedure. A further advantage is that large liver tissues are easy to handle in practical procedures. It can be seen from Figure 4.3, liver segment II/III has up to six different main blood vessels on the cut surface. Such blood vessels can be easily identified and then sutured outside the cannulae, which results in the digestion buffer flowing smoothly and then minimize the buffer loss. When operating on small pieces of liver tissues with unidentified blood vessels, cannulae can only be introduced into liver tissue from the surface. Thus, the isolation procedure could risk an unexpected break of the liver tissue because of the increasing internal pressure. This theory is backed up by the studies of Bhogal et al. and other groups that the isolations carried out using liver wedges with single cut surface gave higher success rate [192-194]. In addition, they also proposed that the optimal liver tissue weight for isolation is approximately 100 gram and 10-120g is more favourable to successful and efficient isolation results [184]. Albert P. Li concurred with this and demonstrated in general large liver fragments (10-300g) gave better results than whole livers and lobes [99, 191, 192]. In this study, the best isolation results came from group C which is within the optimal liver weight range. In practical, according to the experience of the experiments, the liver tissues ranging from 50 g to 100 g are much easier to handle. To sum up, these results demonstrate convincingly that large pieces of liver tissues are ideal for isolation procedure.

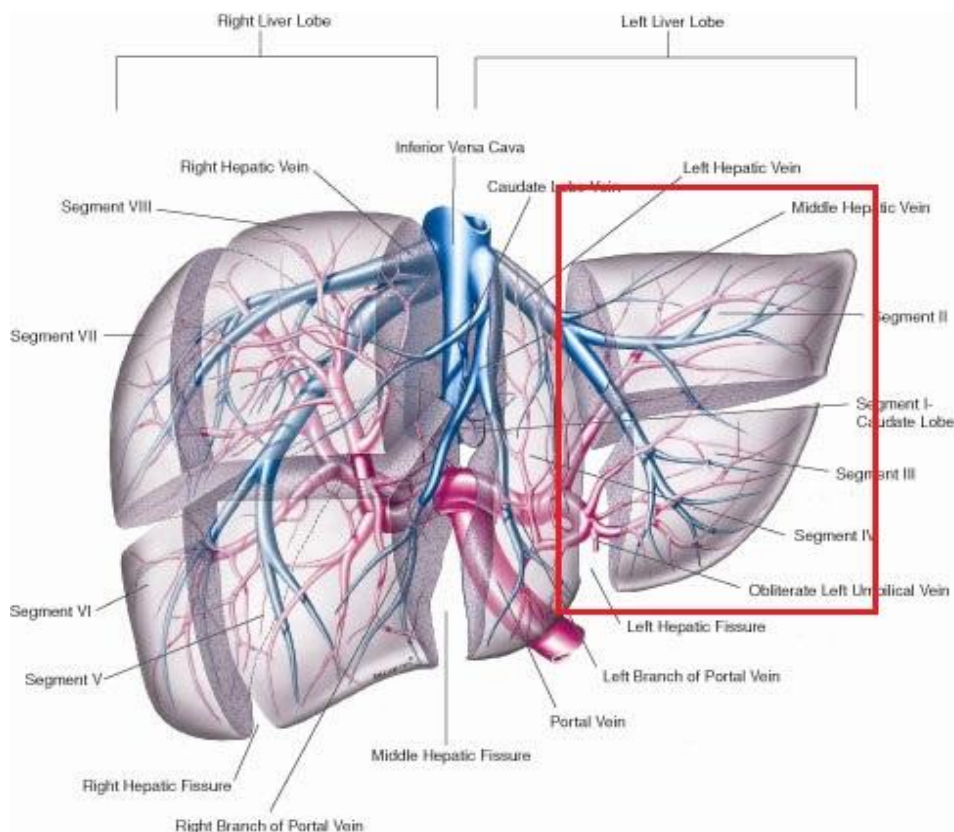


Figure 4. 3 Liver vasculature. Large liver tissues are normally obtained from segment II/III, which was boxed out in red.

4.5.2.3. Effect of Liver Conditions on Hepatocytes Viability and Isolation Efficiencies

By comparing the results between sample 10 and 11 in group D, it is apparent that the condition of the liver tissue is vital to the condition of the isolated cells. According to patient information, the patient of sample 10 and 11 is the receiver of a liver transplant. His severe fatty liver does not function properly, which indicates that the viability and cell function index of hepatocytes isolated from this liver tissue could also be low in. Although the two samples are different in terms of size and isolation method, the results are similar in terms of low cell viability and cell yield rate. Many researchers have drawn the conclusion that high degree of steatosis generally lead to unsuccessful cell isolation [192-194]. Patients,

who were under pre-surgery radiation therapy or chemotherapy, may also provide liver tissue in an unstable or poor condition. It is an obvious fact the radiation therapy and chemotherapy can lower the human immunity to illness and disease, resulting in a higher risk of organ damage [184]. For instance, the patient of sample 9 has undergone radiation therapy before operation and the viability of this sample is as low as 41.7% despite the fairly large tissue size and the use of a proper isolation method.

By observation of the entire isolation procedure of sample 10 and 11, the fat cells can greatly impact the efficiency of hepatocytes isolation. After tissue digestion stage, the collected cell suspension is a mixture of hepatocytes and fat cells, where the fat cells are the majority. At this stage, it is impossible to isolate hepatocytes from the mixture suspension. Table 1 illustrates that hepatocytes, isolated from fatty liver tissues, have an extremely low cell yield rate. Except for fatty liver, there is no evidence that could link other poor conditions of liver tissue to low cell yield rate.

Moreover, most authors have previously made the assumption that the function of isolated human primary hepatocytes from normal liver tissue was consistent between different sources [184]. In light of our findings, this assumption is not entirely accurate. The sample 6 and 9 are all normal liver tissues from surgery for other organs (Cholangiocarcinoma: operating on liver only because the two organs are closed to each other; Renal cancer: operating on liver only because of the kidney is located at the back of the liver). These two samples are similar in term of patient age and sample size. However, the isolation result is totally different. Therefore, further function analyses of human hepatocytes isolated from different liver tissues are definitely necessary. More importantly, a group of comparable

cryopreservation results also rely on the normality of the freshly isolated human hepatocytes. This is the main reason that freshly isolated hepatocytes are more favourable to the study of cryopreservation of human hepatocytes.

To sum up, viabilities of isolated hepatocytes are highly dependent on the conditions of liver tissue. Due to the lack of variety of poor liver tissue condition, only fatty liver was identified to have a low cell yield rate.

4.5.2.4. Effect of Other Factors on Hepatocytes Viability and Isolation Efficiencies

Besides of the three factors listed above, the perfusion time (digestion time) is another factor that could affect the result of the isolation. Other studies reported that the perfusion times could range from 20-50 minutes [184]. Based on the observation in this study, the perfusion time is highly depended on the enzyme types, concentration of the enzyme, sample size and different perfusion protocols. Since the enzyme used during perfusion has the ability to destroy the tissue structure and extracellular matrix of the hepatocytes and then make the tissue sample felt soft [99], the stopping point of the perfusion can be simply determined by softness of the liver tissue.

Another key factor is the time delay between hepatectomy and beginning of liver perfusion. Bhogal et al. reported their findings that the time delay should limited within 3 hours in order to obtained an optimizing isolation results [184]. The data obtained in this study concur with their findings and all the time delays of the experiments are limited to approximately 2 hours.

4.5.3. Function Assays and Cell Morphology of Isolated Human Hepatocytes

4.5.3.1. Function Assays

A series of function assays were performed on human hepatocytes isolated from sample 3. The reason that sample 3 was chosen to be tested is that this was the earliest opportunity that we obtained enough cells from the isolation procedure. All the results are listed below in table 3.

Table 4.3 Results of human hepatocytes function assays

Patient information	Male, 72 years old, cholangiocarcinoma, undergo 1 week of chemotherapy before operation	
	Unit	Data
Post-isolation viability	%	76±3.42
Adhesion efficiency	%	75.65±6.08
Albumin secretion assay	ug/million cells/24h	17.75
CYP1A2 assay	pmol/million cells/min	0.74
CYP3A4 assay	pmol/million cells/min	1.37
UGT assay	pmol/million cells/min	491.29

Data listed in table 3 was obtained by taking the average reading from the corresponding reader and converting to the appropriate data format according to the standard curve (Figure 4.4) respectively.

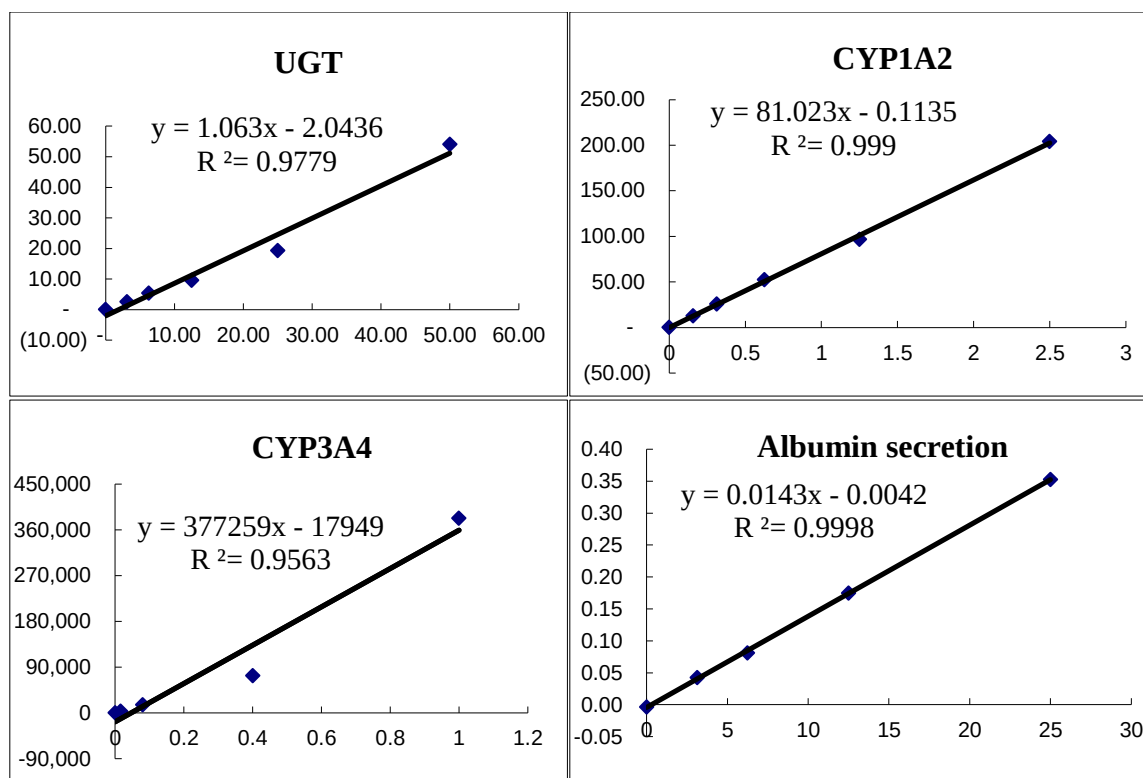


Figure 4.4 Standard Curves of UGT, CYP1A2, CYP3A4 and albumin secretion assays.

Data of UGT, CYP1A2, CYP3A4 and albumin secretion assays are of significant importance to drug metabolism and bioactivation tests. Fold increase/decrease on UGT, CYP1A2 and CYP3A4 after induction and/or inhibition is an important index to characterise hepatocytes functions. In table 3, only the baseline of a series of enzymes activities are listed to represent the functions of the freshly isolated hepatocytes. Despite the high cell adhesion efficiency, the cell function results indicate that the hepatocytes of sample 3 are still functional but in a weak condition and low metabolism abilities were noted. However, the results demonstrate that the isolation procedure was successful and isolated hepatocytes are undamaged during the procedure. The attempt to introduce induction and/or inhibition was also carried out but the outcomes did not meet expectations.

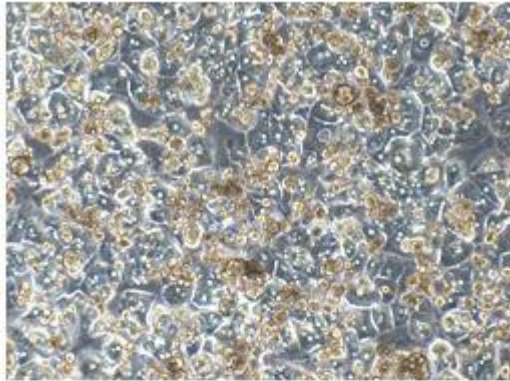
Normally, the fold change of 10 or higher is considered to be the optimal sample for drug testing and other experiments. Due to the poor condition of the cells, the fold changes were limited to 3. Consequently, hepatocytes isolated from sample 3 are expected to have a low cell adhesion rate after the cryopreservation procedure and hence are unsuitable for other experimental purposes.

As a summary, data from table 3 illustrates that the ability of performing fully functional assays to human hepatocytes is obtained. Unfortunately, the second attempt for a successful function assay and further investigation of cell functions were unable to be performed due to the lack of tissue sample. What could be said that a whole set of methodology for the functional assays of human primary hepatocytes have been established.

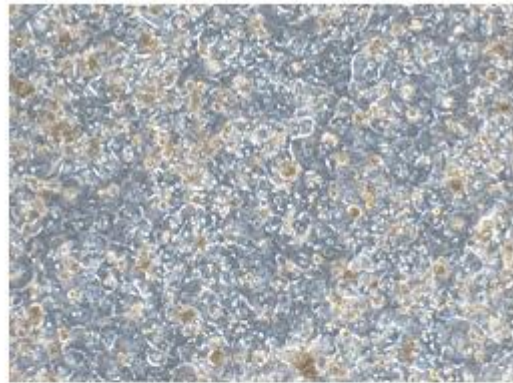
4.5.3.2. Cell Morphology

Cell morphology of isolated human hepatocytes is examined by fluorescence microscopy. Images are showed below. As Figure 4.5 suggests, the cell adhesion rate is high on day 1. Such a high rate indicates that the hepatocytes culture protocol is successful and efficient. It is apparent from Figure day 5 that the spaces between the cells are wider when compared to day 1, demonstrating that with the decrease of cell conditions, the cell adhesion drops due to cell apoptosis. . However, of major interest, is activity in the first three days of the cell culture. Presuming that the cell adhesion rate is maintained to expected high levels up to day 3, we are still able to perform the majority of the cell function assays on these cells. However, the long-term human hepatocytes culture beyond a week is still a challenge.

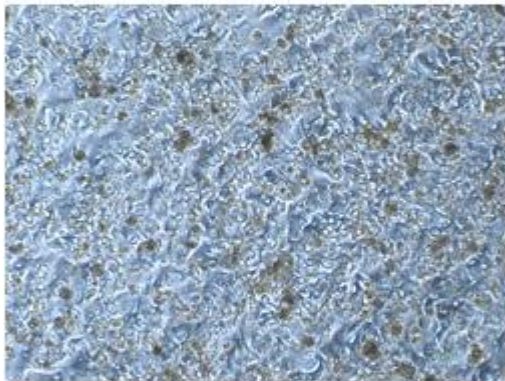
Day 1



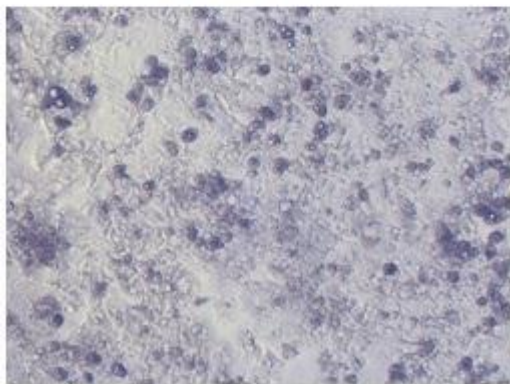
Day 2



Day 3



Day 5



Day 5 (fluorescence)

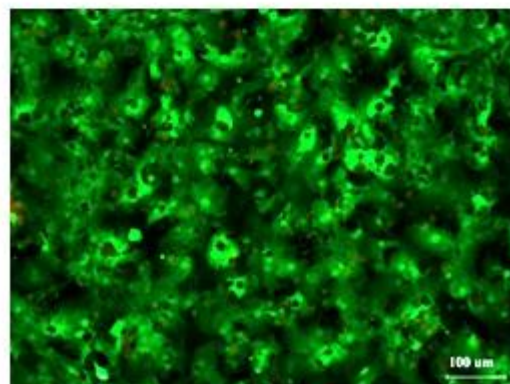


Figure 4.5 Cell morphology of isolated human hepatocytes. Images are captured from day 1 to day 5. Cells were stained by calcein-AM and EthD-1 and fluorescence image was taken on day 5. Scale bar were set to 100 μm .

4.6. Summary

In this chapter, two different protocols for human primary hepatocytes isolation were established, which are direct-digestion method and perfusion method, applicable to different sizes of liver tissue samples. The efficiencies of both methods were examined and compared in terms of cell viability and cell yield rate.

The post-isolation cell viabilities and cell yield rate are lower than the results from some other's studies [190, 191]. There are several potential explanations for this: first of all, most of these experiments were carried out in hospital lab, which have some equipment are more favourable to hepatocytes isolation, i.e. large peristaltic pump or infusion liked circulating system which can achieve high perfusion rate. Secondly, the source of liver tissue that used for isolation may be different. At last, other studies may use different enzyme or enzyme concentration from us. However, when compared to the some studies carried out under similar experimental conditions [181, 184], great improvements were observed in this study. Such improvements demonstrate that the protocol adopted by this study is accurate and efficient.

Moreover, the results of post-isolation cell viability from both isolation methods lead us to conclude that the viabilities of isolated human hepatocytes are highly dependent on the condition of the liver tissue and isolation method has less significant effect on isolated cell viabilities. On the contrary, cell yield rate has a close relationship with the isolation methods. As long as the sample is big enough to introduce two cannulae, the perfusion method should be adopted as the desired isolation method.

Isolation efficiencies of different types and conditions of liver tissue were also compared. Typically, large piece of liver tissues with identified blood vessels lead to high cell yield rate [181, 184], and liver tissues in good condition leads to high cell viability and better cell function.

Finally, a comprehensive cell function assay was performed on liver tissue sample 3 as an example which includes cell adhesion efficiency, CYP1A2 assay, CYP3A4 assay, albumin secretion assay, UGT assay and cell morphology. The results demonstrate that hepatocytes of sample 3 are still functional after isolation and 72 hours culture but the metabolism abilities are low. The major causes of such low cell function are the poor condition of liver tissue and cell apoptosis. Unfortunately, due to the lack of tissue sample and obtained cells, further experiment could not be performed. Nevertheless, the developed protocols were established and proved.

Chapter 5 Cryopreservation of Human Primary Hepatocytes

5.1. Introduction

Human primary hepatocytes are of significant importance to pharmacological research, drug toxicological study and therapeutic purposes. Due to the lack of liver tissue samples and the increasing number of clinical needs, human primary hepatocytes transplantation, as an alternative method to treat many end-stage liver diseases and liver-based metabolic disorders, are being aggressively pursued in modern medical technology [58]. However, the availability of fresh liver tissues suitable for hepatocytes isolation are limited, and the maintenance of human primary hepatocytes cultivation is possible for only several days due to cell apoptosis [195]. Therefore, successful long-term preservation techniques for human primary hepatocytes are in great demand to provide a significant amount of human hepatocytes for in vitro research and clinical purposes. Cryopreservation of freshly isolated hepatocytes from liver tissue samples represents one of the solutions for these purposes. Although progress concerning this technique has been reported [196, 197], the problems, such as low post-thaw cell viabilities, high cell apoptosis rate and limited cell function after recovery, remain to be solved [198].

The selection of cryoprotectants is critical to cryopreservation of hepatocytes. The conventional cryoprotectants that are used by most hepatocytes cryopreservation protocols

consist of UW solution (Belzer, USA), DMSO and fetal calf serum (FCS). The most popular choice of cryoprotectant, DMSO, has the ability of inhibiting intracellular ice formation [58]. The use of FCS has been extensively proven by many researchers, to improve the post-thaw viability and cell attachment rate of hepatocytes [199-203]. Katenz et al. proposed a hypothesis that “FCS protects the cells from injury induced by the generation of free oxygen radicals during the freeze cycle [204]”, however the results are inconclusive. The concentration of DMSO, ranges from 5% to 20%, whilst concentration of FCS is normally adopted as 10%. Another key procedure that affects the outcome of hepatocytes cryopreservation is the freezing protocol. There are three major freezing protocols that were commonly used by researchers, which are slow freezing protocol, fast freezing protocol and step-wise freezing protocol. Since constant freezing rate, regardless if it is fast or slow, normally leads to unsatisfactory post-thaw cell viabilities [205], step freezing protocol, with varying freezing rate at each step was adopted by most researchers.

Vitrification is a similar freezing procedure where ice formation is prevented by using a high concentration of CPA and ultrafast freezing rate. Vitrification, however, can only be applied to small sample size and hence was not explored in this work.

In this study, the step freezing protocol with varying freezing rate was adopted. It is achieved by using a programmed freezer (Kryo 560-16, Planer, USA). The formula of the cryoprotectant was further modified by adding 0.1M sucrose or 0.1M glucose as lyoprotectant in order to provide extra protection to hepatocytes during the freezing process and 100X ROCK inhibitor (Y-27632) at a ratio of 1:1 to overcome cell apoptosis problem. The effects of altering FCS concentration and cell density were also studied. At last, 3D

encapsulation of RGDS-alginate was applied to human hepatocytes as a novel cryopreservation method. The effectiveness of various combinations and protocols was assessed by hepatocyte functional assays, including post-thaw cell viability, cell adhesion rate, phase I and II enzyme activities and albumin secretion, comparing to the cell functions of freshly isolated hepatocytes (before freezing). Results were analysed statistically.

5.2. Ethics Statement

The liver tissue samples were obtained from Tianjin Medical University Cancer Institute and Hospital. The experiments of using liver samples were approved by the Ethics Committee of the hospital following the procedure set up by the national ethic guidelines, with full consents of the patients.

5.3. Patient Information

The patients were screened from pancreatoduodenectomy (whipple procedure), liver cancer, kidney cancer and receiver of liver transplantation. Informed consents were obtained from the patients for the use of liver tissue for research purposes. Basic patient information relevant to the study e.g. sex, age, ethnicity, medication and serological data were recorded. Virological status for HIV1 and HIV2, Hepatitis B (HBV) and Hepatitis C (HCV) was carried out on the patient by serological screening using approved diagnostic kits.

5.4 Materials and Methods

5.4.1. Modified Formulas of Cryoprotective Media

As described above, conventional cryoprotective medium is made of UW solution supplemented with 10% DMSO and 10% FCS. Since 10% DMSO provides the most effective and reliable protection to the cells and has been verified by many researchers, this concentration is also adopted in this study. However, the effectiveness of different concentrations of FCS in cryoprotective media was studied experimentally. Sucrose and glucose were also added to the cryoprotective medium in order to assess and compare their protective abilities during the cryopreservation process as lyoprotectants [61, 66]. The concentrations of these two agents were set to 0.1M. Also, different cell densities of hepatocytes during the cryopreservation procedure were also studied. Due to a limited supply of liver tissue samples, only two different densities, 3 and 6 million cells per ml, were adopted. All modified formulas of cryoprotective media are listed in table 5.1.

Table 5.1 Modified Formulas of Cryoprotective Media

formula 1	UW solution	10% FCS	10% DMSO	0.1M Sucrose	6 million cells/ml
formula 2	UW solution	20% FCS	10% DMSO	0.1M Sucrose	6 million cells/ml
formula 3	UW solution	10% FCS	10% DMSO	0.1M glucose	6 million cells/ml
formula 4	UW solution	10% FCS	10% DMSO	0.1M Sucrose	3 million cells/ml

Rho-associated coiled coil forming protein serine/threonine kinase inhibitor (ROCK inhibitor, Y-27632) has the ability to prevent cell apoptosis. It has been adopted by mammalian cell cultivation of several cell lines. In this study, the cryopreservation outcomes with the presence of ROCK inhibitor were compared with the results where the ROCK inhibitor was not presented. The results were assessed in terms of post-thaw viability and cell adhesion rate.

5.4.2. Cryopreservation Procedure

5.4.2.1. DMSO Loading Procedure

In order to avoid osmotic shock to cells, the DMSO loading process was modified. Differing from the conventional DMSO loading process, DMSO was added to the cryoprotective medium at the initial stage and then mixed with cells, rather than directly added to the cell suspension. The modified DMSO loading procedure is specified as follow. The cryoprotective medium was prepared without the presence of DMSO and then divided into two equal parts. The first part was supplemented with 5% DMSO and mixed thoroughly and with care while the second part was supplemented with 15% of DMSO. The hepatocytes initially were resuspended in the first part of cryoprotective medium and left on ice for 3 minutes. Then, gradually the second part of cryoprotective medium was loaded to the cell suspension and left on ice for a further 5 minutes. While loading, the tube containing the cell suspension was gently shaken by hand in order to prevent the accumulation of concentrated DMSO in certain areas. In this way, the DMSO concentration outside the cells increases gradually and stepwise, to minimise the osmotic shock to the hepatocytes.

5.4.2.2 Freezing Protocol

The adopted freezing protocol is shown in table 5.2. The changes in the freezing rate were controlled by a programmed freezer. This protocol was modified from that developed by Terry et al. [179], which based on the study published by Diener et al. [201]. The freezing protocol was programmed into the freezer control unit; and the freezer was turned on 30 minutes prior to loading the samples in order to cool down the freezing chamber. Once the temperature of the chamber reached the desired starting temperature, the samples were loaded into the chamber and then the chamber door was secured. The thermometer inside the chamber should have contact with the sample in order to maintain control of the freezing rate.

Table 5.2. Freezing Protocol of Human Primary Hepatocytes

Step	Starting Temperature (°C)	Rate (°C/minute)	Time (minutes)	End Temperature (°C)
1	8	-1	8	0
2	0	Hold	8	0
3	0	-2	4	-8
4	-8	-40	0.5	-28
5	-28	-2.5	2	-33
6	-33	2.5	2	-28
7	-28	-1	32	-60
8	-60	-10	4	-100
9	-100	-20	3	-160

5.4.2.3. Recovery Media

The standard recovery medium is Williams Medium E supplemented with 10% FCS. A further modification was performed on this formula by adding 0.1M glucose to the standard recovery medium. Cryopreserved hepatocytes were recovered by both recovery medium and the differences between these two formulas were compared with the use of post-thaw cell viability, adhesion rate, phase I and II enzyme activities and albumin secretion.

5.4.2.4. Thawing Protocol

The thawing protocol was also modified based on standard thawing protocols. After 7 or 30 days storage at -196°C in liquid nitrogen tank, cryovials were quickly thawed in a 37°C water bath with frequent swirling. Vials should be removed from the water bath when the sample was close to being fully thawed with a little ice crystal on the top. Following this, the cell suspension was transferred to a pre-cooled 50ml tube and placed on ice. The cell suspension was diluted by adding 0.5ml, 1ml, 2ml, 4ml of recovery medium every minute to the suspension with constant stirring. The recovery medium was also pre-cooled to 4°C. Finally, the cell suspension was centrifuged at 50g for 5 minutes at 4°C and the supernatant was removed. Cell pellet was resuspended in a known volume of Williams Medium E culture medium for function tests or culture.

5.4.3. Cell Encapsulation

Human primary hepatocytes are encapsulated by both normal alginate and RGDS modified alginate using the same technique as described in Chapter 3. A few changes have been made due to different cell types. As a novel cryopreservation method for hepatocytes

preservation, the optimal cell density needs to be tested. Thus, the encapsulation with cell densities of 1, 3, 6 and 10 million cells per ml alginate solution were all studied. Since human hepatocytes are much smaller and more fragile than hESC, the hepatocyte-alginate suspension was ejected through a 27 gauge needle rather than a 30 gauge in order to decrease the pressure. With bigger needles, the diameter of alginate beads was increased to 1 mm with 0.1mm as standard error. After cryopreservation, cells were recovered from alginate beads by dissolving medium and resuspended in the culture medium for viability assay and function tests.

5.4.4. Viability and Function Assays

The post-thaw viability was analysed by the trypan blue method.

Cell adhesion rate was obtained by counting the dead cell number in the supernatant, in this way calculating the attached cell rate. Differing from other cell adhesion assays, 4×10^5 live cells were seeded in 24 –well plate in order to perform further function assays. Thus, the results were based on attachment rate of live cells rather than the mixture of live and dead cells. Therefore, the cell adhesion rate may exceed the total cell viability.

Phase I and II enzyme activities and albumin secretion assays were carried out following the same protocols as demonstrated in the previous chapter. The results obtained were also compared with the results of cell functions of freshly isolated hepatocytes.

5.4.5. Statistical Analysis

Each experiment was performed in triplicate. Analysis of variance (ANOVA) was adopted as the method to determine the statistical significant differences between groups. The p-values were also calculated using T.TEST function with a two-tailed distribution in Excel. A p-value of less than 0.05 was considered as statistically significant. All data presented is in the format of mean $\pm$ standard deviation.

5.5. Results and Discussion

5.5.1. Freezing Protocol

Numbers of cryopreservation protocols for human primary hepatocytes have been published and most of them have been proven efficient for research or clinical uses [205-210]. Generally speaking, the slow controlled freezing rate protocol demonstrates better results than constant freezing rate protocols [201, 205, 210]. The one adopted in this study is a step-wise freezing protocol with different freezing rate at each step. This protocol is originally created by Terry et al. [179] based on Diener's study on cryopreservation of rat hepatocytes [201]. With detailed consideration and investigation of cryobehaviour of the cells and latent heat of fusion during freezing process, Diener et al. proposed that a rapid decrease of temperature at the supercooling temperature of the suspension, which can promote ice crystallization, is necessary and benefit to cell cryopreservation [208]. The temperature of supercooling is variable between -8°C and -24°C before homogeneous ice

crystallization. With constant $-2^{\circ}\text{C}/\text{min}$ freezing rate, a sudden release of latent heat of fusion can be observed, which will warm-up the sample [201]. Such release of latent heat is deleterious to a successful cryopreservation [211]. In order to overcome this problem, a rapid cooling must be launched between the highest supercooling temperature and homogeneous ice nucleation temperature [201]. Thus, a shock cooling process was started at -8°C and stopped at -28°C in our freezing protocol. Diener et al. also proposed that such shock cooling should be done within 0.1 second [201]. However, it is not possible to achieve $-200^{\circ}\text{C}/\text{min}$ cooling rate in our laboratory. So, the freezing rate of the shock cooling step was modified to $-40^{\circ}\text{C}/\text{min}$.

Although the shock cooling step could help to minimize the sudden release of heat, the latent heat was not eliminated [201]. A freezing process with $-2.5^{\circ}\text{C}/\text{min}$ to -33°C was followed in order to eliminate the latent heat of fusion [201]. Moreover, a slight increase of temperature can ensure a stable and rate-constant decrease of the sample temperature [201]. At the last step, unlike terry et al. [180], all the samples were cooled to -160°C rather than stop at -100°C since the samples were going to store in liquid nitrogen.

5.5.2. Cryopreservation Outcome

To demonstrate the quality of human primary hepatocytes cryopreservation process, the following parameters were examined as indicators: post-thaw cell viability; cell adhesion rate; phase I and II cell functions; albumin secretion and cell morphology. The results of post-thaw cell functions were compared with the results of post-isolation cell functions to demonstrate the successful preservation of cell metabolism abilities.

5.5.2.1 Post-thaw Cell Viabilities and Cell Adhesion Rates

Post-thaw cell viabilities were examined after 7 days cryopreservation and the results showed that $64.73\% \pm 5.27\%$ cells survived the cryopreservation process. Each cell viability assay was repeated three times and the final indicative result is an average reading of the cell viabilities of multiple tissue samples. Compared to the data obtained by Terry et al. published on his most recently paper, our data gives better post-thaw cell survival rate than theirs ($46\% \pm 5\%$) [179]. This significant improvement indicates the successful design of the experiment and cryopreservation protocols.

As Figure 5.1 illustrates, the cell adhesion rate after the cryopreservation process has decreased. The human hepatocytes was preserved in standard cryoprotective medium (formula 1, Table 5.1) and $41.04\% \pm 3.06\%$ cells were successfully attached after thawing. In comparison with the post-isolation cell adhesion rate ($75.65\% \pm 6.08\%$), this decrease is significant. This attachment rate is also compared to the one obtained by Terry et al. and slightly lower than theirs ($48\% \pm 5\%$) [179]. The reason could be various. The most important one is that the results are highly depended on the existing conditions of the cells, which are unable to control in the experiment. But such results could still lead to the conclusion that the cryopreservation protocol requires further modification in order to increase the preservation efficiency.

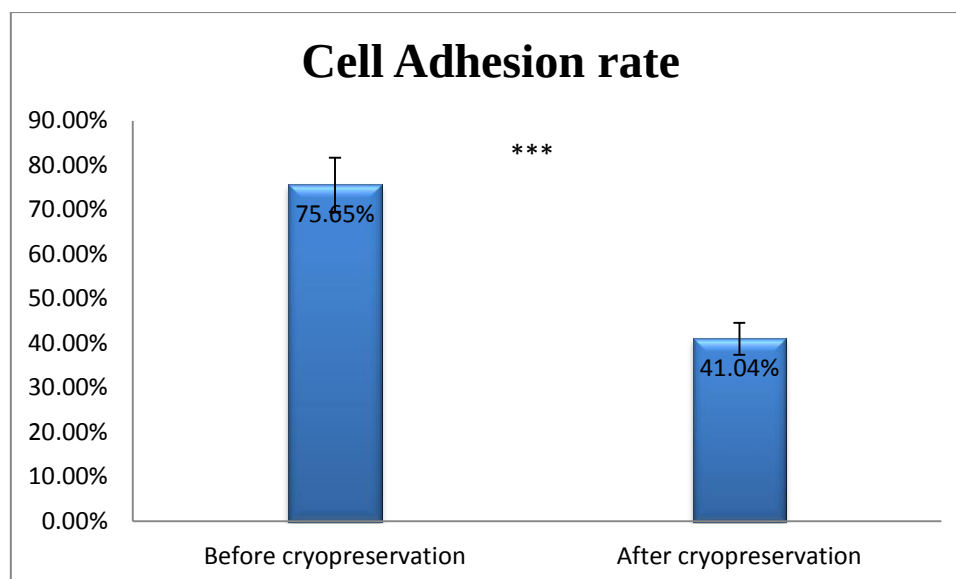


Figure 5.1 Comparison of cell adhesion rate before and after cryopreservation process. Data is given as mean $\pm$ standard deviation, obtained from at least three independent trials. (* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$) {Experiment conditions: operating temperature: 4°C, cryoprotective medium: formula 1 (Table 5.1), preservation time: 7 days}

As discussed above, cell viability and adhesion rate are both highly dependent upon the condition of the tissue sample. The results above indicate that the ability of successfully preserving freshly isolated human hepatocytes is achieved, although there are big scopes to improve further.

5.5.2.2. Cell Function Assays

A comprehensive cell function assay was performed after thawing, which include CYP1A2, CYP3A4, UGT, and albumin secretion. The data tabulated in Table 5.2 is the baseline of each function assay, indicating the basic abilities of the metabolism of hepatocytes. The results were compared with the data gained from post-isolation.

Table 5.3. Comparison of post-isolation and post-thaw cell functions

Patient information	Male, 72 years old, cholangiocarcinoma, after 1 weeks chemotherapy before operation		
	Unit	Post-isolation	Post-thaw
Post-isolation Viability	%	76 ±3.42	64.73 ±5.27
Adhesion Efficiency	%	75.65 ±6.08	41.04 ±3.06
Albumin Secretion	ug/million cells/day	17.75	21.34
CYP1A2	pmol/million cells/min	0.74	2.37
CYP3A4	pmol/million cells/min	1.37	4.72
UGT	pmol/million cells/min	491.29	291.37

It can be observed that the post-thaw phase I and II enzyme activity and albumin secretion levels increased when compared with the results that of fresh cells. Such results appear to be the opposite of the results gained from the cell adhesion rate, but in fact this is not the case. The function assays were performed on the attached cells. In another words, the cells which contributed to the tests have already survived the cryopreservation process and recovered in a relatively good condition. However, the results of post-isolation hepatocytes function assays were based on a mixture of cells in both good and weak conditions. Presuming most of the weak cells were dead during cryopreservation process, the better results of post-thaw hepatocytes function are reasonable. Moreover, these results also demonstrated that the cell functions of human primary hepatocytes were well preserved during the freezing process.

Unfortunately, it was not possible to conduct further attempts to measure post-thaw cell function assay due to lack of tissue samples. In addition, most findings regarding the post-thaw hepatocytes function assays were using rat hepatocytes. Although limited researchers

have published their results using human hepatocytes, there is not one set of data as comprehensive as ours including isolation, cryopreservation, post-isolation function assay and post-thaw function assay. Thus, comparable data that obtained under similar experiment conditions can hardly be found in literatures.

5.5.2.3. Cell Morphology

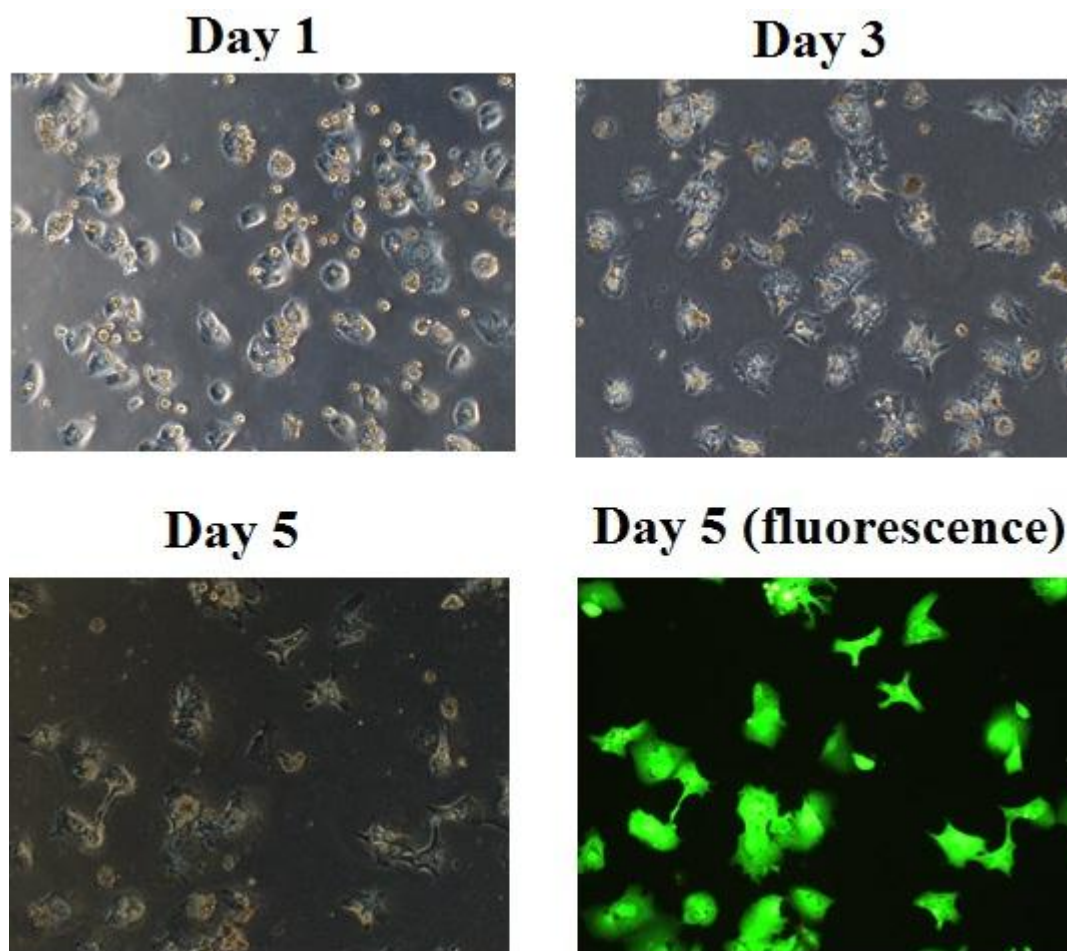


Figure 5.2 Post-thaw human primary hepatocytes morphology. Images are captured from day 1 to day 5. Cells were stained by calcein-AM and EthD-1 and fluorescence image was taken on day 5.

In general terms, cell structures were intact from day 1 to day 5 (Figure 5.2). Dead cells were not located in the fluorescence image since they were in a free-floating form and off-focus on the microscope, but they can be seen from bright field images, i.e. day 1 and day 3. Compared to freshly isolated human primary hepatocytes, post-thaw hepatocytes have lower cell adhesion rate which confirms the data in Figure 5.1. Moreover, a continuous decrease of cell attachment was observed. This is mainly due to the cell apoptosis, which has been addressed by many researchers, stating that the maintenance of human primary hepatocytes cultivation is only possible for several days [212]. Further discussion of the effects of ROCK inhibitor was presented in later discussion.

5.5.3. Modification of Recovery Medium

A hypothesis has been proposed that the hyperosmotic concentration of solutes in the recovery medium could greatly assist post-thaw human hepatocytes qualities [179]. The standard recovery medium is Williams medium E supplemented with 10% FCS. In this study, 0.1M glucose was added into the standard recovery medium and its efficiency was analysed in terms of post-thaw viability and adhesion rate. The results are presented below in Figures 5.3 and 5.4.

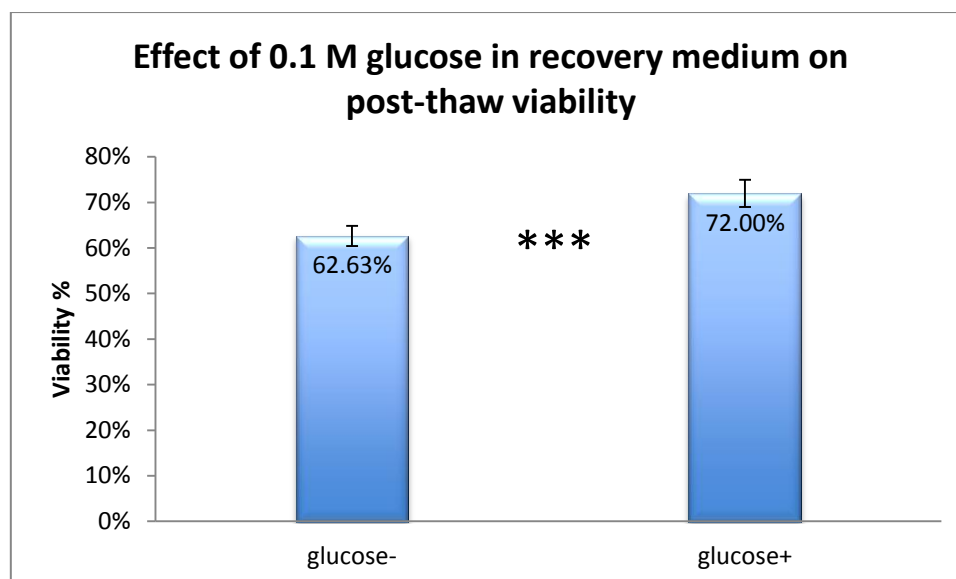


Figure 5.3 Comparison of post-thaw hepatocytes viabilities with/without the presence of 0.1M glucose in standard recovery medium. Data is given as mean±standard deviation, obtained from at least three independent trials. (*p<0.05, **p<0.01, ***p<0.001) {Experiment conditions: operating temperature: 4°C, cryoprotective medium: formula 1 (table 5.1), preservation time: 7 days}

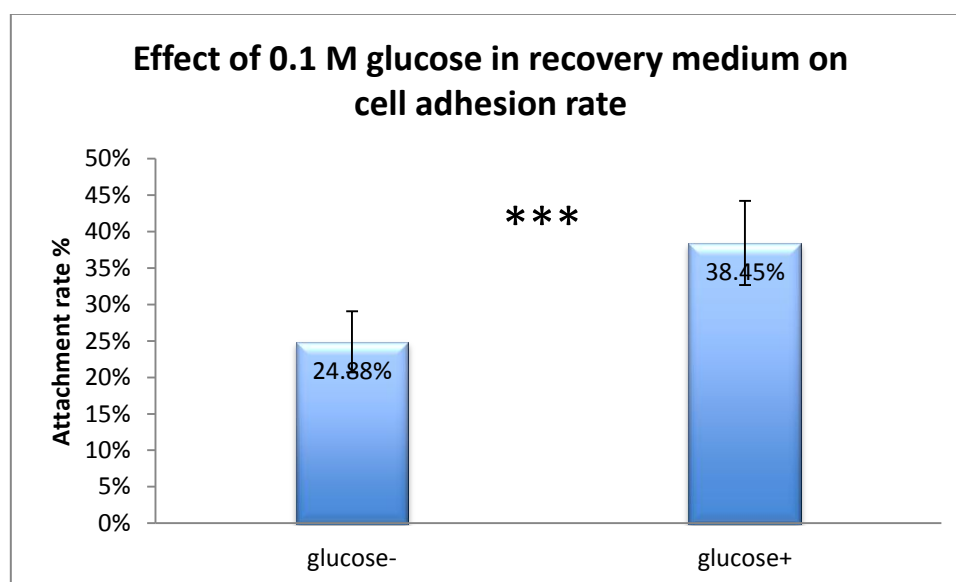


Figure 5.4 Comparison of post-thaw hepatocytes adhesion rate with/without the presence of 0.1M glucose in standard recovery medium. Data is given as mean±standard deviation, obtained from at least three independent trials. (*p<0.05, **p<0.01, ***p<0.001) {Experiment conditions: operating temperature: 4°C, cryoprotective medium: formula 1 (table 5.1), preservation time: 7 days}

As the data suggested in Figure 5.3 and 5.4, both post-thaw viability and adhesion rate benefited from the presence of glucose. Moreover, a significant improvement on post-thaw cell survival rate was also obtained compare to the results of Terry et al. ($52\% \pm 9\%$) [179]. This data confirmed the validity of the hypothesis. During the freezing process, the cryoprotective medium became increasingly concentrated due to the formation of ice crystals. Correspondingly, the cells became dehydrated under such high osmotic pressure. When thawing, hyperosmotic concentration of solutes, such as the addition of glucose, induced less osmotic pressure difference across the cell membrane hence protected the integrity of cell membrane and cell functions [180]. Since the cell functions were well preserved, the cell adhesion rate was also increased. These results demonstrated the successful modification of the recovery medium. This modified formula was adopted for all following experiments.

5.5.4. Comparison of Different Formulas of Freezing Medium

5.5.4.1. Concentration of Fetal Calf Serum (FCS)

The fetal calf serum (FCS) concentration was the first parameter of the formula that was examined. Although FCS cannot be used for clinical applications, but for preclinical and research purpose, FCS has been proven to be effective in cryopreservation of many types of cells. Two different FCS concentrations, namely 10% and 20%, have been adopted by Formula 1 and 2 respectively and the results were compared and analysed. Other parameters of these two formulas were consistent, being UW solution, 10% DMSO, 0.1M sucrose and 6 million cells per ml as cell density (Table 5.1). The comparative results are illustrated below in Figures 5.5 and 5.6.

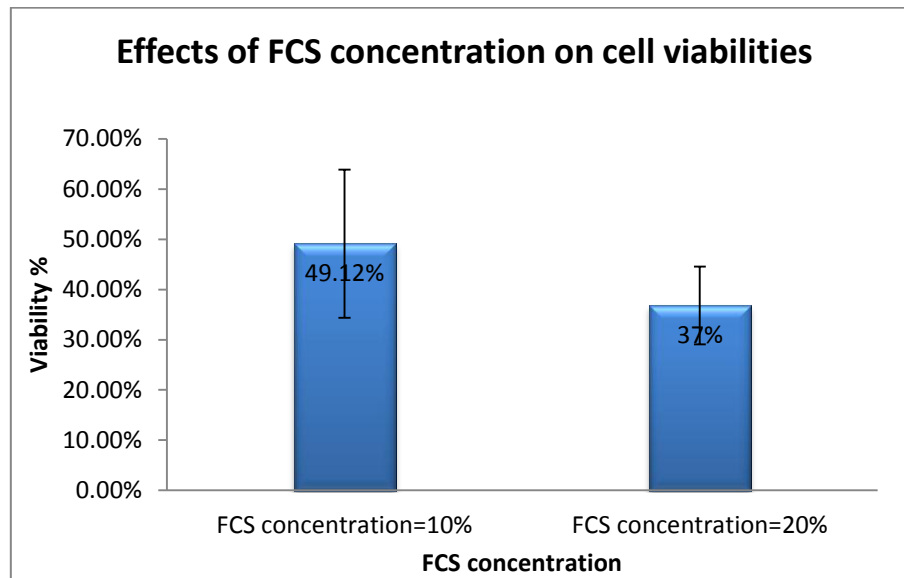


Figure 5.5 Effect of FCS concentration on cell viabilities. Data is given as mean $\pm$ standard deviation, obtained from at least three independent trials. No significant difference between these two groups of data according to two-tailed distribution T-TEST. {Experiment conditions: operating temperature: 4°C, cryoprotective medium: formula 1/formula 2 (table 5.1), preservation time: 7 days}

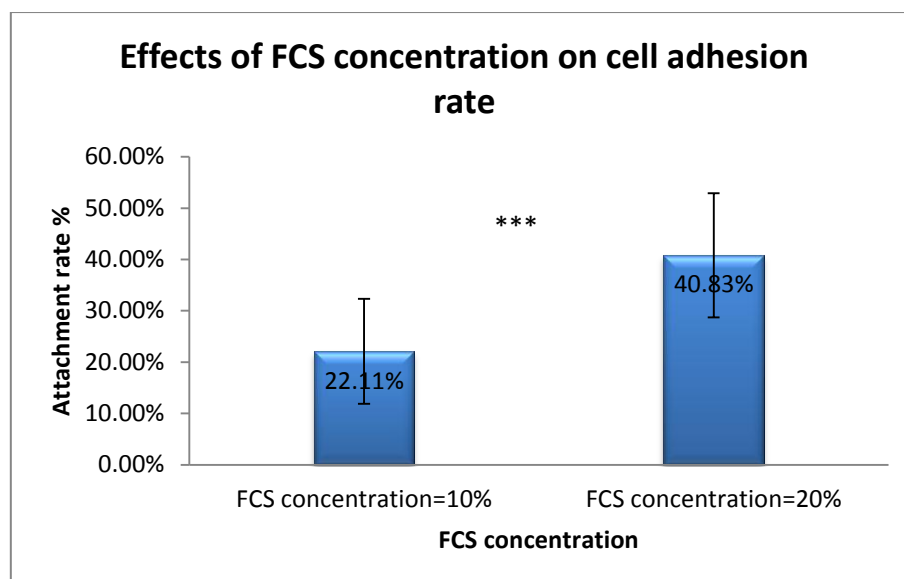


Figure 5.6 Effect of FCS concentration on cell adhesion rate. Data is given as mean $\pm$ standard deviation, obtained from at least three independent trials. (* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$) {Experiment conditions: operating temperature: 4°C, cryoprotective medium: formula 1/formula 2 (table 5.1), preservation time: 7 days}

FCS, as an important component of the cryoprotective medium, was involved in a variety of hepatocytes cryopreservation protocols. The concentration ranges from 10% to 90% [199]. The optimum concentration is inconclusive. As Figure 5.5 illustrates, it appears that 10% FCS provides improved post-thaw viability than 20% as FCS concentration. However, there is no significant difference between these two groups of data ($p = 0.20519$). For cell adhesion rate, 20% FCS concentration gives a better result compared to 10% FCS concentration. In spite of this, there is not enough evidence that can lead us to conclude that higher concentration of FCS in cryoprotective medium can provide increased cell protection. The reasons could be summarised as follows:

- 1) According to findings of various researchers' [199], 90% of FCS in cryoprotective medium has an equivalent result compared to lower serum concentrations.

- 2) FCS has several drawbacks that could interfere with the outcome of experiments [213, 214], such as lack of uniformity in the composition of serum, high risk of contamination and inclusion of some of the growth inhibiting factors.
- 3) It is a trend to adopt serum-free medium in modern day cell culture in order to get more reproducible results with well-defined media [215-217].

Consequently, FCS concentration should be reduced in hepatocytes cryopreservation protocols both on scientific and moral grounds. Therefore, lower concentration of FCS, i.e. 10%, is more favourable in this study. If the preserved cells are for clinical implant to human, FCS has to be replaced completely.

5.5.4.2. Comparison of the Effects of Sucrose and Glucose

It has been reported that different saccharides have the ability to protect the cells against cryoinjury hence increase post-thaw viability [218], such as trehalose and sucrose. Katenz et al. [219] published their findings and stated that the trehalose can improve the post-thaw cell viability by stabilizing cell membranes and proteins during freezing procedures. Similar to the trehalose that contains 2 glucose molecules, sucrose is a disaccharide that contains 1 glucose and 1 fructose molecule. Thus, sucrose has been tested to ascertain if it can achieve the same effect as trehalose. Since glucose was the fundamental component of both trehalose and sucrose, the potential protective ability of glucose was also tested. The results are presented below in Figures 5.7 and 5.8.

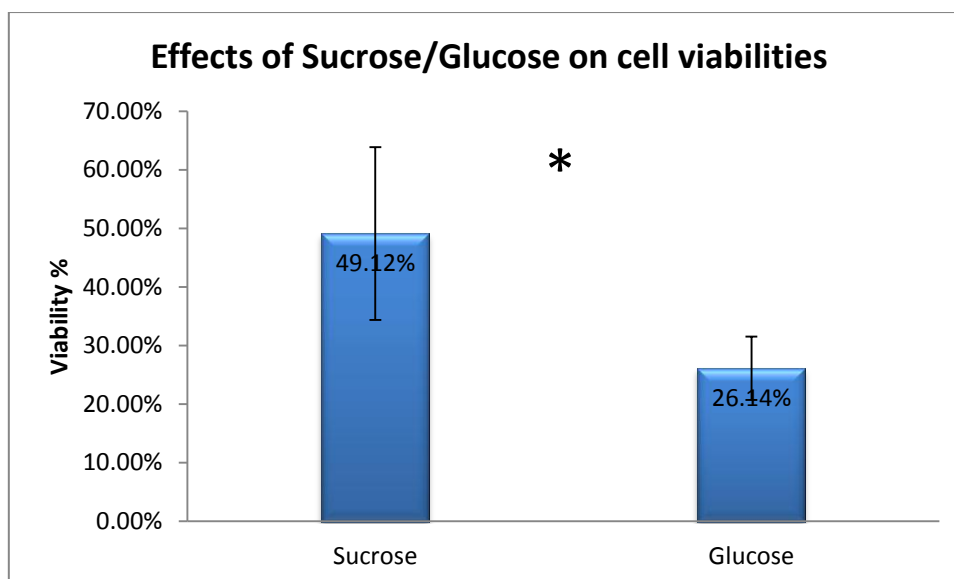


Figure 5.7 Effects of sucrose and glucose on cell viabilities. Data is given as mean±standard deviation, obtained from at least three independent trials. (* $p<0.05$, ** $p<0.01$, *** $p<0.001$) {Experiment conditions: operating temperature: 4°C, cryoprotective medium: formula 1/formula 3 (table 5.1), preservation time: 7 days}

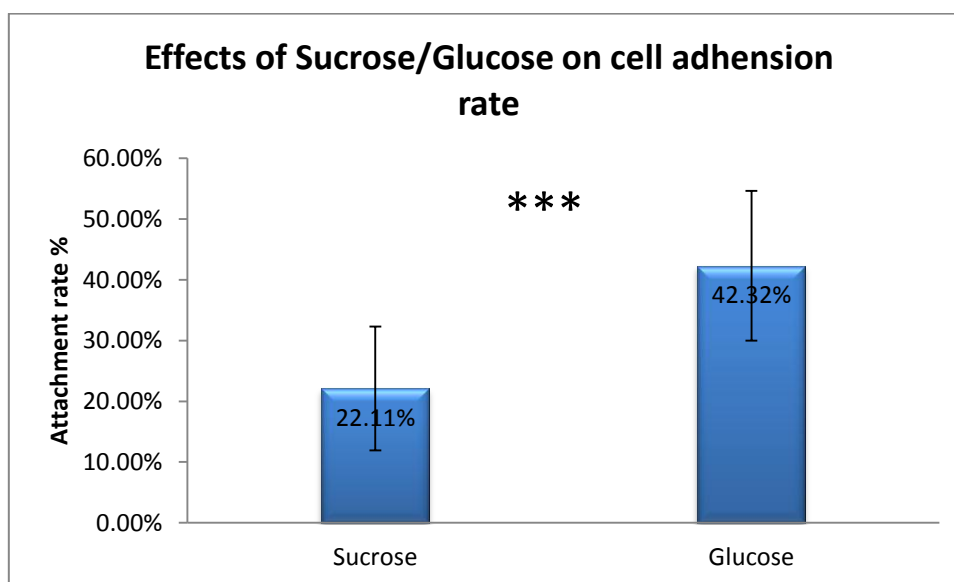


Figure 5.8 Effect of sucrose and glucose on cell adhesion rate. Data is given as mean±standard deviation, obtained from at least three independent trials. (* $p<0.05$, ** $p<0.01$, *** $p<0.001$) {Experiment conditions: operating temperature: 4°C, cryoprotective medium: formula 1/formula 3 (table 5.1), preservation time: 7 days}

As Figure 5.7 illustrates, human hepatocytes cryopreserved with the presence of sucrose had significantly higher post-thaw viability ($49.12\% \pm 14.76\%$), but lower adhesion rate ($22.11\% \pm 10.20\%$) than hepatocytes cryopreserved with the presence of glucose (viability = $26.14\% \pm 5.41\%$, adhesion rate = $42.32\% \pm 12.35\%$). The results satisfied the expected results. Sucrose has been rated as a non-permeable cryoprotectant and widely adopted by different cryopreservation protocols. Similar to trehalose, sucrose can adsorb on the surface of the cells in the form of a viscous layer, causing exosmosis of water from the cell, therefore reducing intracellular ice formation [68, 220]. It also inhibits the rate of extracellular ice growth by increasing solution viscosity and securing the structure of ice amorphous in the close proximity of the cell [65, 220]. This has the effect of protecting the cell from external mechanical injury due to encroaching ice crystals [66]. However, glucose does not possess these abilities. Moreover, glucose is a nutrient for cells. During freezing process, the metabolic ability of the cells is decreasing along with the temperature. So that, the concentration of the nutrient should be strictly regulated [199, 221]. Additional nutrient would be unfavourable for cells during cryopreservation process. As mentioned above, the glucose was normally used in the recovery medium to create a hyperosmotic environment for dehydrated cells whilst thawing occurs. It is this precise factor which explains why human hepatocytes, cryopreserved with the presence of glucose, had a higher adhesion rate. Although human hepatocytes in both groups were in a poor condition to start with, the cryopreservation was benefited by the presence of sucrose rather than glucose.

5.5.4.3. Cell Density

Two different cell densities were tested in this study, which are 2 million cells per ml and 6 million cells per ml. The data is shown below in Figures 5.9 and 5.10.

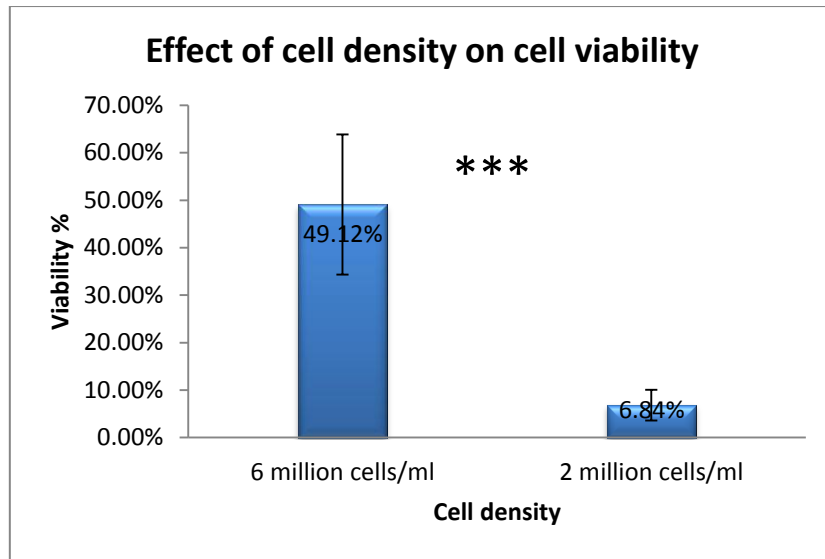


Figure 5.9 Effects of cell density on cell viabilities. Data is given as mean $\pm$ standard deviation, obtained from at least three independent trials. (* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$) {Experiment conditions: operating temperature: 4°C, cryoprotective medium: formula 1/formula 4 (table 5.1), preservation time: 7 days}

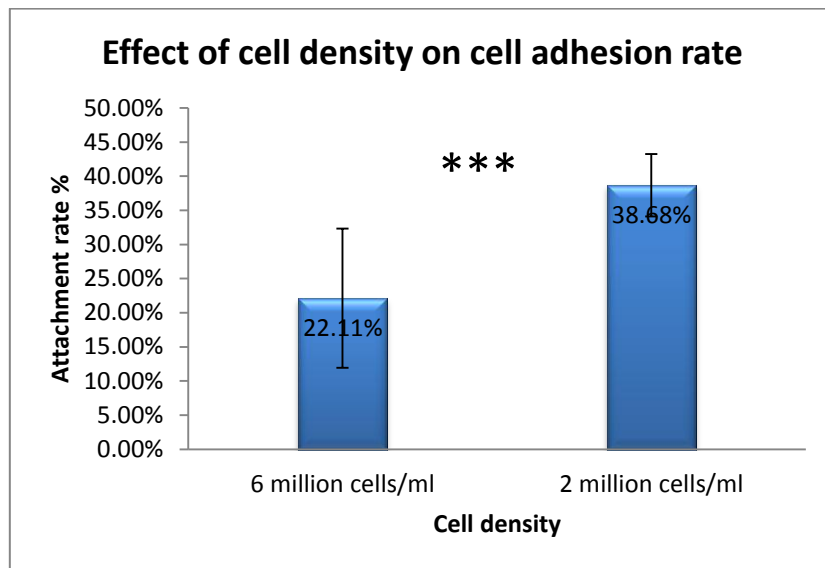


Figure 5.10 Effect of cell density on cell adhesion rate. Data is given as mean $\pm$ standard deviation, obtained from at least three independent trials. (* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$) {Experiment conditions: operating temperature: 4°C, cryoprotective medium: formula 1/formula 4 (table 5.1), preservation time: 7 days}

Human hepatocytes preserved with a cell density of 2 million cells/ml had extremely low viability ($6.84\% \pm 3.25\%$). This indicates the unsuccessful design of the cryopreservation protocol. These findings also confirmed the conclusion summarised by other researchers, such as Terry et al. [180], that samples with low densities (1 and 2 million cells per ml) are not suitable for human hepatocytes cryopreservation.

5.5.4.4. Effects of ROCK inhibitor

The ROCK inhibitor has been widely used in cultivation and cryopreservation of human embryonic stem cells since it has the ability to inhibit cell apoptosis. But this is no one has ever made an attempt to test whether it possesses the same ability that can inhibit the cell apoptosis of human hepatocytes. In this study, the effect of ROCK inhibitor during cryopreservation of human hepatocytes was analysed.

The Formula 1, 2 and 3 of cryoprotective medium and recovery media were further modified by adding 1% of 100-times ROCK inhibitor and the efficiencies of these formulas were tested. The effects of ROCK inhibitor are shown in Figures 5.11 and 5.12.

As the data in the figures below illustrate, human hepatocytes, preserved without the presence of ROCK inhibitor, has similar post-thaw viabilities compared with the cell preserved with ROCK inhibitor and there are no significant differences between the two. However, the cell adhesion rates of human hepatocytes without the presence of ROCK inhibitor were much higher than the other rates. ROCK inhibitor is normally used in culture and cryopreservation of human embryonic stem cell. It has been rated as an efficient chemical compound to inhibit the cell apoptosis hence improves the cell quality during cell

cultivation and cryopreservation processes. Since human primary hepatocytes are different from hESCs, the ROCK inhibitor does not provide the same effects to human hepatocytes as it normally does to hESC. One of the applications of ROCK inhibitor to hepatocytes is to prevent hepatocytes damage in acute liver injury in rat [222]. As the experimental data proves, ROCK inhibitor has the ability to reduce cell apoptosis in vitro since the phosphatidylinositol 3-kinase/akt pathway can be activated by the administration of ROCK [141, 223]. But the experimental data was obtained by using rat liver tissue rather than isolated rat hepatocytes. The ECM provided by liver tissue is totally different from the hepatocytes suspension. Other applications of ROCK inhibitor to human hepatocytes are normally using ROCK inhibitor to treat hepatocellular carcinoma. The positive data obtained indicates that ROCK inhibitor is effective on reducing the rate of intrahepatic metastasis by inhibiting cell motility [224-227]. However, this is no conclusive data that can indicate that ROCK inhibitor have positive effect on reducing the post-thaw apoptosis of human hepatocytes. Thus the significant decrease of cell adhesion rate observed in this study indicates that the ROCK inhibitor was not suitable for the use in cryopreservation of human primary hepatocytes.

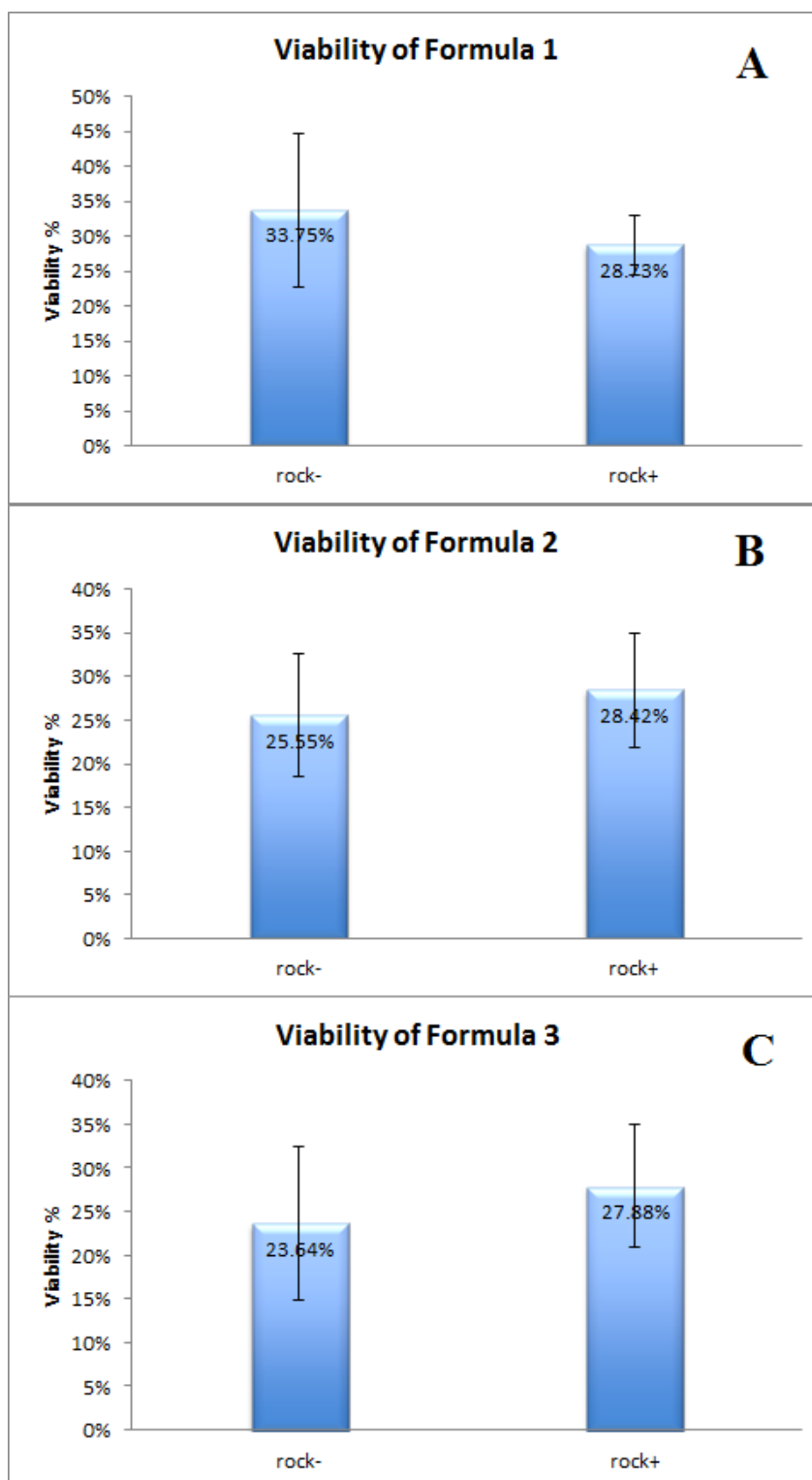


Figure 5.11 The effects of ROCK inhibitor on post-thaw cell viabilities. Data is given as mean $\pm$ standard deviation, obtained from at least three independent trials. No significant difference between these two groups of data according to two-tailed distribution T-TEST.

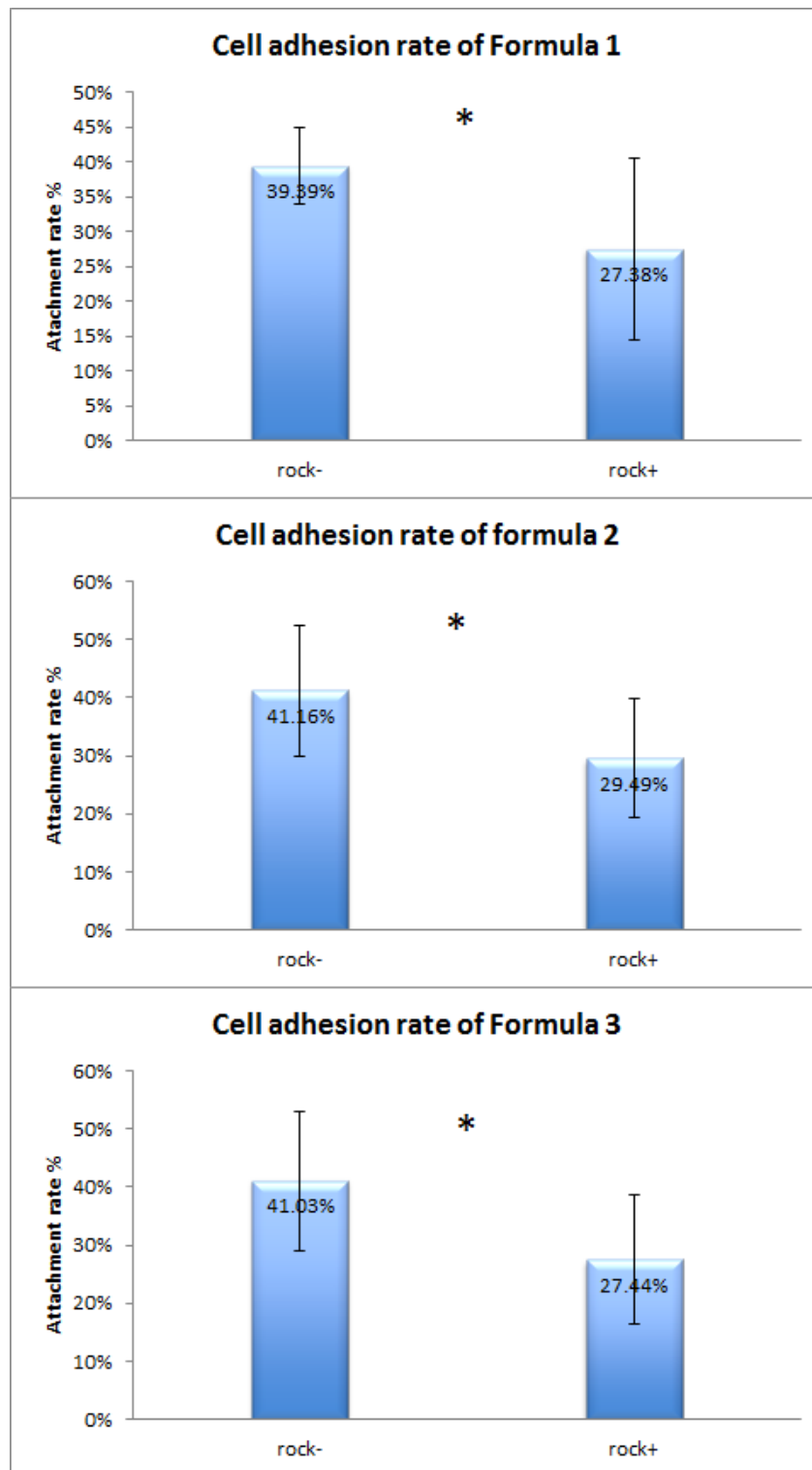


Figure 5.12 The effects of ROCK inhibitor on post-thaw cell adhesion rate. Data is given as mean±standard deviation, obtained from at least three independent trials. Hepatocytes preserved without the presence of ROCK inhibitor had better results (* $p < 0.05$) based on two-tailed distribution T-TEST.

5.5.5. Three-Dimensional Cryopreservation of Human Primary Hepatocytes

Three-dimensional encapsulation is another novel approach for cryopreservation of human primary hepatocytes created in this study. The 3D encapsulation using RGDS modified alginate has been widely adopted by cryopreservation of human embryonic stem cell, but it is “blue sky” in cryopreservation of human primary hepatocyte area,

The protocol of cryopreservation of human primary hepatocytes in RGD modified alginate beads was established in this study as a novel cryopreservation method. Human hepatocytes were encapsulated by RGD-alginate and then cryopreserved by standard protocol. The detailed protocol for RGD-alginate preparation, cell encapsulation and cryopreservation of the alginate beads were presented in Chapter 3.

Unfortunately, the experiment was unsuccessful. Less than 5% post-thaw cell viability was obtained, independent of cell density.

In order to find the flaws of the design, a further experiment was carried out by immediately dissolving the alginate beads containing freshly isolated human hepatocytes in dissolving medium and examining the cell viability. The results showed that only 40.8% cells survive the encapsulation and dissolving process, which is extremely low compared to its post-isolation cell viability (87.3%). Moreover, the viability of encapsulated cells was continually dropping with time. The results indicated that cell preparation and encapsulation, the way it is done, the time it takes, all severely affect the hepatocyte viability, even before cryopreservation.

Such low cell viability of human hepatocytes may be due to the following reasons:

- 1) Human hepatocytes were exposed to shear during ejecting process due to the viscous nature of the alginate. Such shear/pressure would damage the cell integrity, hence lead to cell death.
- 2) The exposure to the dissolving medium with high osmotic pressure would cause the cell to dehydrate, and thus damage the cell membrane [171].
- 3) Human hepatocytes might not attach onto the extracellular matrix that was provided by the RGD modified alginate, unlike other cells. The hepatocytes in free-form are more fragile than the attached cells [228].

Sadly, human liver samples were not available for further optimisation of the protocols and hence it was not possible to provide conclusive results on whether 3D RGD-alginate cryopreservation is effective to protect human hepatocytes in cryopreservation. Further studies are required to test this important hypothesis.

5.6. Summary

This chapter reports the experimental results of cryopreservation of human hepatocytes. Although this has been identified as critically important, experimental data are very limited due to the difficulty in obtain human primary hepatocytes. The experimental data from this study using human primary hepatocytes are hence of great value to the community.

In the experiments, a complete cryopreservation procedure, including freezing and thawing, was performed on freshly isolated human primary hepatocytes. The procedure was followed by a post-thaw viability assay and a comprehensive function assay including cell adhesion, phase I and II functions, albumin secretion and cell morphology. Despite the original poor condition of the isolated human hepatocytes, cells could be well preserved following the cryopreservation protocol at -196°C in liquid nitrogen tank for a minimum of 7 days. The results indicate the success of the cryopreservation procedure and the accurate design of the cryopreservation protocol. For long-term cryopreservation of human primary hepatocytes, although a similar outcome is expected, the efficiency of the current protocol still needs to be assessed and further research required.

Five different formulas of cryoprotective medium were carefully designed in order to determine the optimum parameters of the formula. Similar to the standard formula, 10% of DMSO is adopted by all the formulas since it has been proven to be the optimum concentration. The optimum concentration of FCS, however, is still a matter of some debate. In this study, 20% of FCS in cryoprotective medium provides better result than 10%. However, based on its various drawbacks, such as lack of batch to batch uniformity and high risk of contamination, 10% of FCS was found more effective than 20%. Recent studies have declared that different saccharides possess the capability to protect the cells against cryoinjury and so increase post-thaw viability. The results presented illustrate that sucrose provides a better protection than glucose. This is due to its functional ability of reducing intracellular ice formation and inhibiting extracellular ice growth, a function that glucose does not possess. The cell density of cryopreserved samples should be greater than 6 million cells per ml. Cells with lower densities were proven to possess low post-thaw

viabilities. A possible explanation is that cells at a high density may form a loose aggregate which provides additional protection to each other. The presence of ROCK inhibitor was deemed as unnecessary. As data suggested, ROCK inhibitor did not provide extra protection to human hepatocytes during the cryopreservation procedure. Its ability to inhibit cell apoptosis was not observed in this study.

During thawing, the recovery medium was further modified by adding 0.1M glucose to the standard recovery medium. The result displays significant improvements of the post-thaw cell viability and adhesion rate, which indicates the successful modification of the recovery medium.

Finally, the 3D cryopreservation of human primary hepatocytes using RGD-alginate encapsulation, similar to that reported in Chapter 3, was tested. The human hepatocytes were encapsulated in RGD modified alginate beads and cryopreservation by standard protocol. More than half of the cells in the sample did not survive the encapsulation process. Moreover, the post-thaw viability of the sample was extremely low (less than 5%). The results showed the importance of encapsulation and the fragility of human primary hepatocytes. Further research is required to test the efficacy of 3D cryopreservation of human hepatocytes.

The availability of human liver tissues proved the main limitation of this experimental work. More experimental work can be performed if human liver tissue made available. During the work, freshly isolated rat hepatocytes were used extensively for the training and practice. Extensive data from these experiments were obtained but not included in the thesis.

Chapter 6 Application of Electroporation and Sonoporation Mediated Cell Membrane Transport

6.1. Introduction

In the previous chapters, the experimental results presented demonstrated the challenges and complexities in human cell cryopreservation. One challenge would be how to protect intracellular components and prevent intracellular ice formation. Many effective cryoprotectants, such as sugars, cannot penetrate cells and mediated cell membrane transport must be used to allow their entry to cells. Mediated cell membrane transport is considered to be one of the most important developed techniques in recent years. It has a wide range of uses, particularly gene transfections. Physical methods to create temporary pores on cell membrane, using electronic pulses and ultrasound (i.e. electroporation and sonoporation), are most widely used for mediated cell membrane transport. Here, it is worth exploring their application in cell cryopreservation. With the help of electroporation and sonoporation, non-permeable cryoprotectants, e.g. trehalose, may be physically transported into the cells and provide protection during the freezing process.

The mechanism of electroporation involves a high voltage current being applied causing an interaction between the phospholipids of the bilayer which disrupts their structure for a limited period of time. The molecules of interest, which are normally impermeable to cell membrane, are able to pass through the cell membrane. The pores, created by the high

voltage electric current on the surface of the membranes, remain open for a very short period of time and the membranes recover shortly thereafter. Thus, the integrity of the cell membrane is well preserved and there is neither intracellular material loss nor injury. There have been many researches carried on in this area that involves delivering impermeable CPAs into the cells to promote cryopreservation or freeze-drying and delivering foreign DNA to achieve genetic manipulation or promote DNA transfection [229-234]. Different cell types have been adopted in these researches, such as human red blood cells, human T-lymphocytes cell line Jurkat, human embryonic stem cell line, the murine myeloma cell line, the murine fibroblast cell line, murine embryonic stem cell line, monkey embryonic stem cell line and etc. [231, 235-237]. However, same electroporation protocol cannot be applied to different cell types with different experimental purposes since some of the key experimental parameters are cell type specific such as the resealing time constant and the initial membrane permeability to certain chemicals and electrolytes [231]. Such experimental parameters are vital to a successful electroporation procedure. Thus, each experiment should be carefully designed according to different cell types and experimental purposes.

The mechanism of sonoporation is similar to, and in some cases superior to, electroporation. This technique utilizes high frequency sound or ultrasound in order to make cell membranes temporarily permeable to which they are not normally impermeable; particles such as genes, drugs and other large molecules. Hence, it has a similar property to electroporation, i.e. to transfer large molecules into an intracellular environment. Nonetheless, a successful sonoporation is, to some extent, more difficult to achieve than electroporation in the lab. The sonoporation system requires accurate design of the

impedance matching network circuits, carefully selecting of the ultrasonic transducer (different transducers have different frequencies, focus distance, shape and flat or concave surface), calibration of the transducer with specialized equipment and more equipment to facilitate the system such as oscilloscope and power amplifier. Thus, the sonoporation system is more difficult to control and adjust than electroporation system. Although the sonoporation has been widely used in delivery of gene, drugs, protein and permeable cryoprotectants [238-241], there is no successful case that uses sonoporation to deliver impermeable CPAs into human embryonic stem cell and human hepatocyte to promote cell cryopreservation. This area is definitely worth exploring.

In this study, the feasibility of application of electroporation and sonoporation to cell cryopreservation was studied. Trehalose, as a non-permeable cryoprotectant, was selected to be delivered through the cell membrane. The objective was to establish a practical protocol to promote cell membrane transport on a physical basis with minimum cell membrane damage and maximum cryopreservation efficiency. Human colorectal cancer cell line (DLD1), which is easily available, was chosen for this experiment. The feasibility of transporting trehalose into cells and its effect on cryopreservation outcome was studied. Afterwards, such techniques can be applied to human embryonic stem cells and human hepatocytes.

6.2. Electroporation Mediated Trehalose Transport on Cryopreservation

6.2.1. Materials and Methods

6.2.1.1. Chemicals and Reagents

The following chemicals and biochemical were used in the experiments: RPMI-1640 Medium with Sodium Bicarbonate, 1X Trypsin-EDTA, antibiotic/antimycotic solution, DMSO, Trehalose and Trypan blue were purchased from the Sigma-Aldrich Company Ltd. (Dprestd, UK); fetal bovine serum (FBS) from M.B. Meldrum Ltd. (Bourne End, UK); MTT and PBS from Invitrogen Ltd. (Paisley, UK). Other consumptions used, such as culture flask and pipettes were all purchased from VWR International Ltd (UK).

6.2.1.2. Cell Line

The objective of this chapter is to establish the experimental protocols of cell cryopreservation using DLD1 as the test cells. DLD1 is a human colorectal cancer cell line, shown in Figure 4.1. The cell viabilities after cryopreservation are compared in groups in terms of different CPAs, concentration of CPAs, cooling rate and cooling protocol. Each pool of data will be followed by an analytical discussion.

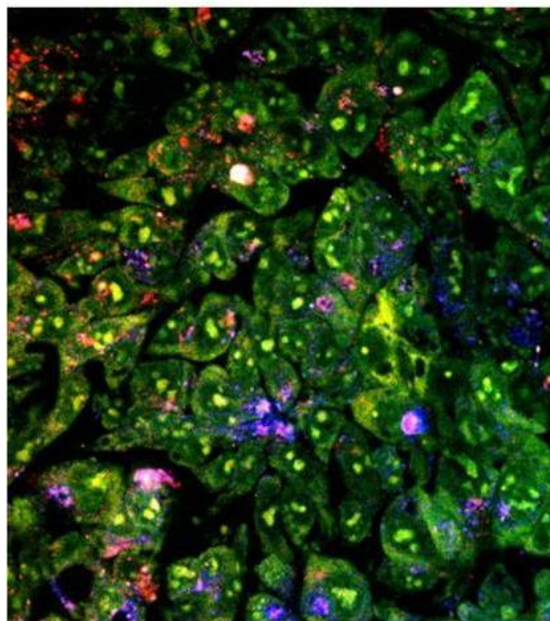


Figure 6.1 Monolayer of DLD1 Cells was stained with acridine orange and the imaged produced with the Oxford University's multiphoton microscope.

Image courtesy of Centre for Tissue Engineering and Bioprocessing

6.2.1.3. Cell Culture Protocols

For maintenance, cell lines were grown as monolayers on the surface of standard T-75 cm² tissue culture plates with RPMI-1640 media (w/Sodium Bicarbonate, supplemented with 10% fetal bovine serum and antibiotics). The flasks were incubated at 37°C in a 5% CO₂ humid incubator. As part of this maintenance, the cells were subcultured twice a week. Subculturing involved trypsinizing confluent monolayers which removed cells from the surface and dispersed the monolayer as single cells.

6.2.1.4. Cryopreservation Methods

Standard methods

For cell sample preparation, cell lines were trypsinized from the tissue flask and resuspended with a cell intensity of 1×10^6 cell per mL. Cell suspensions were loaded into several vessels with a volume of 1 mL and centrifuged for 2 minutes at 10000 rpm. The culture medium was removed and the cells resuspended with different CPAs, then loaded into cryotubes.

The samples were classified into two groups in terms of different freezing protocols. In order to achieve a slow-rate cryopreservation, one group of the samples was cryopreserved by a programmed freezer (Kryo 560-16, Planer, USA). This programmed freezer can provide a wide range of freezing rates, including a very slow freezing rate, which is very difficult to achieve with a normal freezer. The other group of samples was placed into the NALGENE™ Cryo 1 °C freezing container and was exposed to a cooling rate of -1 °C per min in -80 °C freezer. The container uses anhydrous 2-propanol as a cooling buffer to achieve this cooling rate in the freezer.

Freezing protocols were designed as follow:

- 1) Protocol A – a relatively faster cooling rate. The samples were placed in the freezing container at room temperature. The container was then directly placed into a -80 °C freezer. The container secures a -1 °C/min cooling rate. After 2 hours, half of the samples were removed from the -80 °C freezer and placed directly into liquid

nitrogen. All the samples were preserved for 48 hours before the post-thaw viability assay.

- 2) Protocol B – slow cooling rate. The samples were placed into programmed freezer and cooled from 20 °C to 4 °C then held for 5 minutes. After that, the samples were cooled from 4 °C to -7 °C with -1 °C per minute cooling rate and then to -80 °C with -0.3 °C/min cooling rate without any pauses. When the samples reach -80 °C, half of the samples were moved from the programmed freezer and placed into -80 °C freezer. The rest of the samples were cooled from -80 °C to -120 °C with -1 °C/min. Finally, these samples were directly placed into liquid nitrogen. All samples were preserved for 48 hours before post-thaw viability assay.

Electroporation method

Before all of the experiments, DLD1 cells were trypsinized from a tissue flask and counted using a haemocytometer. Thereafter, 2×10^6 cells were resuspended in 400 μ L PBS or PBS containing 0.2 mol/L trehalose and loaded into an electroporation cuvette.

Each cuvette was clearly marked, placed between electroporation electroporators and then exposed to a single voltage pulse with field strength of 625 V/cm^2 at room temperature. The capacitance at low voltage mode is 1050 μ F and the application time was 25 ms. After the exposure to electric pulses, each sample was allowed to remain in the buffer for 20 minutes and then loaded into vessels. The vessels were centrifuged and removed from the buffer. The cell pellets were resuspended in 1 mL culture medium for viability assay before cryopreservation. The cell suspension was loaded into cryo-vessels with different CPAs

and cryopreserved using $-1\text{ }^{\circ}\text{C}/\text{min}$ freezing rate. The post-thaw cell viabilities were measured after 24 hours preservation in $-80\text{ }^{\circ}\text{C}$ freezer.

The electroporation cuvettes (VWR, UK) were molded with aluminium electrodes and medical grade polycarbonate with a gap size of 4mm and 800 μL maximum volume (Figure 6.2). The cuvettes with a 4mm gap between electrodes could generate a fairly low field strength, which is much more suitable for mammalian cells.



Figure 6.2 The electroporation cuvettes

The ECM 399 Electroporation system (BTX-Harvard Apparatus, USA) was adopted in this study. The ECM 399 is an exponential decay wave electroporation system specifically designed to deliver the field strengths and pulse lengths required for the simple transformation of bacterial and yeast cells. In a low voltage mode, which is the mode we were using, the ECM 399 has been tested as effective for transfections of mammalian cells. This electroporation system benefits from its simple design and easy operation. However, the efficiency of this system is limited due to several unchangeable physical parameters, such as capacitance and application time.

Storage temperature

As the standard freezing protocols stated, two different cryopreservation temperatures were tested in this study. The samples were preserved in -80 °C and -196 °C, achieved by using the -80 °C freezer and liquid nitrogen tank respectively. For the electroporation method, cells were preserved at -80 °C for 24 hours before viability assay.

Cryoprotectants (CPAs)

Two types of cryoprotectants (CPAs) were adopted in this study, DMSO as the permeable CPA and Trehalose as the non-permeable CPA. Their cryoprotective efficiencies were assessed by post-thaw viability assay after they were preserved for 24 or 48 hours.

Following a standard method, the efficiencies of different CPA concentrations were analysed and compared using trypan blue exclusion method. The concentrations of DMSO with the highest cryopreservation efficiency were selected from 5%, 10% and 20%. The results with optimum concentration were compared with the results obtained by using trehalose as CPAs. Two trehalose concentrations were tested, which are 0.5M and 1M.

The standard CPA formula was modified into a further 5 sub formulas, presented in table 6.1. Sample 1, the control group, was treated with 0% CPA. Sample 2 was treated with 10% DMSO and the results obtained were used as a reference to compare and cross reference the results of the standard cryopreservation method with the other 5 groups in order to investigate the effects of the electroporation process.

Table 6.1. Modification of CPA formulas

Sample	DMSO in freezing medium	Trehalose in electroporation buffer (concentration)	Trehalose in freezing medium (concentration)
Sample 1	0	0	0
Sample 2	10%	0	0
Sample 3	10%	0	0.2 M
Sample 4	0	0	0.2 M
Sample 5	0	0.2 M	0.2 M
Sample 6	10%	0.2 M	0.2 M
Sample 7	0	0.2 M	0

Thawing

The samples were removed from the freezer or liquid nitrogen tank after 48 hours cryopreservation and quickly thawed in a 37 °C water bath. After the samples completely thawed, the cell suspension was transferred in to tubes and the cryoprotective medium was removed. The cell pellet was resuspended in fresh culture medium and the viability assay was carried out.

6.2.1.5. Statistical Analysis

Each experiment was performed in triplicate. Analysis of variance (ANOVA) was adopted as the method to determine any significant statistical differences between the groups. P-values were also calculated using T.TEST function with a two-tailed distribution in Excel.

P-values of less than 0.05 and were considered as significant for statistical purposes. All data was presented is in the format of mean $\pm$ standard deviation.

6.2.2. Results and Discussion

6.2.2.1. Analysis of Standard Cryopreservation Method

DMSO Concentration

DMSO is considered as one of most effective CPAs and commonly adopted by most researchers during cryopreservation. The cell viability, with differing concentration of DMSO as CPA, is shown in Figure 6.3.

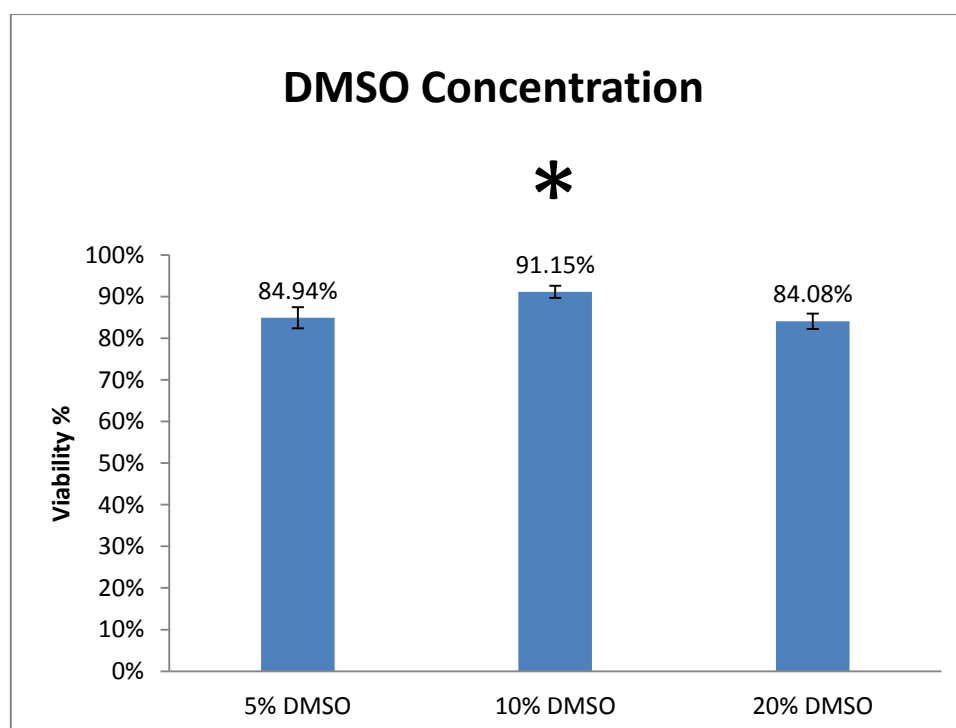


Figure 6.3 Post-thaw cell viability of samples preserved using different DMSO concentrations. Data is given as mean $\pm$ standard deviation, obtained from at least three independent trials. (* $p < 0.05$) {Experiment

conditions: operating temperature: 20°C, cryoprotective medium: culture medium supplemented with DMSO, preservation time: 2 days}

As the data in Figure 6.3 illustrates, post-thaw cell viability attained a peak value among the three tested DMSO concentrations, which indicated that the cryoprotective medium, supplemented with 10% DMSO, provided maximum protection to cells during the freezing process. Having identified the optimum DMSO concentration, 10% DMSO was adopted for the following experiments.

Cryoprotective efficiencies of DMSO and trehalose with different cooling rate

As the data illustrates in Figure 6.4, trehalose demonstrated little protection during the cryopreservation process when compared with DMSO. However, approximately 14% of the cells survived the freezing process because of the protection of trehalose. This is due to its functional abilities of reducing intracellular ice formation and inhibiting the rate of extracellular ice growth. Since trehalose is impermeable to cell membrane, its protective ability is limited.

Cooling rate is another key parameter of cryopreservation. As illustrated in Figure 6.4, a cooling rate of -1°C/min shows better result than -0.3°C/min, but there is no significant difference between the two groups. However, -1°C/min is much easier to achieve than -0.3°C/min in practice. Thus, -1°C/min was adopted as the optimum cooling rate.

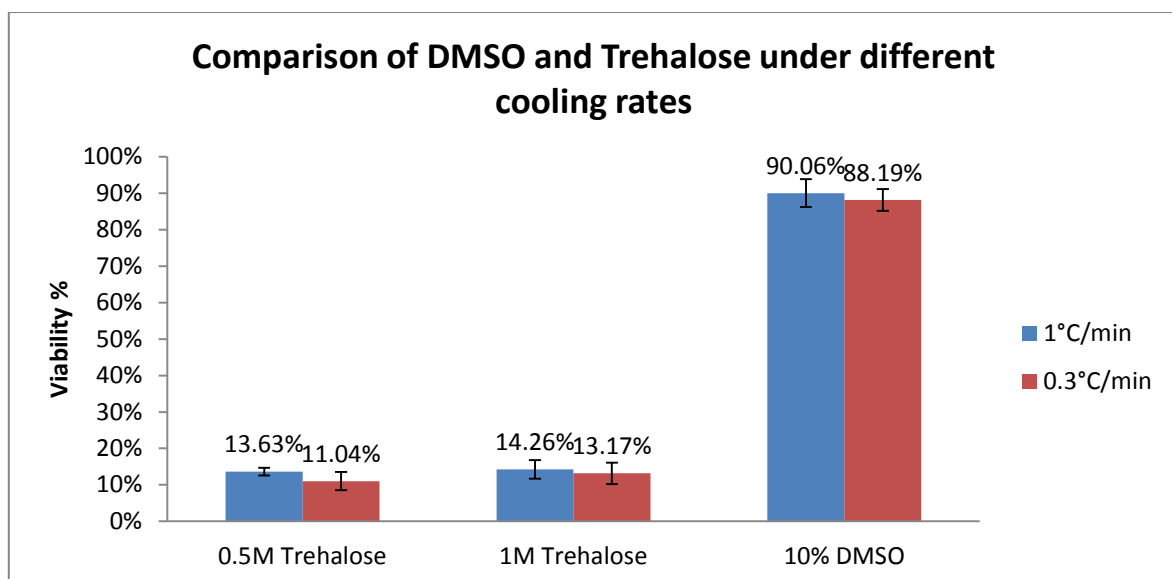


Figure 6.4 Comparison of DMSO and Trehalose under different cooling rates. Data is given as mean $\pm$ standard deviation, obtained from at least three independent trials. . No significant difference between the two groups of data according to two-tailed distribution T-TEST {Experiment conditions: operating temperature: 20°C, cryoprotective medium: culture medium supplemented with 0.5M Trehalose, 1M Trehalose and 10% DMSO respectively, preservation time: 2 days}

Comparison of different storage temperatures

The comparison of post-thaw viabilities under different preservation temperatures is shown in Figure 6.5. The results satisfy the expectations. At the lower preservation temperature in liquid nitrogen, the post-thaw cell viability with DMSO as CPA ($74.60\% \pm 4.12\%$) is significantly lower in comparison ($90.06\% \pm 5.85\%$) at -80°C . For trehalose, both concentrations demonstrated little protection in both conditions. Generally, for long-term cell cryopreservation, cells can be preserved efficiently in liquid nitrogen rather than the -80°C freezer. Thus, the decrease of the post-thaw viability is a drawback, but this is acceptable when seeking long-term cell preservation.

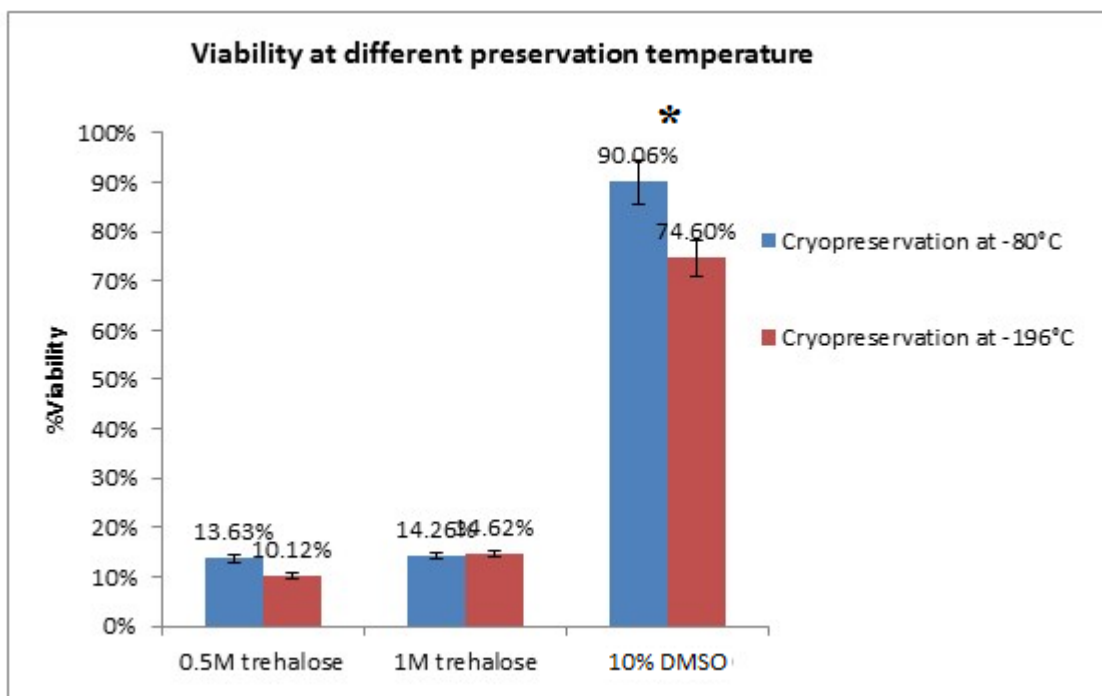


Figure 6.5 Post-thaw cell viabilities under different storage temperatures. Data is given as mean±standard deviation, obtained from at least three independent trials. (* $p < 0.05$) {Experiment conditions: operating temperature: 20°C, cryoprotective medium: culture medium supplemented with 0.5M Trehalose, 1M Trehalose and 10% DMSO respectively, preservation time: 2 days}

6.2.2.2. Analysis of Application of Electroporation to Cryopreservation

Cells were classified into 7 different groups for differing testing purposes. Samples in different groups were cryopreserved with different CPAs and the results were analysed and compared in order to verify the feasibility of the application of electroporation to cell cryopreservation.

Sample 1, as the control group, was cryopreserved without any CPA. The results showed that only 1.54%±1.03% cells survived the freezing process. Such low post-thaw viability indicates that the cryoinjury is lethal to the cells without cryoprotectant.

The electroporation procedure was applied to sample 2, before cryopreservation, with 10% DMSO. The results were compared with the data obtained from the standard method in order to demonstrate the damage of the electroporation procedure to cells. From the data shown in Figure 6.6, there is an approximate 5% difference between these two groups of data. Such close post-thaw viabilities indicate that the electroporation procedure did not cause major damage to cells. Compared to cryoinjury, induced by the freezing process, the damage from the electroporation process can be ignored.

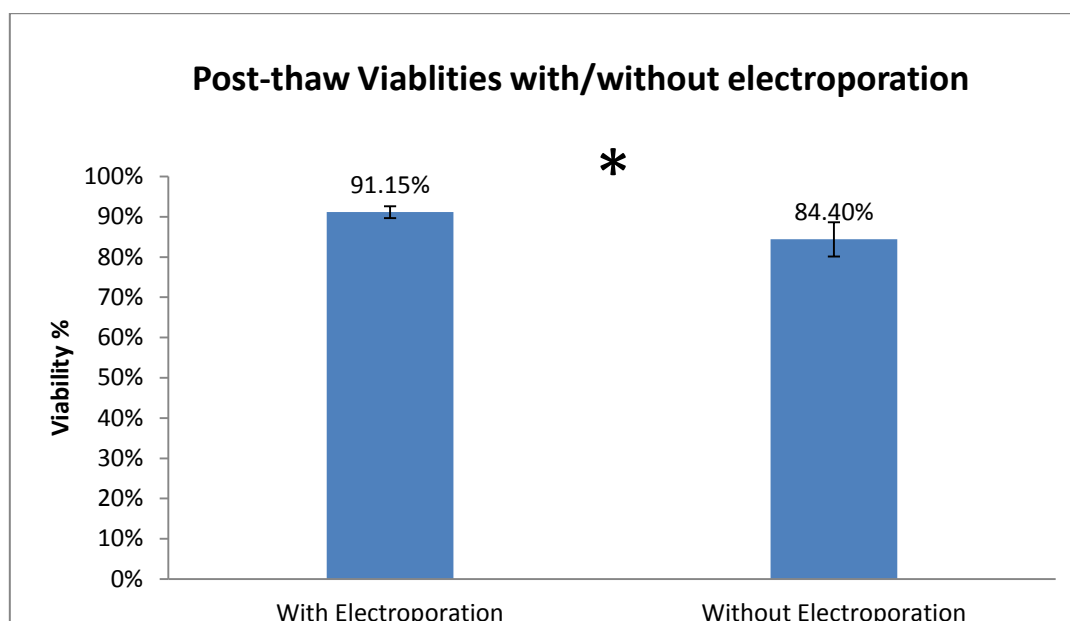


Figure 6.6 Comparison of post-thaw cell viabilities with or without electroporation. Data is given as mean $\pm$ standard deviation, obtained from at least three independent trials. (* $p < 0.05$) {Experiment conditions: operating temperature: 20°C, cryoprotective medium: culture medium supplemented with 10% DMSO, preservation time: 2 days}

Sample 3 was cryopreserved with 10% DMSO and 0.2M trehalose whilst sample 4 was preserved with only 0.2M trehalose. As Figure 6.7 illustrates, when both DMSO and trehalose were present in the freezing medium, DMSO was the most effective one.

Moreover, the data also confirmed that without the aid of electroporation, trehalose was impermeable to cell membrane.

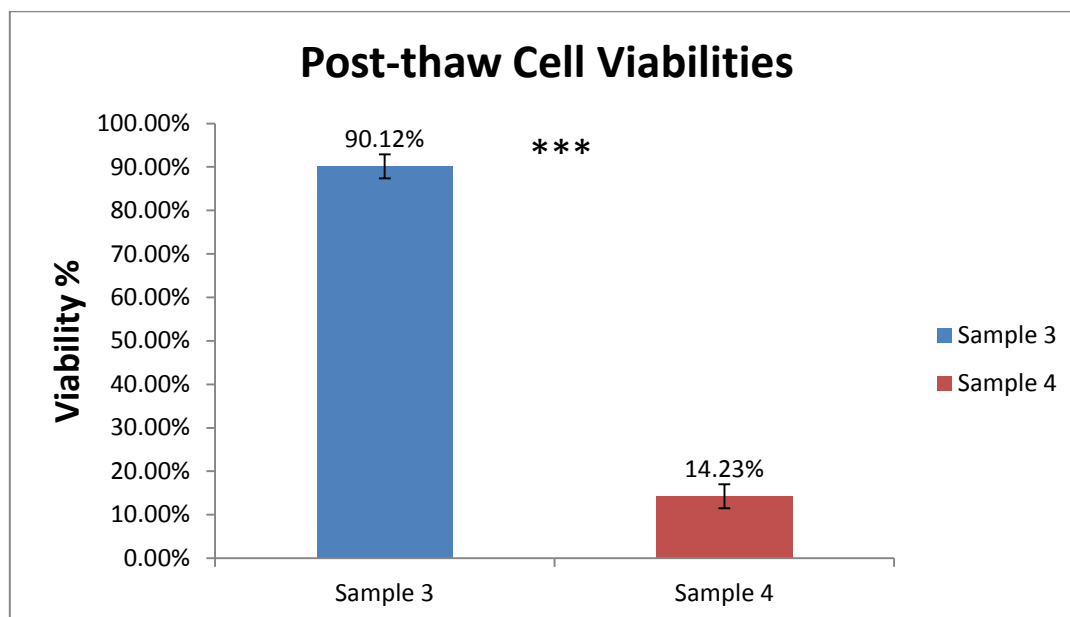


Figure 6.7 Comparison of post-thaw cell viabilities of sample 3 and 4. Data is given as mean $\pm$ standard deviation, obtained from at least three independent trials. (***) ($p < 0.001$) {Experiment conditions: operating temperature: 20°C, cryoprotective medium: culture medium supplemented with 10% DMSO+0.2M trehalose and 0.2M trehalose only, preservation time: 2 days}

For sample 5 and 7, 0.2M trehalose was added into the electroporation buffer before electroporation. The results of post-thaw viabilities were compared with the data gained from sample 1 and 4 (Figure 6.8).

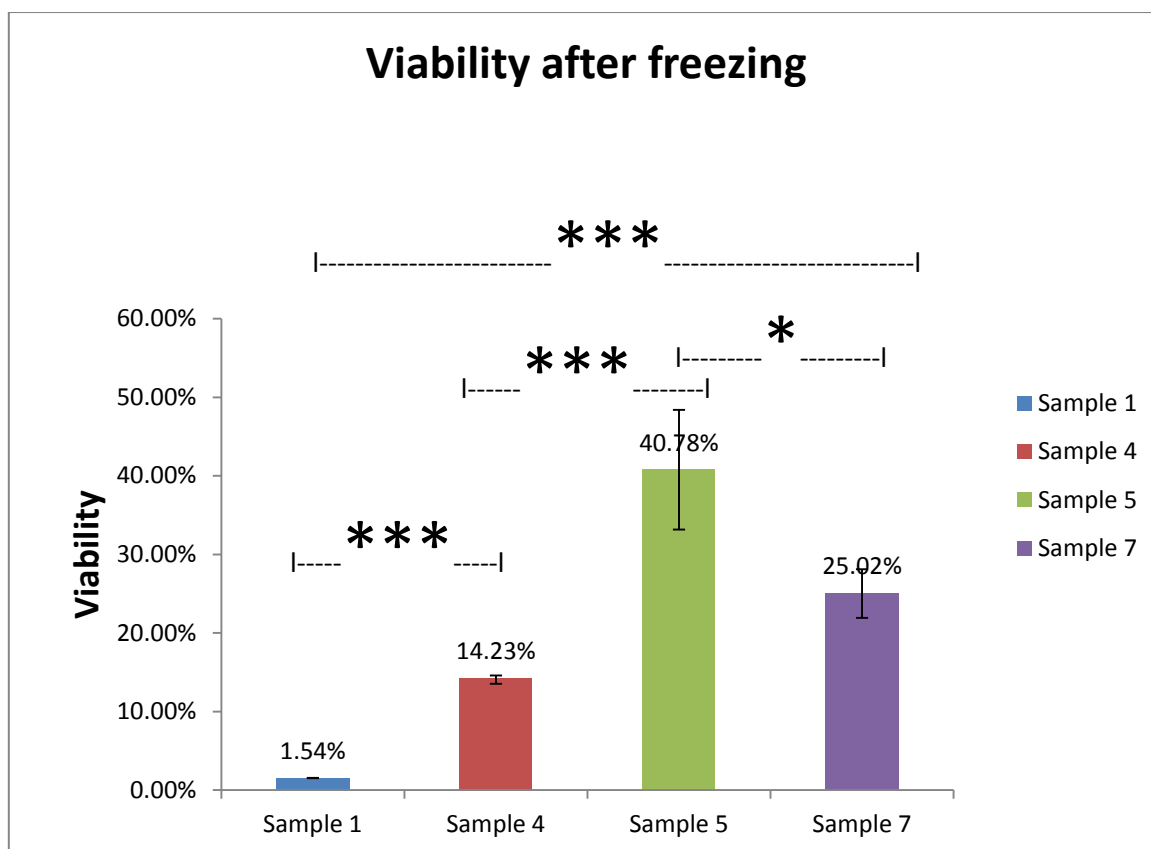


Figure 6.8 Comparison of post-thaw cell viabilities of sample 1, 4, 5 and 7. Data is given as mean±standard deviation, obtained from at least three independent trials. (* $p<0.05$, ** $p<0.01$, *** $p<0.001$) {Experiment conditions: operating temperature: 20°C, cryoprotective medium: refers to table 6.1, preservation time: 2 days}

According to table 6.1, the difference between the freezing protocol sample 1 and sample 7 was the 0.2M trehalose in the electroporation buffer. A significant improvement of cell viability between sample 1 and 7 was found in Figure 6.8. In addition, sample 4 achieved the same viability improvement as sample 1. As post-thaw viability of sample 4 suggests, trehalose works insufficiently as a cryoprotectant when it is impermeable to cell membrane. Theoretically, trehalose could form some hydrogen bonds with proteins or cytoplasm to support their structures, hence preserve their functions. Consequently, when trehalose penetrates the cell membrane, in addition to its function of cryoprotectant, it can act as

lyoprotectant to protect the proteins and cytoplasm against cryoinjury. Considering the significant increase on post-thaw viability, the only explanation is that the trehalose was successfully delivered into the cells by the electroporation procedure so it can work efficiently as a cryoprotectant and lyoprotectant.

Moreover, there is another problem which can be identified from Figure 6.8 - the standard deviations of the viabilities of sample 5 and 7 are relatively high. This phenomenon indicates that the electroporation was not stable and the efficiency of electroporation was far from promising. These problems might due to the following reasons: First of all, the ECM 399 electroporation system was specifically designed for bacterial and yeast cells, it may only work for some types of mammalian cells at a low voltage mode. Generally, low voltage long pulse produce high rates of transient poration, while high voltage short pulse provides a higher rate of stable poration. Secondly, the electroporation system is too simple and much of its physical parameters cannot be altered. Since it is impossible to adjust these parameters, such as voltage, capacitance value, and pulse lengths, the intrinsic parameters could lead the electroporation to be inefficient. Finally, due to the high passage number of the DLD1 cells, it is possible that the cells' activities and capabilities to process chemicals have already decreased. The dysfunction of DLD1 cells might be a further explanation of the unstable electroporation results.

6.3. Sonoporation – Principle and System Design

6.3.1. Principles of the System Design

The sonoporation system is designed to achieve successful delivery of trehalose through cell membranes. An ideal design aims to maximize the reparable sonoporation and minimize the lethal. As described in the previous chapter, both reparable and lethal sonoporation are induced by the inertial cavitation effect [242]. Thus the design of the ultrasound system at the initial stage aims to enhance the inertial cavitation effect in order to obtain higher sonoporation levels. Lethal sonoporation could be minimized by other means in the future, such as the addition of other chemicals.

The major exposure systems, adopted by most researchers in this area are shown in Figure 2.3. After careful consideration and evaluation, the rotating tube system is utilized. Moreover, several modifications have been made to optimize the exposure system. Specifically, the modifications include a second ultrasound transducer and the addition of a network analyser. Further modifications are needed based on observations.

The vessel containing the experimental material can significantly influence the bioeffects and efficiency of the exposure system. In this study, a polypropylene tube is chosen as the cell suspension container.

The rotating exposure system of this project is shown in Figure 2.7 no. 3. There are two complementary rotation-effect models which could potentially explain the interaction of lytically effect bubbles and cells in the rotating exposure tubes. The first one is the standing

wave model. Standing wave, known as stationary wave, refers to a wave that remains in a constant position. Like the design in Figure 2.7 No.2, the standing wave usually forms in the polypropylene tube whilst placed in a water bath under a continuous wave. Without rotating, cells congregated at the pressure minima of the standing wave, while lytically effective bubbles congregated at pressure maxima. Such conditions will minimize or even eliminate the cell lysis due to the segregation of bubbles capable of lysing the cells. Since the cells are trapped by a relatively weaker force, tube rotation could stir the suspending medium, thereby increasing the probability of association of lytically bubbles and cells. This hypothesis is supported by many researchers and their work, for example [243]. The second model is the bubble returning model. When the microbubbles grow larger than its resonant size, they will implode violently and then cavitation occurs. In the stationary system, however, the transducer will push the bubbles, which are smaller than the resonant size, to the far side of the tube. Furthermore, as long as the ultrasonic wave is present, the bubbles cannot travel back to the bulk of the suspending medium under such a force from the transducer. This phenomenon limits the potential interaction of cells and bubbles. If the rotation method is used, it will move the bubbles at the furthest side of the tube around to the position nearest the transducer; hence the bubbles can be swept back through the cell suspension. The entire trace of the bubble with the presence of rotation is similar to drawing a closed half cycle starting from the furthest side of the tube. Undoubtedly, this can contribute to the enhancement of cell-bubble interaction.

6.3.2. The Design of the Sonoporation System

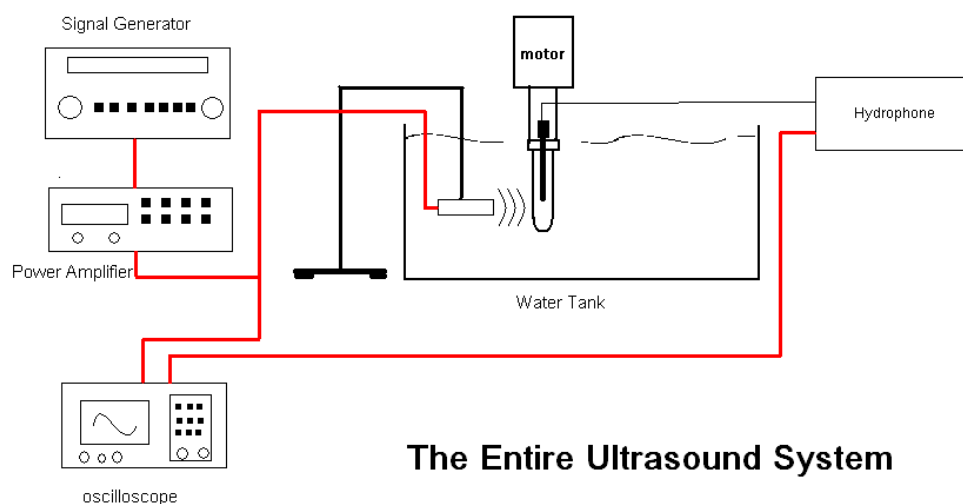


Figure 6.9 The design of the sonoporation system

As shown in Figure 6.9 above, the complete sonoporation system is composed of a signal generator, a power amplifier, a water bath, the exposure system, an oscilloscope and a hydrophone. The transducer used in this design is a videoscanner flat-plate-focused immersion transducer with centre frequency of 2.25 MHz and focus distance of 1 inch, purchased from OLYMPUS industrial. A needle type hydrophone is placed inside the tube through a small hole at the top of the tube cover. This kind of hydrophone can be screw-connected to the preamplifier base unit and transmits the signal, which detected from the inside of the tube, to the oscilloscope. Thus, the acoustic pressure inside the tube can be measured and monitored.

At the beginning of the experiment, a network analyser was involved the Agilent/HP 8753C, this is a vector network analyser which can measure both amplitude and phase properties associated with the reflection and transmission of RF electrical signals. The

8753C is one of the most widely used network analysers which offers high accuracy in measuring S-Parameters, reflection and transmission parameters. This network analyser has a working frequency range from 300 kHz to 3 GHz which covers all the frequencies required in this project. For implementation, the network analyser could work as a substitute for the signal generator.

Another important part of the design is the impedance matching network circuits, as shown in Figure 6.10. These circuits are placed between the signal generator or network analyser and the ultrasound transducer. The impedance matching network was used to match the input impedance of an electrical load with the output impedance of its corresponding signal source in order to maximize the power transfer and/or minimize reflections from the load. Without this, most of the power from the signal generator will be reflected back to the source and the output power at the transducer will be extremely low. In this case, the cell suspension could only receive a very low acoustic pressure which leads to an inefficient sonoporation.

Other selections of measurement equipment are based on testing during experiments.

LOWPASS Hi-Low MATCHING NETWORK

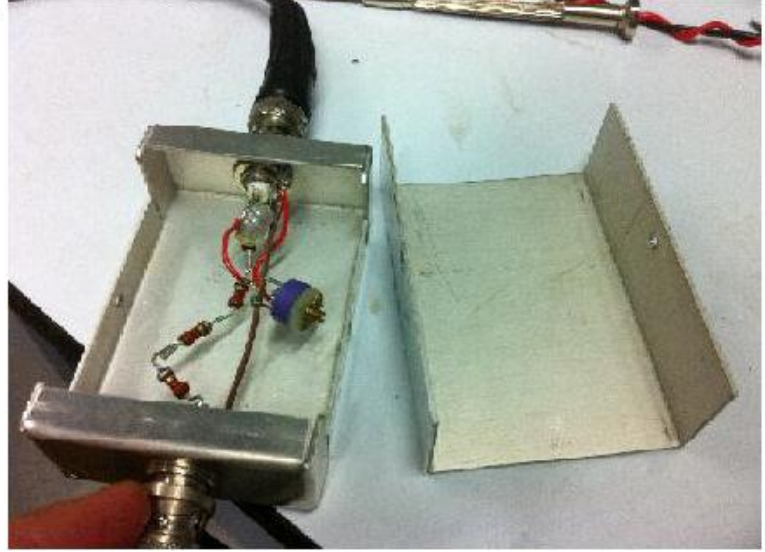
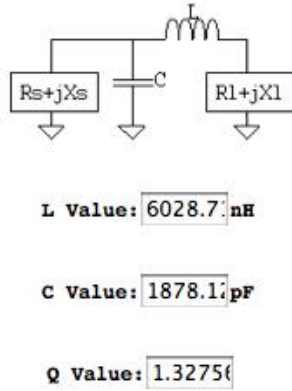


Figure 6.10 The design of impedance matching network.

6.3.3. Key Physical Parameters

The violently imploding ultrasound-induced microbubbles results in inertial cavitation, which is the fundamental mechanism of the sonoporation [242]. These bubbles can grow due to a process called rectified diffusion, which is the process of gas diffusing into and out of the bubble whilst an acoustic pressure cycle is applied to the medium via ultrasound. For non-inertial cavitation to occur, a threshold condition must be satisfied i.e. when the flow rate of gas into the bubble is equal to the flow rate of gas out of the bubble. Equation 1 expresses the rarefaction process and denotes the mass flow rate of gas diffusing into the bubble.

$$\frac{dm}{dt} = \frac{8\pi}{3} DC_{\infty} R_o \left(\frac{P_A}{P_o} \right)^2 \left(1 + \frac{2\sigma}{R_o P_A} \right) \quad (1)$$

Where P_A is the amplitude of the acoustic pressure, P_o is the ambient pressure, R_o is the initial radius of the bubbles, D is the diffusion constant, σ is the surface tension of the bubble and C_∞ is the concentration of gas dissolved in the liquid ignoring the gas bubbles.

Similarly during compression, equation 2 denotes the mass flow rate of the gas diffusing out of the bubble. Where C_o is the saturation gas concentration.

$$\frac{dm}{dt} = -4\pi DC_\infty R_o \left(1 + \frac{2\sigma}{R_o P_A} - \frac{C_\infty}{C_o} \right) \quad (2)$$

Thus when the equation 1 and 2 [129] are equal, the threshold for non-inertial cavitation is reached and stable cavities will occur in the liquid. Equation 3 describes this stipulation when the threshold is met.

$$\left(\frac{P_A}{P_o} \right)^2 = \frac{3}{2} \left[1 - \frac{C_\infty}{C_o} \left(1 + \frac{2\sigma}{R_o P_A} \right)^{-1} \right] \quad (3)$$

For inertial cavitation there are two threshold conditions an upper and a lower limit in which inertial cavitation can occur. These two thresholds, along with the non-inertial cavitation threshold, were plotted in Figure 6.11 to demonstrate the relationship between the acoustic pressure and the bubble radius.

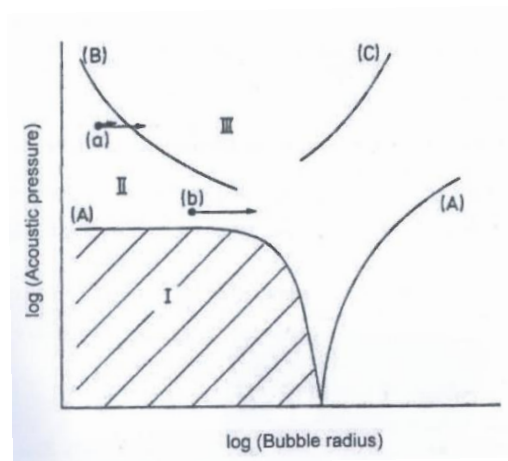


Figure 6.11 Cavitation Threshold Conditions: (A) Non-inertial Cavitation Threshold Condition (B, C) Upper and Lower Threshold Conditions For Inertial Cavitation, respectively[129].

Bubbles in region I represent the stable cavities that lead to non-inertial cavitation. Bubbles in region II represent non-inertial cavities that have the potential to grow unsteadily and consequently collapse. Once the bubble grows from region II into region III as shown by the arrow (a) the bubble is certain to collapse under the threshold conditions. This collapse will lead to a high energy release in the form of a shock wave; this mechanism could then lead to an increase in cell permeability. Other bubbles in region II will grow by rarefaction until they are large enough to rise to the surface of the liquid due to their buoyancy; this is indicated by arrow (b) [129]. Thus the ultrasound transducer must produce the inertial cavities that satisfy the threshold condition. In sonoporation, for this to occur, bubbles must follow a similar path as arrow (a) in Figure 6.11.

Typically, the extent of sonoporation is evaluated by the mechanical index (MI). The MI contains several key physical parameters, such as peak negative pressure (PNP) and the

centre frequency of the transducer. The PNP can be measured by the voltage of hydrophone. The following equations were used to convert the voltages recorded to the acoustic pressure.

$$A = 20 \log_{10} \left(\frac{S}{S_o} \right) \quad (6)$$

$$S = V/P \quad (7)$$

Where A is the hydrophone sensitivity, V is the recorded hydrophone voltage and P is the acoustic pressure requiring calculation. Each hydrophone has its own sensitivity chart which gives us the precise number of S_o and S . The calculation of PNP is of vital importance in sonoporation, because the bubbles form and grow at the negative cycle of the ultrasonic wave. On the basis of prior researchers' design, MI equal to 1.5 is the accepted starting point.

6.3.4. Evaluation Protocol of Cell Resealing Time after Sonoporation

In order to obtain further understanding of sonoporation, cell resealing time should be considered. The accessibility of pore resealing time can be assessed using FITC dextran (70 kDa, approximate 6 nm Stokes's radii), which is extremely difficult to pass through the cell membrane. The experiment could follow these subsequent steps:

- (1) Apply ultrasound to the cell suspension in the presence of FITC dextran, confirm the effect of ultrasound and the uptake of large molecules with the fluorescence microscope.

- (2) Apply ultrasound to several cell suspension samples with the same cell concentration but with the absence of FITC dextran, with all the parameters the same as the previous stage. Then add FITC dextran at 0, 2, 5, 10, 25, 50 seconds to the samples respectively after sonoporation.
- (3) Examine the extent of the uptake of the fluorescent dye

The time point, with a significant decrease of FITC-dextran-positive cell number, indicates the “close” time of the pore induced by ultrasound.

6.4 Summary

This chapter presented a feasibility study of mediated cell membrane transport to enhance cell cryopreservation by creating transient pores on cell membrane, using physical methods – electroporation and sonoporation. These pores are big enough to allow non-permeable cryoprotectants, such as trehalose, enter the cells, but small enough not to kill the cells.

Electroporation was tested using human colorectal cancer cell line (DLD1). Following the establishment of a fairly standard cryopreservation protocol, trehalose was added into the cryoprotective medium. Electroporation was applied using a selected electronic field strength and application time, and cells treated without trehalose, with trehalose but not electroporation, with trehalose with electroporation were cryopreserved. The viability of each group was compared. It was found that the electroporation mediated trehalose indeed improve the cell viability during cryopreservation process.

Sonoporation was also explored. The principle of the design was presented considering the control on the focus and strength of ultrasound. A prototype of sonoporation system was designed and assembled.

The presented applications of electroporation and sonoporation to cell cryopreservation of human embryonic stem cells and human primary hepatocytes are, to some extent, state-of-art in the cryopreservation field. Although many researches regarding the application of electroporation and sonoporation have been done in this area [229-237], the applications of such techniques to hESCs and hepatocytes are rarely achieved. Moreover, such applications require original designs of the systems and experimental protocols since some of the key parameters of the experiments are cell type specific. Hence, this area is definitely worth exploring. Unfortunately, the electroporation and sonoporation system have not been applied to human embryonic stem cells and human primary hepatocytes in this study due to lack of time and experimental equipment. But the feasibility study has achieved and some of the experimental parameters have been selected. Further studies using human embryonic stem cells and human primary hepatocytes can be performed in future.

Chapter 7 Conclusions and Future Work

7.1 Conclusions

The objectives of this study are to develop new protocols to improve the cryopreservation outcome of human embryonic stem cells and human primary hepatocytes and to develop novel methods for cell cryopreservation. Based on the experimental study, the following conclusion can be derived.

1. 3D cryopreservation of human embryonic stem cells using RGDS modified alginate was proven to be more effective compared to conventional cryopreservation. An improvement of post-thaw cell survival ($70.76\% \pm 3.25\%$) over that of conventional cryopreservation ($62.78\% \pm 2.45\%$) was obtained. A reduction of post-thaw cell apoptosis of 3D cryopreservation was also gained. These findings were visually observed by Calcein-AM and EthD-1 staining.
2. The key parameters affecting hESC cryopreservation were studied and optimised. 10% DMSO was identified as the most effective CPA concentration which can lead to maximum cell survival. The $-1^{\circ}\text{C}/\text{min}$ was shown to be the optimal cooling rate, while 30 minutes was considered as the optimal CPA loading time.
3. The RGDS peptide modification protocol was established and the bonding efficiency was quantified by FTIR and combustion analysis. As experimental data illustrated, 0.48% wt. RGDS peptide was successfully attached onto the alginate

backbone. This is comparable to the result given for commercially available RGDS-alginate from NovaMatrix (0.8% wt.).

4. The effect of RGDS modified alginate on 3D cryopreservation of hESC was compared to that of unmodified alginate. It can be concluded from the obtained data that the modification of alginate is necessary in order to achieve an efficient 3D cryopreservation process and can also lead to a more satisfactory outcome compared to unmodified alginate.
5. The effect of ROCK inhibitor was also tested. As the results demonstrated, the efficiency of hESC cryopreservation without the presence of ROCK inhibitor was low. Furthermore, the post-thaw apoptosis rate of cells preserved without ROCK inhibitor was consistently higher than cryopreserved with ROCK inhibitor after thawing. It can be concluded that the addition of ROCK inhibitor to cryoprotective medium and recovery medium is necessary.
6. The isolation protocols of human primary hepatocytes were established. Following these protocols, human primary hepatocytes were successfully isolated from fresh liver samples with a fairly high cell viability and preserved cell function. This conclusion was confirmed by cell morphology and function assays.
7. The large liver-wedges samples were identified as the ideal liver samples for isolation procedure with highest cell yield rate.
8. When human primary hepatocytes isolated from liver sample had a with low cell viability, purification with Percoll was required. This procedure could purify the obtained human hepatocytes by removing the majority of the dead cells from the cell suspension.

9. The perfusion isolation method of isolating hepatocytes should be adopted if possible. As the result demonstrates, the perfusion method achieved high cell yield rate while the direct-digestion method always led to low cell yield rate. However, it should be noted that it is not always possible to apply the perfusion method to small pieces of liver sample. The liver sample requires blood vessel network within the tissue and at least 1 suitable vessel on the cut surface that can allow the cannulae to suture. The liver samples that do not meet these requirements are considered to be isolated using the direct-digestion method.
10. The post-thaw cell survival assay and a comprehensive function assay, including cell adhesion, phase I and II functions, albumin secretion and cell morphology, were performed to cryopreserved human hepatocytes after thawing in order to analyse the cryopreservation outcome and optimize the parameters. The results indicate the success of the cryopreservation procedure and the optimisation of the cryopreservation protocol. 10% of FCS was found more effective than 20%. Considering the drawbacks of using FCS in cryoprotective medium, such as lack of batch to batch uniformity and high risk of contamination, 10% was utilized as the optimum FCS concentration. Sucrose was demonstrated to provide extra protection to human hepatocytes during the freezing and thawing process. 6 million cells/ml was selected as the optimum cell density for the cryopreserved sample, since lower densities proved to lead low post-thaw cell survival. Unlike the result of hESC cryopreservation, the presence of ROCK inhibitor was deemed as ineffective.
11. The electroporation was applied to cell cryopreservation in order to enhance the cryopreservation outcome by physically delivering trehalose, as cryoprotectant, into cells. The experimental data demonstrated by using DLD1 cells suggests that the

electroporation procedure was successful with limited efficiency and the electroporation mediated trehalose indeed improve the post-thaw cell viability.

12. Based on the study of the principle of the sonoporation technique, a prototype of sonoporation system was designed and assembled. Further study using human embryonic stem cells and human primary hepatocytes can be performed in the future.

7.2. Original Contributions

The original contributions of the presented work can be summarized as follow:

- The normal alginate was successfully modified by RGDS peptide in the laboratory. The Bonding of the RGDS peptide was confirmed and analysed by FTIR and relatively high bonding efficiency was achieved.
- 3D encapsulation of human embryonic stem cell using RGDS modified alginate was achieved. The encapsulated hESCs was successfully cryopreserved in 3D manner and better cryopreservation outcome than the one using suspension or normal alginate was obtained.
- The feasibility of 3D cryopreserving hESCs in RGDS-alginate beads without the presence of ROCK inhibitor was investigated. Moreover, the feasibility of using RGDS-alginate encapsulation to reduce the doses of DMSO was also studied.
- An efficient laboratory-standard human hepatocyte isolation protocol was established in this study. Viable human primary hepatocytes were successfully isolated from

various types and conditions of liver tissue, which includes small liver pieces, large liver wedges, and fat liver.

- The freshly isolated human primary hepatocytes were successfully cryopreserved. Each composition of the cryoprotective medium and recovery medium was studied in detail, such as concentration of the FCS, concentration of the glucose or sucrose, cell density and doses of ROCK inhibitor. The optimal ones were selected. A series of complete and detailed data related to the cryopreservation of human primary hepatocytes was obtained, including the patient specific data of the liver tissue, tissue conditions and size, post-isolation cell viability and function assay, culture method, post-thaw cell viability and function assay and information of subsequent culture. Most importantly, each series of data was obtained from same liver tissue, which ensures the uniformity of the samples and the comparability between different benches.
- The feasibility of using RGDS-alginate encapsulation and ROCK inhibitor to improve the outcome of the human hepatocytes cryopreservation were investigated.
- A prototype of sonoporation system which specialized in delivering trehalose into cells was designed and assembled.
- The feasibilities of using electroporation and sonoporation to deliver impermeable CPA, i.e. trehalose, into hESCs and human hepatocytes in order to improve the cell cryopreservation were carefully studied.

7.3. Future Research

Based on the findings of this study, the following areas of future research were identified:

1. The possibility of replacing the addition of ROCK inhibitor to cryoprotective medium with 3D RGDS-alginate encapsulation should be studied. Due to the high passage number of the human embryonic stem cell samples, the data from this study showed inconclusive results.

First of all, the hESC samples with low passage number are certainly required. Secondly, the RGDS peptide modification protocol need to be further modified in order to obtain RGDS-alginate with better qualities, i.e. higher RGDS peptide bonding efficiency and higher dialysis efficiency, in order to provide better 3D environment for cell attachment and higher protective ability to cells during freezing process. Thirdly, the encapsulation process needs to be further modified, aiming to find a more suitable size of alginate beads for cell encapsulation which can balance the diffusion rate of culture medium/CPAs (small size of alginate beads) and shear force during alginate bead formation. Finally, each step of cryopreservation process needs to be more precisely controlled, for example, the exposure time to CPAs and the CPA removing process after thawing.

2. The cryopreservation protocol for human primary hepatocytes should be further modified. More parameters are due for testing, such as the seeding procedure, seeding temperature and freezing and thawing protocols. The application of 3D encapsulation to human hepatocytes cryopreservation should be further studied. Due to the limited availability of liver samples, the optimum parameters for human

primary hepatocytes cryopreservation are inconclusive and the optimization for 3D encapsulation is definitely required.

3. Based on the results of application of electroporation to DLD1 cells, the effect of electroporation to the cryopreservation of human embryonic stem cells and hepatocytes should be analysed. However, the ECM 399 Electroporation system is not good enough for hESCs and hepatocytes. A better electroporation system with improved control is required. Moreover, such fragile cell types as hESCs and hepatocytes require a new set-up of all physical parameters for the electroporation process in order to obtain maximum electroporation efficiency and high cell survival rate. The design of the experiments may start based on previous setting obtained from DLD1 cells but a thorough optimisation of parameter settings is necessary. As a novel method to aid cell cryopreservation, the results should be compared to conventional cell cryopreservation methods
4. Following the design and assembly of the sonoporation system, the application of sonoporation technique to the cell cryopreservation should be studied in the future. The system design certainly requires further modification based on the experimental data. For instance, the focus distance of each ultrasound transducer is due for calibrating by professional equipment and the powers (Mechanical index) of the transducers need to be precisely controlled.

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Appendix 1

Typical Infrared Absorption Frequencies						
Functional Class	Stretching Vibrations			Bending Vibrations		
	Range (cm ⁻¹)	Intensity	Assignment	Range (cm ⁻¹)	Intensity	Assignment
Alkanes	2850-3000	str	CH ₃ , CH ₂ & CH 2 or 3 bands	1350-1470 1370-1390 720-725	med med wk	CH ₂ & CH ₃ deformation CH ₃ deformation CH ₂ rocking
Alkenes	3020-3100 1630-1680 1900-2000	med var str	=C-H & =CH ₂ (usually sharp) C=C (symmetry reduces intensity) C=C asymmetric stretch	880-995 780-850 675-730	str med med	=C-H & =CH ₂ (out-of-plane bending) cis-RCH=CHR
Alkynes	3300 2100-2250	str var	C-H (usually sharp) C≡C (symmetry reduces intensity)	600-700	str	C-H deformation
Arenes	3030 1600 & 1500	var med-wk	C-H (may be several bands) C=C (in ring) (2 bands) (3 if conjugated)	690-900	str-med	C-H bending & ring puckering
Alcohols & Phenols	3580-3650 3200-3550 970-1250	var str str	O-H (free), usually sharp O-H (H-bonded), usually broad C-O	1330-1430 650-770	med var-wk	O-H bending (in-plane) O-H bend (out-of-plane)
Amines	3400-3500 (dil. soln.) 3300-3400 (dil. soln.) 1000-1250	wk wk med	N-H (1°-amines), 2 bands N-H (2°-amines) C-N	1550-1650 660-900	med-str var	NH ₂ scissoring (1°-amines) NH ₂ & N-H wagging

						(shifts on H-bonding)
Aldehydes & Ketones	2690-2840(2 bands) 1720-1740 1710-1720 1690 1675 1745 1780	med str str str str str str	C-H (aldehyde C-H) C=O (saturated aldehyde) C=O (saturated ketone) aryl ketone α , β -unsaturation cyclopentanone cyclobutanone	1350- 1360 1400- 1450 1100	str str med	α -CH ₃ bending α -CH ₂ bending C-C-C bending
Carboxylic Acids & Derivatives	2500-3300 (acids) overlap C-H 1705-1720 (acids) 1210-1320 (acids) 1785-1815 (acyl halides) 1750 & 1820 (anhydrides) 1040-1100 1735-1750 (esters) 1000-1300 1630- 1695(amides)	str str med- str str str str str str str	O-H (very broad) C=O (H-bonded) O-C (sometimes 2-peaks) C=O C=O (2-bands) O-C C=O O-C (2-bands) C=O (amide I band)	1395- 1440 1590- 1650 1500- 1560	med med med	C-O-H bending N-H (1 _j -amide) II band N-H (2 _j -amide) II band
Nitriles	2240-2260	med	C $\equiv$ N (sharp)			
Isocyanates, Isothiocyanates, Diimides, Azides & Ketenes	2100-2270	med	-N=C=O, -N=C=S -N=C=N-, -N ₃ , C=C=O			