Developing a cryopreservation protocol for human umbilical vein endothelial cells (HUVECs) in monolayers

by

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Abstract

Cryopreservation is a process by which biological materials (cells and tissues) are cooled down to cryogenic temperatures (such as the temperature of liquid nitrogen –196 °C), are maintained at this temperature for a long period of time and then thawed to resume their normal functions [1]. At cryogenic temperature, all biological and chemical activities are stopped [1], [4], [15] allowing very long storage times. In order to minimize damage due to cryoinjury, the use of cryoprotectants (CPAs) in cryopreservation solutions was developed [24], [25], [26]. Dimethyl sulfoxide (DMSO) and hydroxyethyl starch (HES) are two examples of penetrating and non-penetrating cryoprotectants respectively.

In contrast to cell cryopreservation in suspension, cryopreservation of cells in a monolayer, a two-dimensional structure of closely packed cells attached to a substrate, has always been challenging and the cryopreservation protocol for cells in suspension is generally not applicable to cells in monolayers. Cryopreservation of monolayers of cultured human umbilical vein endothelial cells (HUVECs) are of interest as a model for cryopreservation of intact endothelial monolayers that cover the inner surface of blood vessels and also as a model for corneal endothelium.

In this thesis, cryopreservation of human umbilical vein endothelial cells (HUVECs) in monolayers was studied leading to development of a protocol for cryopreservation of HUVECs in monolayers. For this purpose, several different factors had to be considered.

This study showed that the substrate affects viability of HUVECs in cryopreservation. The best cryoprotectant solution for HUVECs in monolayer was a combination of 5% DMSO, 6% HES and 2% chondroitin sulfate. A lower cooling rate of 0.2 °C/min compared with 1 °C/min resulted

in higher viability for plunge-thaw samples for all experimental temperatures. While CPA removal is common after thawing, this study showed that CPA removal 24 hours after thaw resulted in higher viability for HUVECs compared with CPA removal immediately after thaw. This study showed that the highest viability after cryopreservation of HUVEC monolayers was achieved for cells cultured on Rinzl coverslips for 7 days, cooled at a rate of 0.2 °C/min in the presence of 5% DMSO, 6% HES and 2% chondroitin sulfate, plunged into liquid nitrogen from –45 °C and the CPA removal 24 hours after thawing. The relative and absolute viabilities for this protocol were 81.7 ± 9.1% and 70.7 ± 8.9% respectively. These cells can be used as a model for cryopreservation of intact monolayers that cover the inner surface of blood vessels and also as a model for corneal endothelium.

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List of Abbreviations

CS – Chondroitin sulfate

CPA – Cryoprotectant

DMSO – Dimethyl sulfoxide

EGM – Endothelial growth Medium

FBS – Fetal bovine serum

HES – Hydroxyethyl starch

HUVEC – Human umbilical vein endothelial cell

Chapter 1: Introduction

1.1 Cryopreservation

Cryopreservation is an effective method for long-term storage of biological materials (such as cells and tissues) at very low temperatures with the aim of preserving viability and function of cells [1].

1.2 Cryopreservation principles

Cryopreservation consists of two main processes in terms of the water phase transition: the freezing process involves cooling down to very low temperatures such as the temperature of liquid nitrogen (–196 °C) and the transition of water from liquid to solid state (ice), and vice versa is the thawing process [1]. During the cooling process, ice is initially formed in the extracellular environment. It is proposed that the presence of cell membranes prevents the growth of ice crystals into the intracellular environment [1]. Alternatively, the lack of intracellular ice can be due to lack of nucleating agents, which facilitate ice formation. The freezing point of cytoplasm is –0.6 °C. Depending on the cooling rate, it may be possible for the cytoplasmic solution to be in a supercooled (unfrozen) state where it is 5 to 15 °C below its freezing point [1].

Under isotonic conditions, cells are in equilibrium with the extracellular environment. As temperature decreases, ice grows in the extracellular environment [1]. The formation of ice increases the concentration of solutes in the unfrozen fraction of the extracellular environment [2]. This change in concentration of solutes disturbs the osmotic balance between the intra- and extracellular environments, and drives water movement across the membrane, which is the first consequence of extracellular ice formation [2]. Several factors affect the rate of water movement across the membrane: cell membrane permeability, osmotic pressure difference between the cell

and the extracellular environment, and the surface-to-volume ratio, which were first described in a mathematical model by Mazur [3].

Mazur hypothesized that damage during freeze–thaw processes was due two factors: solution effects and intracellular ice formation, which depend on the rate of cooling and permeability of the cell membrane to water. Mazur's two-factor hypothesis was first developed based on the study of red blood cells and yeast [4]. A study of Chinese hamster fibroblasts confirmed that, based on cooling rate, freezing injury is the result of these two distinct classes of factors, and that hypothesis is applicable to mammalian nucleated cells [5]. Solution effects occur during slow cooling and intracellular ice formation occurs during fast cooling [5].

1.2.1 Slow cooling

If cells are cooled slowly, the increase in concentration of solutes in the extracellular environment causes a driving force for water to move out of the cells [5]. If the permeability of the cell membrane is high and the cooling rate slow enough, water will leave the cells before it is supercooled significantly. Slow cooling leads to cell dehydration, which prevents ice formation in the cells [5]. If cells are exposed to this environment for a long period of time, they may be damaged by an increase in the concentration of solutes in both the intra- and extracellular environment [6]. This was described by Lovelock who reproduced the damage that occurred in freeze–thaw processes from a given temperature by exposure of red blood cells to strong NaCl solutions [7]. Hemolysis occurred to red blood cells exposed to solutions stronger than 0.8 M NaCl and re-suspended in 0.15 M NaC1 solution. This study by Lovelock showed that this is the concentration of electrolytes which affects red blood cell viability in the freeze–thaw process when cells are exposed to this environment for a long time, leading to denaturation of lipid–protein complexes and changes in pH [7]. Moreover, his study showed that there is a critical

region for temperature between –4 °C and –40 °C that has a profound influence on the damage incurred by the cells depending on the time of exposure to this region [7]. Cells need to pass this critical region twice, once during cooling and once during warming.

1.2.2 Fast cooling

If cells have a very low permeability to water or when the cooling rate is too high, water does not have enough time to leave the cells and is supercooled during cooling [8]. An increase in the degree of supercooling results in intracellular ice nucleation, which results in formation of many small ice crystals because of rapid cooling [8]. Intracellular ice formation is associated with lethal injury to cells. Several mechanisms have been proposed for intracellular ice formation. One mechanism for intracellular ice formation is that ice crystals can grow into the cytoplasm of the cells through the aqueous pores in the membrane [9],[10]. Another potential mechanism for cryoinjury is the osmotic rupture hypothesis, which explains intracellular ice formation as the result of plasma membrane rupture [11]. Formation of ice in the extracellular environment causes increases in the concentration of solutes, which increases the osmotic pressure gradient across the plasma membrane resulting in membrane rupture. Consequently, the exposure of the plasma membrane to extracellular ice results in intracellular ice nucleation [11],[12]. Another mechanism proposed is that intracellular ice can be nucleated by the effect of external ice on the plasma membrane, which is called surface catalysed nucleation [13]. Another study showed that intracellular ice formation in attached Human Umbilical Vein Endothelial Cells is directional and is initiated at the cell membrane near the nucleus and grows toward the nucleus [14].

A study of Chinese hamster fibroblasts showed that both fast and slow cooling resulted in poor cell viability and that a maximum recovery was achieved at an optimum cooling rate which is

neither too fast to causing intracellular ice formation nor too slow causing cell dehydration and shrinkage. The optimum cooling rate is specific to each cell type [5].

In addition to intracellular ice formation and solution effects, extracellular ice formation may also damage cells due to direct mechanical interaction with the cell membrane consequently causing cell death. During the cooling process, as temperature decreases, formation of extracellular ice limits the space available. Physical damage occurs when cells are sequestered into channels of the unfrozen fraction [15].

1.2.3 Warming

The response of cells during the thawing process depends on the cooling rate during the freezing process. Intracellular ice formation manifests its effect through the warming process, by a process called recrystalization. If rapidly cooled cells are thawed slowly, small ice crystals, which have formed during the cooling process, have enough time to grow and form large ice crystals that cause damage to cells [16]. A study of Chinese hamster fibroblasts in the presence of 5% DMSO v/v by McGann *et al.* showed that the survival of cells warmed slowly was dependent on the cooling rate [17]. Survival was lower for the cells cooled rapidly compared with the cells cooled slowly. However, rapid warming did not have a significant influence on the cells cooled either rapidly or slowly. Rapid thawing has been an appropriate approach for rapidly cooled cells in order to minimize recrystalization [17].

1.3 Cryoprotectants (CPAs)

While the optimum cooling rate can increase cell survival, the presence of cryoprotectants in the cryopreservation solution is essential in order to minimize damage in freeze–thaw processes. Polge *et al.* discovered the cryoprotective activity of glycerol accidentally during spermatozoa

freezing [18]. The optimal concentration of glycerol was 5%, which could preserve sperm motility. Lovelock demonstrated the protective activity of glycerol during freezing and thawing of human red blood cells in suspension and that this protection was maximum when glycerol was present in both the intra and extracellular environment [19]. Glycerol exerted its protective effect by reducing the solute concentration, which damages the cells when they are exposed to the critical temperature region –4 °C to –40 °C [19]. Lovelock also reported the protective activity of dimethyl sulfoxide (DMSO) in human and bovine red blood cells. Compared with glycerol, DMSO penetrated more rapidly; however, less protection was offered by DMSO [20].

CPAs are added to cells before freezing. CPAs are divided into two classes: penetrating and non-penetrating, based on their ability to penetrate the cell membrane. Penetrating cryoprotectants are present at multimolar concentrations. They are non-ionic small molecular weight molecules such as DMSO, methanol, ethylene glycol, 1,2-propanediol and glycerol. They are able to decrease the freezing point of the solution and reduce cryoinjury due to solution effects. They decrease the concentration of other solutes within the cells by their colligative properties [21] and reduce the amount of ice [22]. They increase the viscosity of the solution by formation of hydrogen bonds with water molecules [21]. The most commonly used CPA in cryopreservation protocols is DMSO.

The criteria for penetrating cryoprotectants are low toxicity at high concentrations and high penetration rate into cells [23]. DMSO and dimethyl sulfone ($DMSO_2$) are both similar compounds but DMSO is more effective in terms of cryoprotection because $DMSO_2$ precipitates at subzero temperatures in both intra- and extracellular environments, which causes damage to cells. In addition, compared with DMSO, $DMSO_2$ does not show colligative properties because its concentration does not increase with a reduction in the temperature [24]. It is proposed that

penetrating cryoprotectants also act as membrane stabilizing agents [25]. Moreover, they may offer protection by stabilizing proteins [26].

On the other hand, non-penetrating cryoprotectants such as carbohydrates: sucrose, hydroxyethyl starch (HES), trehalose, and glucose, and alcohols are large molecules, and thus are only present in the extracellular environment. Their presence in cryopreservation solutions causes an osmotic imbalance drawing water out of the cells and avoiding or minimizing intracellular ice formation [27]. Therefore, they prevent cryoinjury due to rapid cooling by cell dehydration before or early in the freezing process [27]. Hydroxyethyl starch is nontoxic and has been used for cryopreservation of keratinocytes in suspension and monolayers [28],[29], HUVECs [30], red blood cells [31], and dermal fibroblasts [32]. Another mechanism of HES action is based on absorbing water molecules and keeping them in a glassy state without any phase transition. Therefore, HES influences the viscosity of the solution [33]. A combination of HES and DMSO has been studied for cryopreservation of different cell types. Cryopreservation of rat mesenchymal cells was not successful in the presence of HES alone, however in combination with DMSO in the cryopreservation solution, HES could provide good protection and reduced the concentration of DMSO required in the cryopreservation solution to 5% [34]. Cryopreservation of unfractionated bone marrow cells in the presence of 5% DMSO and 6% HES led to higher recovery compared with using 10% DMSO [35]. Cryopreservation of HUVECs in suspension in the presence of 5% DMSO and 6% HES resulted in higher viability (87.7 ± 0.8%) compared with 10% DMSO as the only CPA in the cryopreservation solution (67.4 ± 1.9%) [30].

Although the presence of CPAs in a cryoprotectant solution minimizes cryoinjury, their introduction before the freezing process and their removal after the thawing process introduce

osmotic stress on cells [36]. Apart from cooling rate and warming rate, for successful cryopreservation, an appropriate introduction of cryoprotectants is essential, especially in the case of penetrating cryoprotectants, since their introduction and removal cause osmotic volume excursions that may be damaging to cells. For this purpose, the hydraulic conductivity and cryoprotectant permeability of the cell membrane and the tolerable osmotic limits of cell volume need to be considered [37]. The common procedure in order to avoid excessive volume excursion due to osmotic pressure differences across cell membranes is stepwise addition [38]. Cryoprotectant concentration, cryoprotectant exposure time and temperature are also other factors, which need to be considered since cryoprotectant toxicity is affected by these factors [39]. Concentration-dependent toxicity of DMSO has been reported by Fery *et al.* for cryopreservation of cord blood mononuclear cells; high concentration or long exposure time to DMSO reduced viability of these cells [40].

1.4 Current research on cryopreservation of cells

Cell cryopreservation in suspension has been widely studied for a variety of cell types such as stem cells [41], hepatocytes [42], and human umbilical vein endothelial cells (HUVECs) [30]. These studies have helped toward understanding the mechanisms of cryoinjury, which occur in slow and fast cooling processes and response of cells to these processes. While cryopreservation of cells has been successful for cells in suspension, cryopreservation of tissue has always been a challenge. This is because tissues are complex systems composed of a variety of cell types, which have different permeabilities to water and cryoprotectants, and in addition the presence of cell–cell and cell–matrix interactions affect cells responses to cryopreservation [15].

1.4.1 Monolayer cryopreservation applications

Cells can be cryopreserved in two configurations: in suspension (single cells) or in a monolayer (a closely packed layer of cells attached to a substrate). Cryopreservation of cells in monolayers has different applications. For instance, cryopreservation of hepatocyte cells in monolayers would be a practical means for long-term storage, providing a source of cells for *in vitro* toxicity studies, drug metabolism and cell line development [43].

Apart from that, some cells (such as hepatocytes) when cryopreserved in suspension upon thawing are not able to attach to the surface for post-thaw culture and there is less chance of recovery of viable cells in culture. Therefore, an alternative approach is cryopreservation of these types of cells in a monolayer (in an attached state) [44].

Cryopreservation of neuronal networks in monolayers facilitates their long term storage and transportation of these networks, which are widely used in toxicity studies and drug discovery [45].

Another application of monolayer cryopreservation is developing cell constructs that are used for drug testing. Since supply of fresh human tissue is unpredictable, these constructs provide a source of cells which can be used for research. For instance, hepatocyte cells cryopreserved on a film of poly-ethylene terephthalate (PET) have been studied as a source for liver cell-based constructs, which can be stored for a long period of time and used for study of cell response to drugs developed for liver diseases or quality control testing of the cell-based products [75].

1.4.2 Challenge in monolayer cryopreservation

It seems that the methods developed for cryopreservation of isolated cells are not optimal for attached cells. One of the challenges in cryopreservation of cells in monolayers is cell detachment. Detachment has been shown to affect the survival of the cells. A study by Corsini *et al.* for cryopreservation of five adherent mammalian cell types in the presence of 10% DMSO v/v and phosphate buffered saline (PBS) showed that most of the cells were detached from the flask in post-thaw culture [46]. A study by Ebertz *et al.* showed cell detachment after cryopreservation of a monolayer of human corneal endothelial cells (HCEC) in the presence of DMSO and propylene glycol [47].

Different strategies have been applied in order to improve post-thaw cell attachment and viability in monolayer cryopreservation. Human skin fibroblasts were cryopresereved on a collagen gel in monolayers (cooling rate:1°C/min) in the presence of 10% DMSO, 20% glycerol or 10% ethanediol separately; for all three experimental conditions the viability was less than 20% (viability was measured by trypan blue exclusion assay immediately after disaggregation of the collagen gel) [48]. Cryopreservation of keratinocytes seeded on an appropriate matrix showed the highest absolute viability in the presence of 10% HES compared with other CPAs and a cooling rate of 3 °C/min, which was 72% after 24 hours recovery (viability was assessed by dye exclusion assay) [29]. Cryopreservation of mouse neuroblastoma cells (N2a) entrapped beneath a layer of alginate hydrogel in the presence of 10% DMSO in culture medium led to 63% cell recovery immediately after thaw, which was accompanied by loss of viability in the first 24 hours post-thaw (it is not known whether viability was measured before or after CPAs removal) [49]. Attempts have been made to cryopreserve rat hepatocytes on a collagen layer [43]. Microcarrier beads have also been used for cryopreservation of an immortalized human

endothelial cell line (ECV304) in monolayers. The recovery following cryopreservation with DMSO after CPA removal based on total leucine uptake was 70% at a cooling rate of 1 °C/min and increased to 85% at 0.3 °C/min [50].

Modification of the surface for cell cryopreservation has affected cell attachment. For instance, mouse fibroblast cells (NIH-3T3) fixed to glass and silicon substrates had preserved attachment after the freeze–thaw process, which resulted in high post-thaw viability [51]. Cryopreservation of hepatocyte cells (HepG2) in the presence of different concentrations of DMSO (10, 20, and 30%) on two different substrates (glass and polydimethyl siloxane) coated with poly-L-lysine showed that most of the cells maintained their attached state. The maximum viability was achieved in the presence of 20% DMSO regardless of the type of the substrate, which was approximately 90%. It is not stated whether this viability was before or after CPA removal, immediately or after 24 hours recovery (in this study cells were cooled to –80 °C at a cooling rate of 20 °C/min and viability assessment was based on cell staining by Calcein-AM and propidium iodide (PI)) [52].

1.4.3 Intracellular ice formation in monolayers

Extracellular/intracellular ice formation and CPA toxicity are some of the factors that have been identified as contributing to cryoinjury. Cell survival in monolayers needs coordination between adjacent cells and cell–matrix interactions. One of the main concerns in cryopreservation is avoiding intracellular ice formation, which has been shown to be lethal to cells in suspension [12].

A study of intracellular ice formation in various physiological states (single cells, single cells attached to the surface, and attached cell colonies with cell–cell and cell–surface interactions) by Acker *et al*. showed the physiological state influences freezing behavior [53]. At a constant sub-

zero temperature, the prevalence of intracellular ice formation is higher in cell colonies with cell–cell and cell–substrate interactions compared with single cells attached to the substrate [53].

Prevalence of intracellular ice formation is affected by intracellular ice propagation in confluent monolayers [76]. The effect of gap junctions on intracellular ice propagation was first demonstrated by Acker *et al.* in Madin Darby Canine Kidney (MDCK) epithelial cells in monolayers [76]. In comparison with confluent V-79W hamster fibroblast cells that do not form gap junctions, MDCK cells showed higher ice propagation in monolayers due to the presence of gap junctions as inhibition of gap junction by low Ca^{+} media in MDCK cells decreased the incidence of intracellular ice propagation [76]. In the case of monolayers, the protective effect of intracellular ice was presented by Acker *et al.* for Madin Darby Canine Kidney (MDCK) epithelial cells in monolayers [54]. Assessment of post-thaw recovery showed that the majority of cells in monolayers were viable following intracellular ice formation, which shows that intracellular ice conferred protection. This protection arises from preventing osmotic damage during slow cooling. No driving force exists for water to move out of the cells when intracellular ice is formed [54]. A study by Zhurova *et al.* showed that intracellular ice formation in monolayers of dental pulp stem cells offered protection to cells [55]. After 100% intracellular formation in suspension, the majority of cells (85.9 ± 1.7%) were damaged; however, after 100% intracellular ice formation in monolayers, a lower number of cells (25.5 ± 5.5%) were damaged. Confluent monolayers also showed expression of gap-junction protein, and it is proposed that ice propagation through gap junction is responsible for high recovery [55].

1.5 Cryopreservation of endothelial cells

Cryopreservation of endothelial cells has been studied in monolayers and cell suspensions. One of the sources for endothelial cells is umbilical veins. Umbilical veins are readily available [71]. These veins provide a sufficient number of cells including endothelial cells, smooth muscle cells, and fibroblasts (the usual length of an umbilical cord is 20–22 inches long). Human umbilical vein endothelial cells (HUVECs) are adherent primary cells (non-immortalized) [71],[73] and have limited proliferative ability [71]. These cells have shown synthesis of extracellular matrix proteins. In addition, endothelial cells isolated from umbilical veins have shown a cobblestone phenotype in *in vitro* culture [71]. The first successful *in vitro* culture of HUVECs was reported in 1973 when these cells formed a single layer of flat and polygonal shaped cells with centrally located nuclei, which allowed the study of their functions [72].

HUVECs have been used as a model for vascular tissue engineering [56],[57],[58],[59] and the study of angiogenesis [60],[61]. HUVECs have been cryopreserved in suspension [62],[30],[63]. A comparison of human umbilical vein endothelial cells cryopreserved in different states: cryopreservation of whole umbilical vein, cryopreservation of freshly extracted cells in suspension, and cryopreservation of endothelial cells after subculture, revealed that the freshly harvested endothelial cells were not a good candidate for cryopreservation and it was proposed that treatment with proteolytic enzyme and exposure to liquid nitrogen resulted in low viability [64]. The most effective method for cryopreservation needed endothelial cells from primary culture [64]. Cryopreservation of HUEVCs in suspension showed that a higher cooling rate (1 °C/min) resulted in higher viability compared with cells frozen at 0.2 °C/min. The maximum viability achieved for cryopreservation of HUVECs in suspension was in the presence of 5% DMSO and 6% HES with a cooling rate of 1 °C/min (87.7 ± 0.8%) [30].

Another study of endothelial cells in monolayer and suspension was by Pegg *et al.* for cryopreservation of the immortalized human endothelial cell line (ECV304) in the presence of 10% DMSO at different cooling rates [50]. Results showed that the response of cells was different between monolayer and suspension in terms of viability. At cooling rates of 0.3, 1.0, and 10 °C/min, 45–50% of cells in suspension were alive, whereas monolayers showed higher survival when cooling rate decreased (at a cooling rate of 1 °C/min viability was 70% and at 0.3 °C/min viability was 83%) after cryoprotectant removal [50].

1.5.1 Importance of endothelial cell cryopreservation in monolayers

Endothelial cells cover the lumen of blood vessels and form a continuous monolayer [68]. These cells link together by different forms of cell–cell junctions, which mediate adhesion and communication between cells [69]. These junctions form by transmembrane adhesive proteins [68]. These cells are in direct contact with blood components and regulate vascular homeostasis [68],[70].

Cryopreservation of endothelial cells in monolayer has been studied as a model for cryopreservation of endothelial monolayers of blood vessels and cornea.

Cryopreservation of blood vessels has high importance in transplantation for patients without sufficient autologous graft material [63]. For pharmacological research, development of drugs for cardiovascular research needs a source of *in vitro* cells from fresh blood vessels [65]. Since fresh tissues have a short life span, development of a reliable storage method for cryopreservation of blood vessels can provide a source for *in vitro* studies. However, cryopreservation of blood vessels was accompanied by loss of endothelial cell functions. The presence of an intact continuous endothelial layer is essential after cryopreservation of blood vessels as endothelial cells act as a regulator of homeostasis [65].

The efficiency of corneal transplants depends on preservation of endothelial cell function. Since the endothelial layer maintains transparency of the cornea, preservation of this layer is the primary aim in preservation of cornea. Endothelial cells do not proliferate and are not replaced by mitotic division *in vivo* and their number decreases by an increase in age. Moreover; it has been reported that human endothelial cells are difficult to grow in culture due to lack of proliferative activity. In addition, damage/diseases also accelerate loss of endothelial cells [66].

Therefore, preservation of these cells is essential in order to have enough cells which can be used for cell therapy in order to treat corneal dysfunction. Developing a protocol for cryopreservation of endothelium will offer unlimited storage of these cells [66].

1.6 Effect of substrate on attachment of cells after monolayer cryopreservation

The coefficient of thermal expansion describes the change in the size of an object with a change in temperature. A substrate experiences contraction and expansion during freeze/thaw processes. In monolayer cryopreservation cells are attached and fixed to the substrate. In a study of osteoblast cells frozen on glass coverslips and hydroxyapatite disks, Liu *et al*. concluded that the difference between thermal expansion of the cells and substrate is one of the factors which may affect cell survival during cryopreservation and proposed that a difference of coefficient of thermal expansion between a cell and substrate may affect cytoskeleton structure and lead to disruption of cell structure [67].

In a preliminary study in our research group, it was previously hypothesized that the substrate affects the cell attachment in monolayer cryopreservation [74]. This hypothesis was based on the difference in coefficient of thermal expansion between the substrate and ice which forms in the

extracellular environment. While both the substrate and ice experience contraction and expansion during freeze–thaw processes, a lower difference between the coefficient of thermal expansion of ice and substrate is expected to cause less cell detachment [74]. This hypothesis was tested by a former student in our lab for Chinese hamster fibroblast cells on glass and Rinzl plastic coverslips which were frozen at a specific temperature (–9 °C). The thermal expansion coefficient of Rinzl is similar to that of ice. Cells frozen on Rinzl plastic coverslips showed that the majority of cells maintained their attachment to the surface after freezing at (–9 °C) on Rinzl coverslips (97.9 ± 1.4%) compared with cells frozen on glass coverslips (77.9 ± 8%); it was proposed that detachment from the glass coverslip is because of the mismatch between the coefficients of thermal expansion of ice and glass [74]. The report showed that the substrate affected cell detachment after freezing.

1.7 Scope of this thesis

The aim of this thesis is developing a cryopreservation protocol for HUVECs in monolayers with the aim of preserving attachment and viability. For this thesis, it is hypothesized that the type of substrate will affect not only attachment, but also the viability of the cells after cryopreservation. This study was performed by freezing HUVECs seeded on two different types of substrates (glass and Rinzl) in the absence and presence of cryoprotectants. In this study, also the effects of cooling rate, the presence of chondroitin sulfate, and the removal of CPA 24 hours after thawing were assessed.

1.7.1 Objectives of this thesis

- To characterize the proliferative activity of HUVEC on glass and Rinzl substrates.

  This aim was achieved by culturing cells over a period of 10 days and obtaining a growth curve. Cell density, morphology, and formation of one of the components of tight junctions (Claudin–5) were assessed (Chapter 2).

- To assess the effect of substrates (glass and Rinzl) on cell viability and attachment after freezing at a specific temperature. This aim was achieved by seeding HUVECs on two different substrates (glass and Rinzl plastic coverslip). Confluent monolayers of HUVECs were frozen at –9°C and viability and attachment were assessed (Chapter 3).

- To develop a protocol for cryopreservation of HUVECs in monolayers. For this purpose, the response of cells to graded freezing on two different substrates was assessed in the absence and presence of cryoprotectants. Cells were subjected to graded freezing at 1 °C/min (cooling rate) in the absence or presence of 10% DMSO, 20% DMSO, and a combination of 5% DMSO and 6% HES and viability and attachment were assessed (Chapter 4).

- To further optimize the cryopreservation protocol for cryoprotectant removal in order to maintain high post-thaw viability. The effect on HUVEC viability of CPA vehicle solution (CryoStor), addition of chondroitin sulfate (CS) to the CPA solution, lower cooling rate (0.2 °C/min) and delayed removal of cryoprotectant were assessed (Chapter 5).

1.8 References

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Chapter 2: HUVEC growth and culture

2.1 Introduction

Since HUVECs are adherent primary cells, the first step for cell growth and migration on a substrate is attachment [1]. This attachment is mediated by extracellular matrix (ECM) proteins. The surface coating to use for monolayer culture of HUVECs has been studied before [1],[2]. HUVECs are able to attach to the surface in the presence of fibronectin, gelatin, or collagen [3]. In the experiments described in this thesis, coverslips were coated with fibronectin before cell seeding. HUVECs attach to the extracellular matrix through cell adhesion receptors (integrins) [3]. Integrin binds to the Arg-Gly-Asp (RGD) sequence on fibronectin. These receptors act as a bridge that connects the cells to the extracellular matrix through their cytoskeleton elements [3]. Coating with a protein from the ECM such as fibronectin facilitates cell adhesion, spreading and proliferation of cells [4],[5],[6],[7]. It is proposed that fibronectin mimics ECM support for these cells and allows them to attach and facilitates formation of new ECM [7].

When the cells divide in culture, their number increases and in the attached state (monolayer) cell number per area is referred to as cell density. The period of time that it takes for the cell population to double in culture is defined as the doubling time (DT) [9]. These cells after many population doublings become senescent (irreversible growth arrest) in culture. The main difference between senescent and non-senescent cells are changes in morphology and function [8]. Therefore, the maximum doubling number for HUVECs is considered to be 15 in order to avoid cellular senescence (recommended by the manufacturer's protocol [17]). Since the aim of this project is cryopreservation of HUVECs in monolayers, the first step before cryopreservation is investigating HUVEC function, which is characterized by cell growth, morphology and viability of the cells.

The first step of the growth curve is characterized by cell attachment to the surface or substrate and reconstruction of the cytoskeleton, which takes up to 48 hours (lag phase) [9]. The second phase is defined as the exponential phase in which cells proliferate and cell density increases. At this stage, the doubling time is calculated. As the cell number increases, cells pack closer together and spread over the surface of the substrate [10]. The third phase (plateau phase) is a period during which cells have a growth rate near zero; the growth has stopped because of very dense cell population, and growth factors in the medium having been exhausted [10]. Measurement of cell viability has been widely studied and different approaches have been used for viability assessment including dye uptake assays such as trypan blue or fluorescent compounds [9]. Dye uptake assays measure plasma membrane integrity. In this work, in order to differentiate live and dead cells, cells were stained with SYTO13/GelRed. GelRed is a fluorescent nucleic acid stain, which has been designed as a replacement for Ethidium Bromide (EtBr), which is a toxic dye and potential mutagen [11]. GelRed interacts with DNA and, according to the manufacturer's protocol [12], it has the same excitation and emission as EtBr (excitation and emission: 518/605 nm). Because GelRed is incapable of crossing the membrane in living cells with intact membranes, it stains only dead cells. SYTO13 is a fluorescent dye, which exhibits a green fluorescence under a fluorescent microscope [13]. SYTO13 can penetrate both live and dead eukaryotic cells and binds to DNA and RNA (excitation/emission: 488⁄509 nm, DNA) [13].

Communication between endothelial cells occurs through cell–cell junctions: tight junctions (TJ), adherence junctions (AJ) and gap junctions [1]. Both tight and adherence junctions are composed of transmembrane proteins and intracellular components [1]. Tight junctions are responsible for adherence of adjacent membranes and regulating the transport of substances through the space

between two adjacent membranes. Presence of these junctions is necessary for vascular integrity as loss of tight junctions is associated with many pathological disorders [8],[14]. Some components of tight junctions have limited distribution, such as Claudin-5, which has shown expression in HUVECs [8]. In this work, in order to determine the integrity of the junctions between cells, localization of Claudin-5 was examined by immunocytochemistry in HUVECs over a period of 10 days.

The objectives of this chapter are

- To perform a growth curve for HUVECs over a period of 10 days
- To assess HUVEC morphology by phase contrast microscopy over a period of 10 days
- To assess formation of tight junctions among HUVECs by immunolabeling of Claudin-5

2.2 Materials and methods

Human umbilical vein endothelial cells (HUVECs) were used as a model system for this project. All processes including cell thawing, preparation of media and cell culturing, were performed in a biological safety cabinet level 2.

2.2.1 HUVEC storage

Human Umbilical Vein Endothelial Cells, Pooled Donors (HUVECs) (C2519A, LONZA, Walkersville, MD USA) were shipped in dry ice (Cedarlane, Burlington, On, Canada). Cryovials were transferred to liquid nitrogen (–196 ºC) immediately upon arrival. Cryovials were kept in liquid nitrogen until they were used for experiments.

2.2.2 Preparation of culture medium

Cell growth components for HUVECs consist of Endothelial Cell Media BulletKit™ Medium - 500 ml (CC-3156, LONZA) and EGM-2 bullet kits (CC-4176). Upon arrival, EBM™-2 Basal

medium was stored at (2–8 °C) and the Single Quots™ Kit was kept at ≤ –20 °C. To prepare endothelial cell growth medium (EGM™-2 Medium), the contents of the EGM™-2 SingleQuots™ Kit (Lonza Catalog No. CC-4176 containing human Epidermal Growth Factor [hEGF], Vascular Endothelial Growth Factor [VEGF], R3-Insulin-like Growth Factor-1 [R3-IGF-1], ascorbic acid, hydrocortisone, human Fibroblast Growth Factor-Beta [hFGF-β], heparin, Fetal Bovine Serum [FBS]) were thawed (external surfaces of all vials, including the medium bottle were wiped with 70% ethanol) and added to the EBM™-2 Basal medium. Complete growth medium was stored at 2–8 ºC. Cell culture was performed in the absence of antibiotics in the medium.

2.2.3 HUVEC culture

To culture HUVECs, a cryovial (≥ 500,000 cells) from liquid nitrogen was thawed in a water bath at 37 °C until ice crystals disappeared. The cryovial was wiped with 70% ethanol before opening. The contents of the vials (1 ml) were carefully mixed with 5 ml of pre-warmed (37 °C) growth medium (EGM-2) using a micropipette and the cell suspension was transferred equally into two T-75 tissue culture flasks (353136, Corning, NY 14831, USA). Each flask contained 15 ml of pre-warmed growth medium. According to the manufacturer's protocol (LONZA, Clonetics™ Endothelial Cell System Technical Information & Instructions), the recommended seeding density for initial plating after cryopreservation is 2500/cm^2. The flasks were incubated at 37 °C ± 1 °C, 5% CO_2, and 90 % ± 2% humidity. Since HUVECs were initially cryopreserved in 1 ml of growth medium supplemented with 10% DMSO and 10% fetal bovine serum (FBS), the medium was refreshed after 24 hours to remove DMSO, non-adherent cells and debris. Growth medium was replaced every other day thereafter.

2.2.4 Sub culturing (passaging)

Cell health and growth depend on renewing the growth medium and sub culturing at regular intervals. Sub culturing was performed according to the manufacturer's protocol (LONZA, Clonetics™ Endothelial Cell System Technical Information & Instructions). HUVECs were sub cultured when they reached 70–85% confluency (surface coverage), as assessed by phase contrast microscope. After aspirating the growth medium by vacuum, HUVECs were rinsed with 15 ml of pre-warmed HEPES Buffered Saline Solution (HEPES-BSS, CC-5024, LONZA, Walkersville, MD USA). After the HEPES-BSS was aspirated, cells were covered with 6 ml 0.025% Trypsin/0.01% EDTA solution (CC-5012, LONZA, Walkersville, MD USA). The flask, with the lid tightened, was placed in a 37 °C humidified incubator for 2–3 minutes. Cell dissociation was assessed by phase contrast microscopy (rounded cells were floating). Trypsin was neutralized by addition of 12 ml trypsin neutralizing solution (CC-5002, LONZA, Walkersville, MD USA). Harvested cells were transferred to a 50 ml centrifuge tube (430290, Corning, NY 14831, USA). The tissue culture flask was washed with 6 ml HEPES-BSS to remove remaining cells and they were transferred to the same tube. Cells were centrifuged in an Eppendorf 5810R tabletop centrifuge (Eppendorf AG, Hamburg, Germany) for 5 min at 1000 rpm, 20 ºC. The supernatant was aspirated and the cell pellet was suspended in EGM-2 (between 1 to 3 ml, depending on the size of pellet) 100 µl of cell suspension was diluted in 10 ml of Isoton II diluent (Beckman, Coulter, Inc.) and used for cell counting with a Coulter® Z2™ particle count and size analyzer (Beckman Coulter, Mississauga, ON, Canada).

2.2.5 Doubling number calculation

After each sub culturing, it is possible to calculate the number of doublings (population doubling number). The maximum population doubling for HUVECs was considered to be 15 (manufacturer protocol). All experiments were performed with doubling number less than 15. For all of the experiments in this study, HUVECs were used from passage 1 or 2. In every passage, after cell counting, the doubling number was calculated as below [21]

$$population\ doubling\ number = \frac{\ln(\frac{final\ cell\ number}{number\ of\ \ seeded\ cells})}{\ln(2)} \qquad (\text{Eq. } 2.1)$$

The final cell number is the number of cells that is calculated after counting.

2.2.6 Coverslip sterilization

HUVEC growth curve experiments were performed for a period of 10 days on two different substrates, glass (clear white borosilicate glass) and Rinzl (made from clear vinyl plastic) coverslips. Rinzl plastic coverslips (72261-18 with a size of 18×18 mm) and glass coverslips (72190-09 with a size of 9×9 mm) were purchased from Electron Microscopy Sciences (Hatfield, PA 19440, USA). Rinzl coverslips were cut into 9×9 mm pieces. Both coverslips (glass and Rinzl) were sterilized using 70% ethanol in separate petri dishes (100×15 mm, 8-757-13, Fisher Scientific) in a biological safety cabinet for 30 minutes. After sterilization, coverslips were transferred into another Petri dish and washed with 25 ml Phosphate Buffered Saline (1X PBS, 100-10-023, Life Technologies) for 15 minutes. After 15 minutes, both glass and Rinzl coverslips were placed at the bottom of a 24-well plate (Cellstar, 662160). Excess PBS on coverslips was removed by vacuum.

2.2.7 Fibronectin treatment

To promote attachment of HUVECs to coverslips, coverslips (glass and Rinzl) were treated with fibronectin solution from bovine plasma (F-1141-2MG, Sigma-Aldrich) at a concentration of 2.5 μg/cm^2 (recommended concentration: 1–5 μg/cm^2 or 0.5–50 μg/ml) for at least 30 minutes. Fibronectin was prepared in PBS (18 μl of fibronectin in 982 μl of PBS in a microtube). 140 μl of fibronectin solution was added to each coverslip in a 24-well plate in order to cover the entire coverslip (centre and corners). The 24-well plate was kept at room temperature for 30 minutes, and then the fibronectin solution was aspirated from each well by vacuum. A washing step was not necessary after fibronectin removal.

2.2.8 HUVEC growth curve

HUVECs were cultured on glass and Rinzl coverslips, and cell viability, morphology, formation of intracellular junctions (Claudin-5) and cell density were assessed every day. Day 0 was the day of cell seeding. HUVECs were used from passage 1 for the growth curve experiments in order not to exceed 15 doublings by the end of 10 days. As described in section 2.2.4, HUVECs at 70–85 % confluency in T–75 flasks were trypsinized, centrifuged, and seeded at a density of 10,000/cm^2 in a 24-well plate (recommended seeding density) containing fibronectin-coated Rinzl or glass coverslips in a total volume of 500 μl (each well of a 24-well plate with 2 cm^2 surface area needs 2.0×10^4 cells). Growth medium was replaced every other day. For each day of a growth curve experiment, three coverslips were stained and assessed for viability and cell density. The results in section 2.3 are the average of three independent growth curves on glass and Rinzl coverslips.

2.2.9 HUVEC morphology assessment

HUVEC morphology was analyzed by a Labovert phase contrast microscope (Leitz, Los Angeles, CA, USA) equipped with a Pixera camera every day (magnification 10X).

2.2.10 Viability assessment (membrane integrity assay)

HUVEC viability was determined by membrane integrity over a period of 10 days. HUVECs were stained with Syto13 (S7575, Molecular Probes, Eugene, OR, USA) and GelRed (41003, Biotium, CA, USA) every day. Viability was assessed under a fluorescent microscope using the Viability3 program (Version 3.2, the Great Canadian Computer Company, Spruce Grove, AB, Canada). For day 0, staining was performed 4 hours after cell seeding. Cells were stained with SYTO13/GelRed. 10 µl GelRed from stock (10,000X in water) and 10 µl SYTO13 (5 mM) were added to 262.5 µl of PBS in a microtube (final concentrations: 380X GelRed and 0.19 mM SYTO13). The solution was kept on ice in a dark place during staining. 10 µl of staining solution was added to each well containing 190 µl of growth medium and was incubated for 4 minutes in a dark place. After 4 minutes, coverslips were transferred onto a slide with fine tweezers (cell side down) and excess media was removed using Kimwipe tissues. Coverslips were observed under the fluorescent microscope at 10X magnification (Leitz, Dialux 22). The same fluorescent microscope settings were used for capturing images from coverslips every day (Table 2.1). Images were captured with an Infinity3 camera and Infinity Capture software (Lumenera Corporation, Ottawa, ON, Canada). 12 images were captured for each coverslip (covering the entire surface including the corners and center) (Figure 2.1).

Table 2.1: Fluorescent microscope settings

Microscope settings	
Preview resolution	1393 × 1040
Capture resolution	1280 × 1024
Exposure	100 mm
Gain	1.65
Light source type	fluorescent
Light source frequency	60 Hz
Contrast	10
Brightness	0
Gamma	1
Hue	0
Saturation	0
Red	1.19
Green	1.00
Blue	2.50

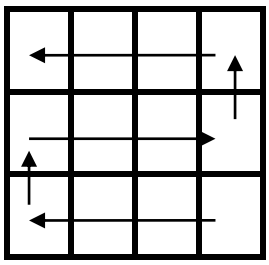

Figure 2.1: Schematic image of a coverslip under a fluorescent microscope to capture images. Each coverslip was divided into 12 parts in order to capture images covering the corners and centre.

Each image contained green (live) cells and red (dead) cells. HUVEC viability in each captured image was measured with the Viability3 program. This program gives information about the total number of cells, number of green cells, number of red cells and pixel number of red and green in each image (Parameters used: upper Th = 255, lower Th = 100 cumul Th = 85%).

Each image consists of green (live) and red (dead) cells. Relative cell viability was calculated by:

$$Relative\ cell\ viability = \frac{number\ of\ green\ cells\ in\ 12\ images}{number\ of\ total\ cells\ in\ 12\ images} \times 100 \qquad \text{(Eq.2.2)}$$

2.2.11 Cell density /mm² calculation

Images were taken with a resolution of 1280 × 1024 under the fluorescent microscope. A further image was taken by bright field microscopy (magnification also 10X) to calculate the area which covered the capture resolution: 1280 × 1024 under the fluorescent microscope. This resolution covers the surface area of one square of a hemocytometer. The surface area of this square is equal to 1 mm^2 (Figure 2.2).

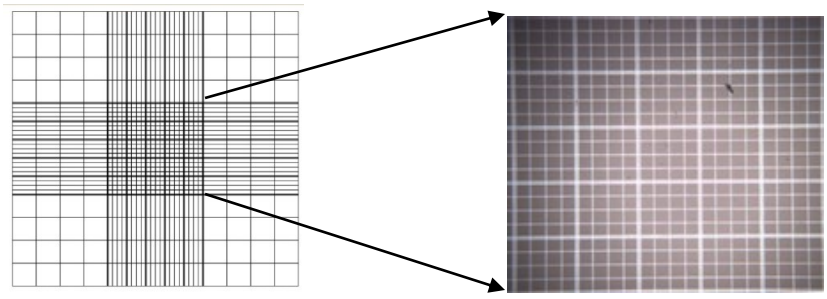

Figure 2.2: Hemacytometer image taken by bright field microscopy (capture resolution: 1280×1024)

2.2.12 Immunofluorescence detection of Claudin-5 in HUVEC monolayers

In order to assess formation of intracellular junctions, Caludin-5 formation was assessed over a period of 10 days for HUVECs on glass coverslips. On each day, one coverslip was transferred from the incubator to a 24-well plate and fixed with 500 µl of methanol at –20 °C for 20 minutes. After methanol removal, the coverslip was washed with phosphate buffered saline (500 µl).

Non-specific staining was blocked by incubation with 400 µl of blocking buffer (10% normal goat serum in 1X PBS) for 45 minutes at room temperature.

After removal of the blocking buffer, cells were stained with Claudin-5, Mouse, MAb-Alexa fluor 488 (invitrogen 352588; stock concentration 500 mg/ml). Claudin-5 antibodies were diluted in a buffer containing 1% bovine serum albumin, 1% normal goat serum (PCN-5000, Invitrogen, USA) in 1X PBS (manufacturer recommended dilution: 5–10 µg/ml; 50–100X dilution). 2.5 µl of stock solution was added to 247.5 µl of buffer (final concentration: 5 µg/ml, 100 x dilution). This solution was kept on ice in a dark place until the time of the experiment.

The coverslip was placed into a petri dish (100 ×15 mm), and the entire surface of the coverslip was covered with 100 µl of antibody solution and incubated overnight at 2–8 °C. The following day, the antibody solution was removed and the coverslip was washed twice with 500 µl of wash buffer (0.1% bovine serum albumin in 1X PBS). The coverslip was placed on a microscope slide with fine tweezers with the cell side down (excess liquid was removed by Kimwipe) and was observed under the fluorescent microscope (magnification: 25X). The microscope was adjusted for green fluorescent imaging (see Table 2.1, section 2.2.10 for fluorescent microscope settings).

2.3 Results

HUVECs were cultured over a period of 10 days on both glass and Rinzl plastic coverslips. The viability assessment showed cells had a very high viability on both coverslips over 10 days in culture (Figure 2.3). The growth pattern of endothelial cells and maturation (differentiation to cobblestone phenotype) was monitored daily by phase contrast microscopy (Figures 2.4 and 2.5). Fluorescent images of cells on day 0 (cell staining after 4 hours of seeding) showed single cells were completely attached, and spread out and a few were in contact with each other on both glass and Rinzl coverslips (Figures 2.6 and 2.7). Comparison of cell density showed that there was an increase in the number of the cells within the first 24 hours on both glass and Rinzl coverslips, which shows that HUVECs have a very short lag phase (a few hours) and started proliferation within the first 24 hours. Analysis of the growth curves revealed an increase in doubling number from day 1 to day 5 that shows rapid proliferation (Figure 2.8). The plateau phase is characterized by no significant difference in cell density from day 5 to day 10 on glass and Rinzl plastic coverslips (Figure 2.8). Kinetics of cell growth (doubling number) over the final 6 days showed that the rate of growth was very slow or stopped (Tables 2.2 and 2.3). Immunolabeling of Claudin-5 (a component of tight junctions in endothelial cells), over a period of 10 days revealed the formation of cell–cell contact among HUVECs (Figure 2.9).

2.4 Discussion

Comparison of results for glass and Rinzl cover slips showed that HUVECs were able to form a confluent monolayer on both coverslip materials. Phase contrast images showed that cells began to show the morphology characteristics of endothelial cells (flat and polygonal phenotype) [4] on the second day in culture. This is during the second stage of the growth curve (exponential phase) in which HUVECs were dividing actively but they were sub–confluent [13]. An increase in cell

density was followed by transition to the confluent monolayer state from day 5 and the cell number reached the growth plateau [13],[14].

Differentiation to the cobblestone phenotype started as cells reached confluence. Monitoring cell morphology by phase contrast microscopy showed that as they got confluent, cells were remarkably smaller in size and packed tightly together (day 6 to day 10 compared to the first 4 days). This confluent monolayer was also characterized by formation of cell–cell junctions by immunocytochemistry, which is essential for endothelial cell integrity in a monolayer. Comparison of images shows that formation of Claudin-5 requires cell–cell contact. Starting from day 3 where some cells were clustered, Claudin-5 was featured at the cell periphery as a distinct cell border (green line). This cell–cell contact was especially well-defined on days 6 to 10 in a fully confluent monolayer between packed cells which shows that Claudin-5 expression is very clear when cells are attached together. Immunofluorescent images provide additional evidence for HUVEC differentiation to the cobblestone phenotype especially from day 6 to day 10; it has been proposed that differentiation usually occurs when cells are in the plateau phase [16]. Since the aim of this project is cryopreservation of HUVECs in monolayers, HUVECs need to be fully confluent and tightly packed. Experiments for the rest of this thesis were performed on day 7 as cells are fully confluent and immunolabeling showed the presence of Claudin-5 localized at cell–cell contact areas.

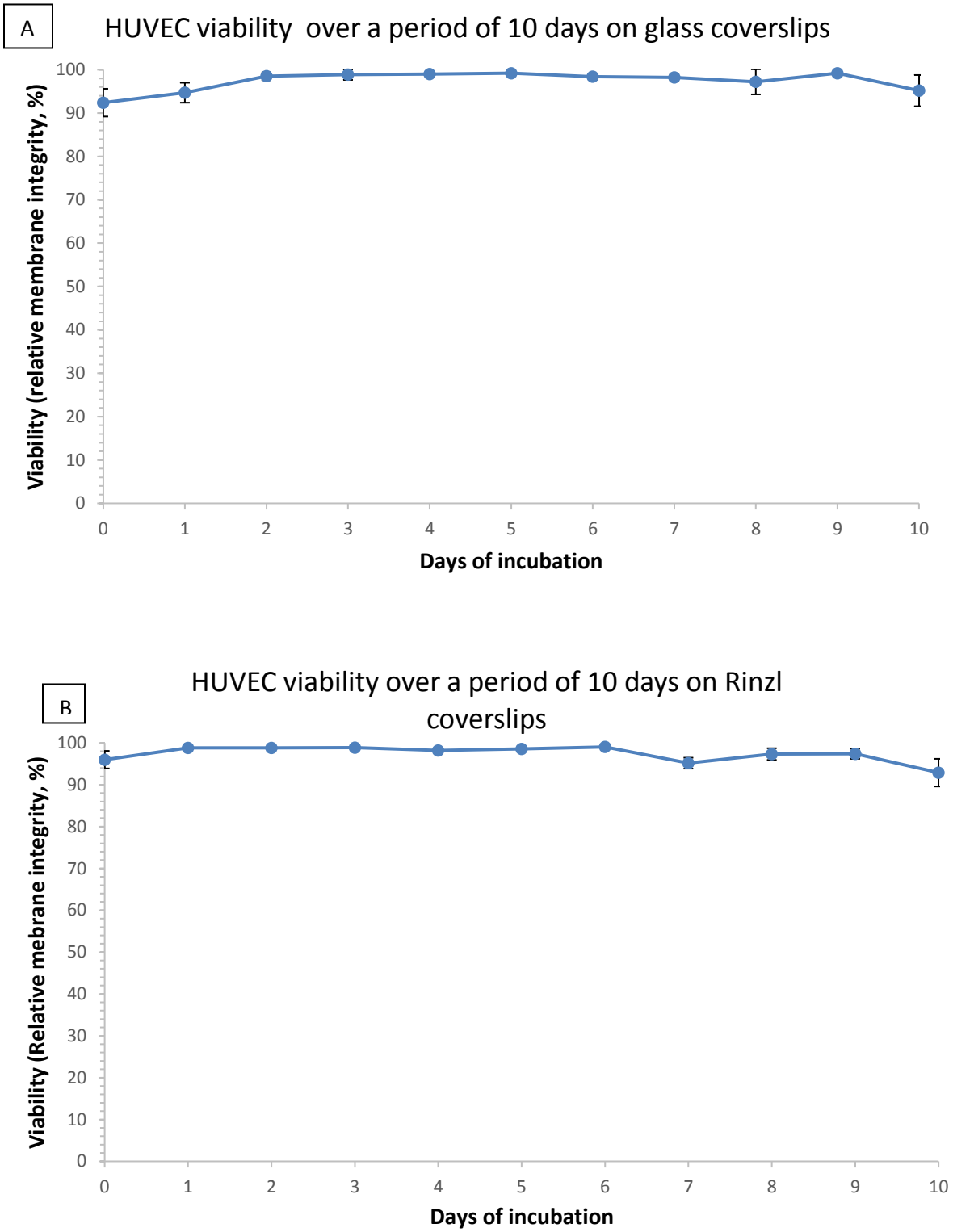

Figure 2.3: HUVEC viability over a period of 10 days on A) glass and B) Rinzl coverslips. HUVECs were seeded at a density of 10,000/cm^2 on coverslips. Every day (starting from day 0), cells were stained with SYTO13/GelRed and observed under a fluorescent microscope (magnification 10X). Data are the average of three independent experiments ± standard error of the mean (SEM).

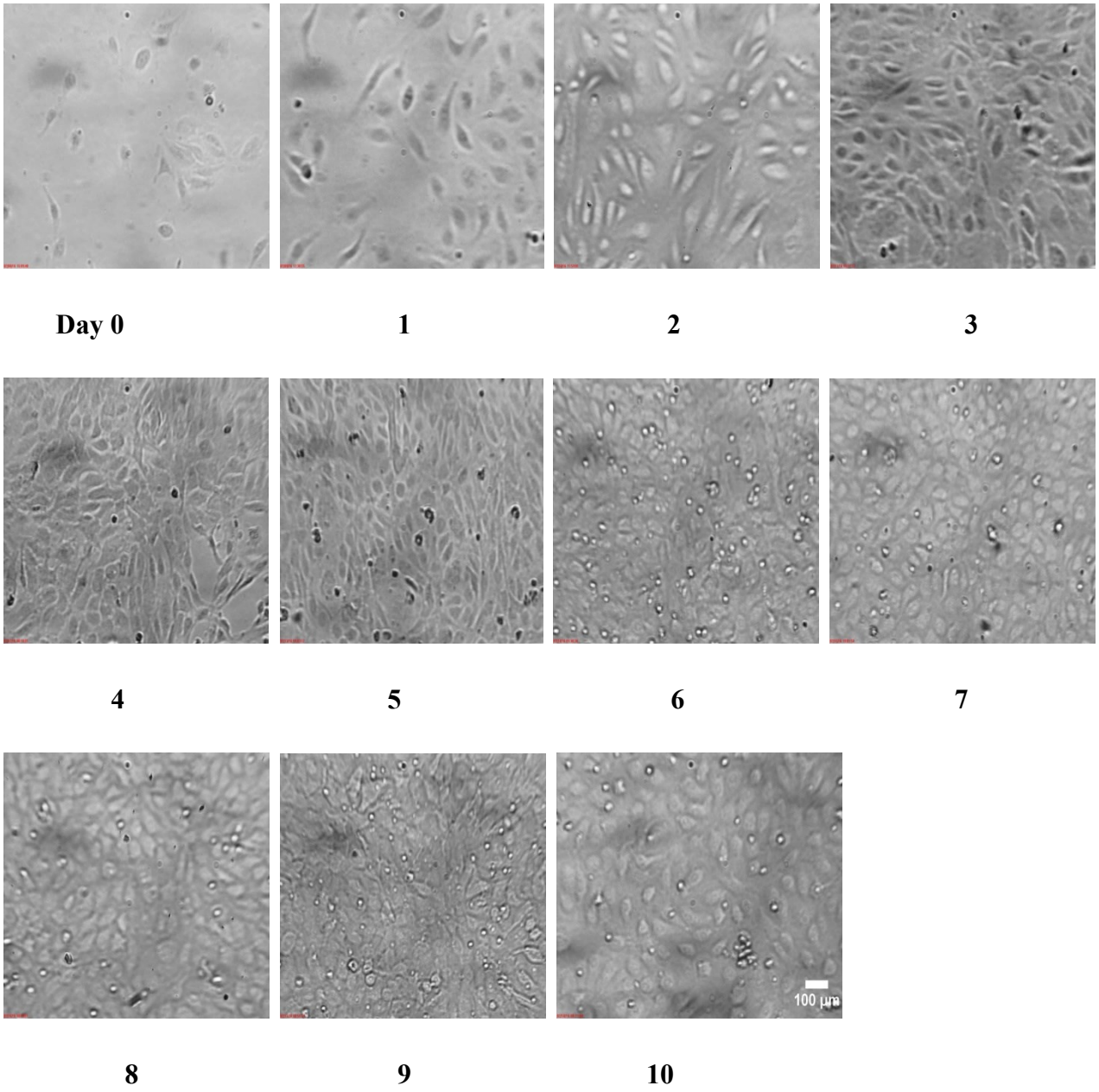

Figure 2.4: Morphological assessment of HUVECs on glass coverslips by phase contrast microscopy over a period of 10 days. HUVECs were seeded at a density of 10,000/cm^2 on coverslips and observed under the phase contrast microscope every day (10 X magnification). Scale bar represents 100 µm.

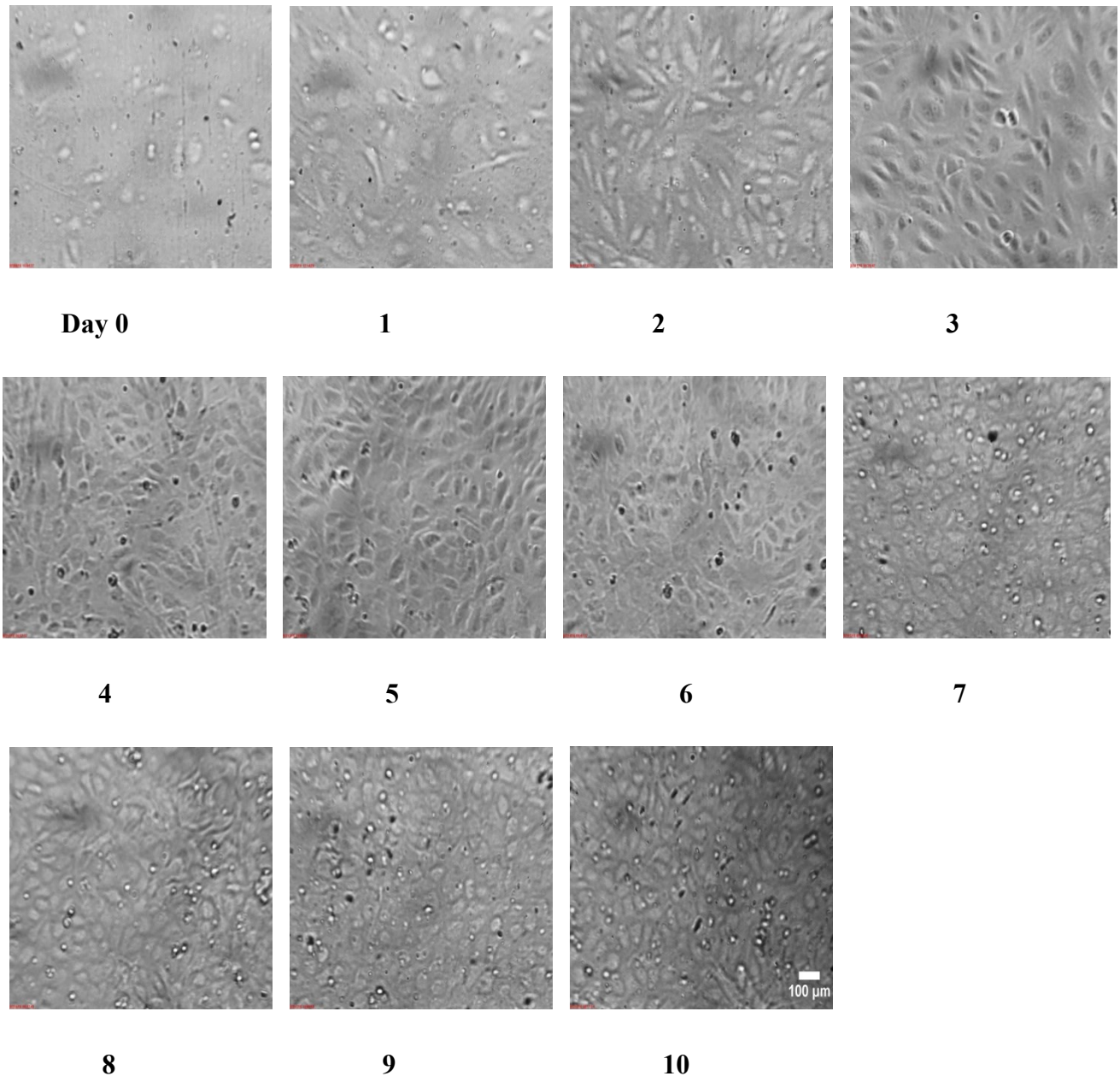

Figure 2.5: Morphological assessment of HUVECs on Rinzl coverslips by phase contrast microscopy over a period of 10 days. HUVECs were seeded at a density of 10,000/cm^2 on coverslips and observed under the phase contrast microscope every day (magnification: 10 X). Scale bar represents 100 µm.

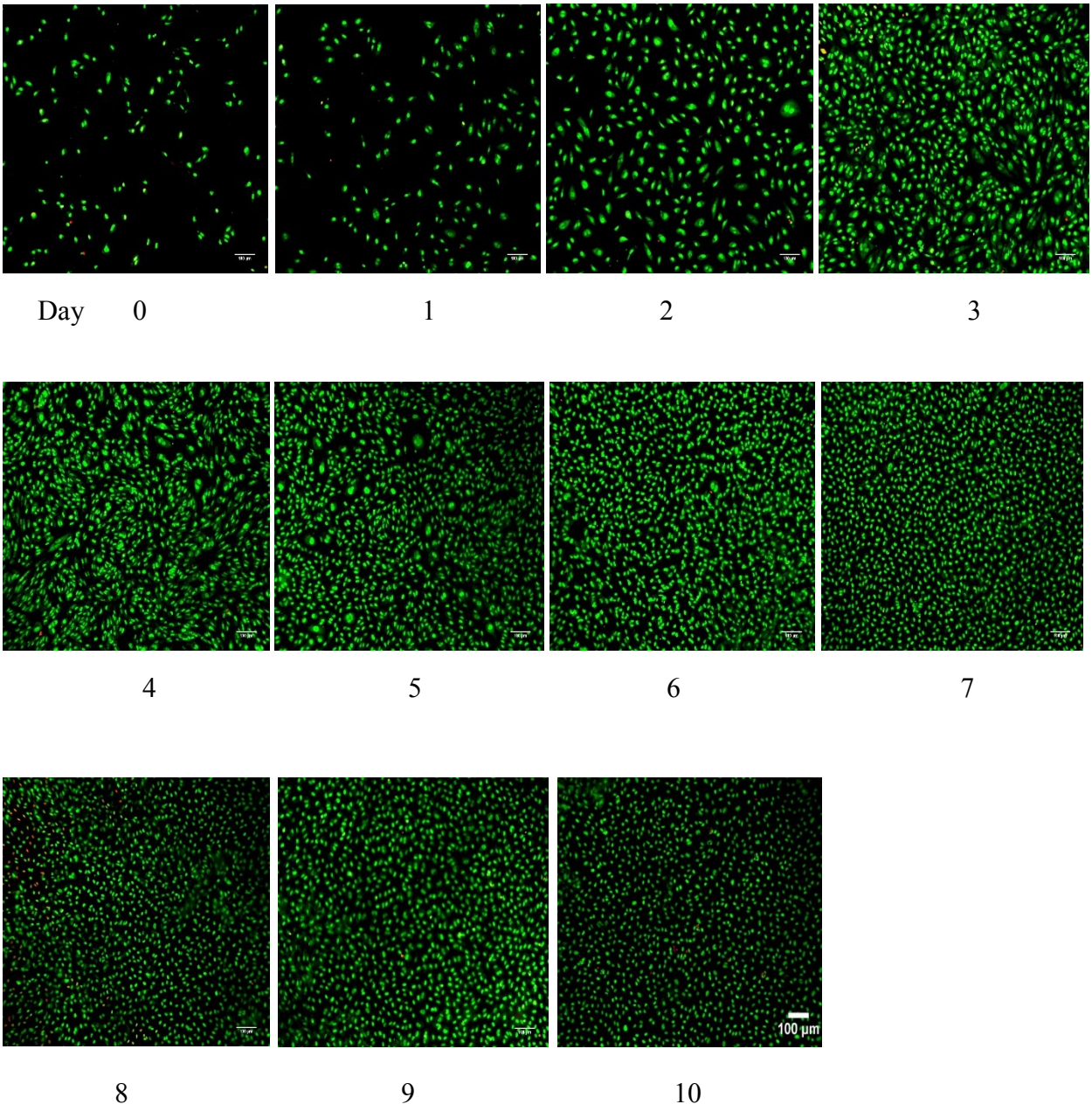

Figure 2.6: Viability assessment of HUVECs over a period of 10 days on glass coverslips. Cells were seeded at a density of 10,000/cm^2 and were stained with SYTO13/GelRed. Cells were observed under the fluorescent microscope (magnification: 10X) and viability was assessed using the Viability3 program. Scale bar represents 100 µm.

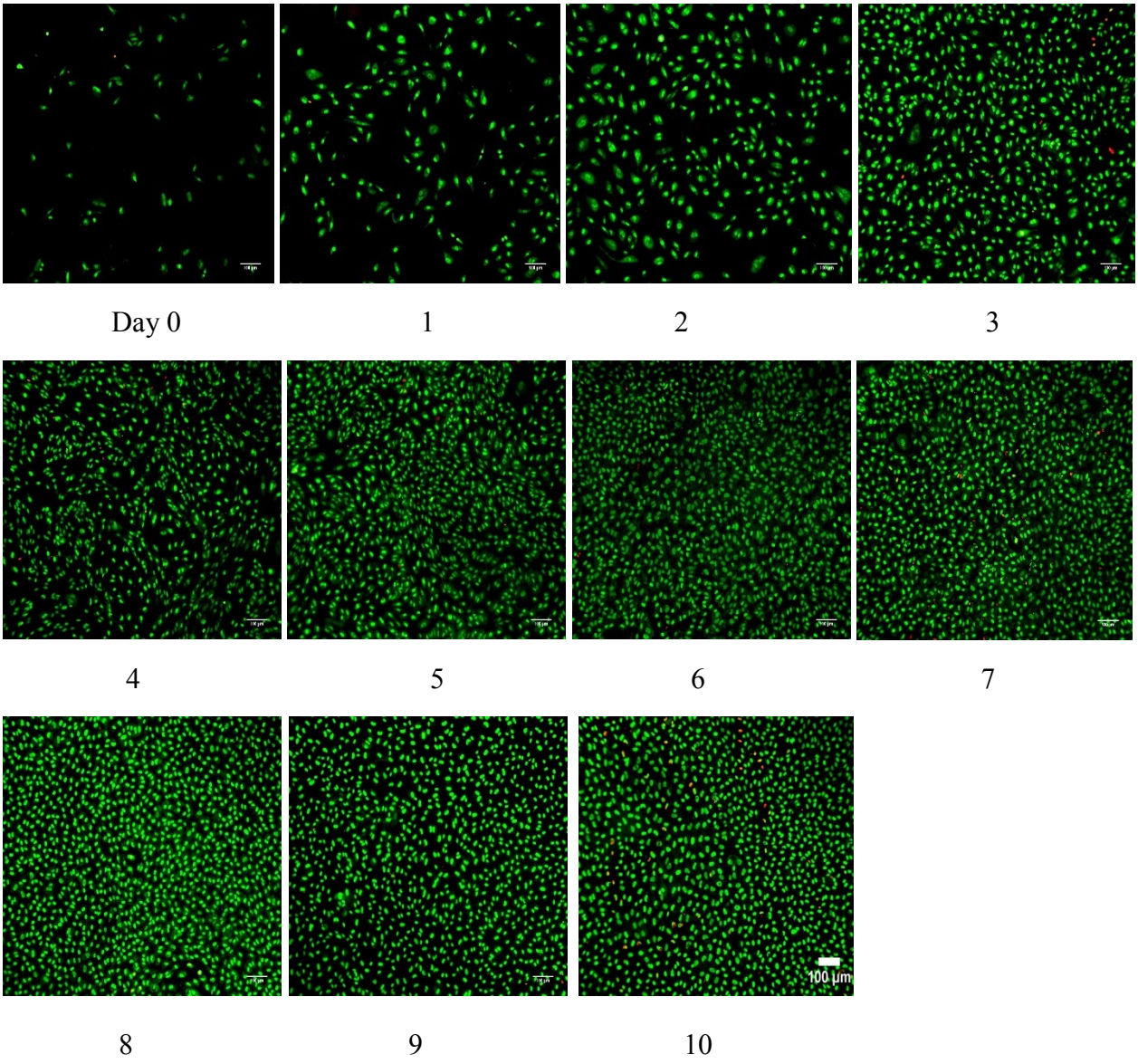

Figure 2.7: Viability assessment of HUVECs on Rinzl coverslips over a period of 10 days. Cells were seeded at a density of 10,000/cm^2 and were stained with SYTO13/GelRed. Cells were observed under the fluorescent microscope (magnification: 10X) and viability was assessed using the Viability3 program. Scale bar represents 100µm.

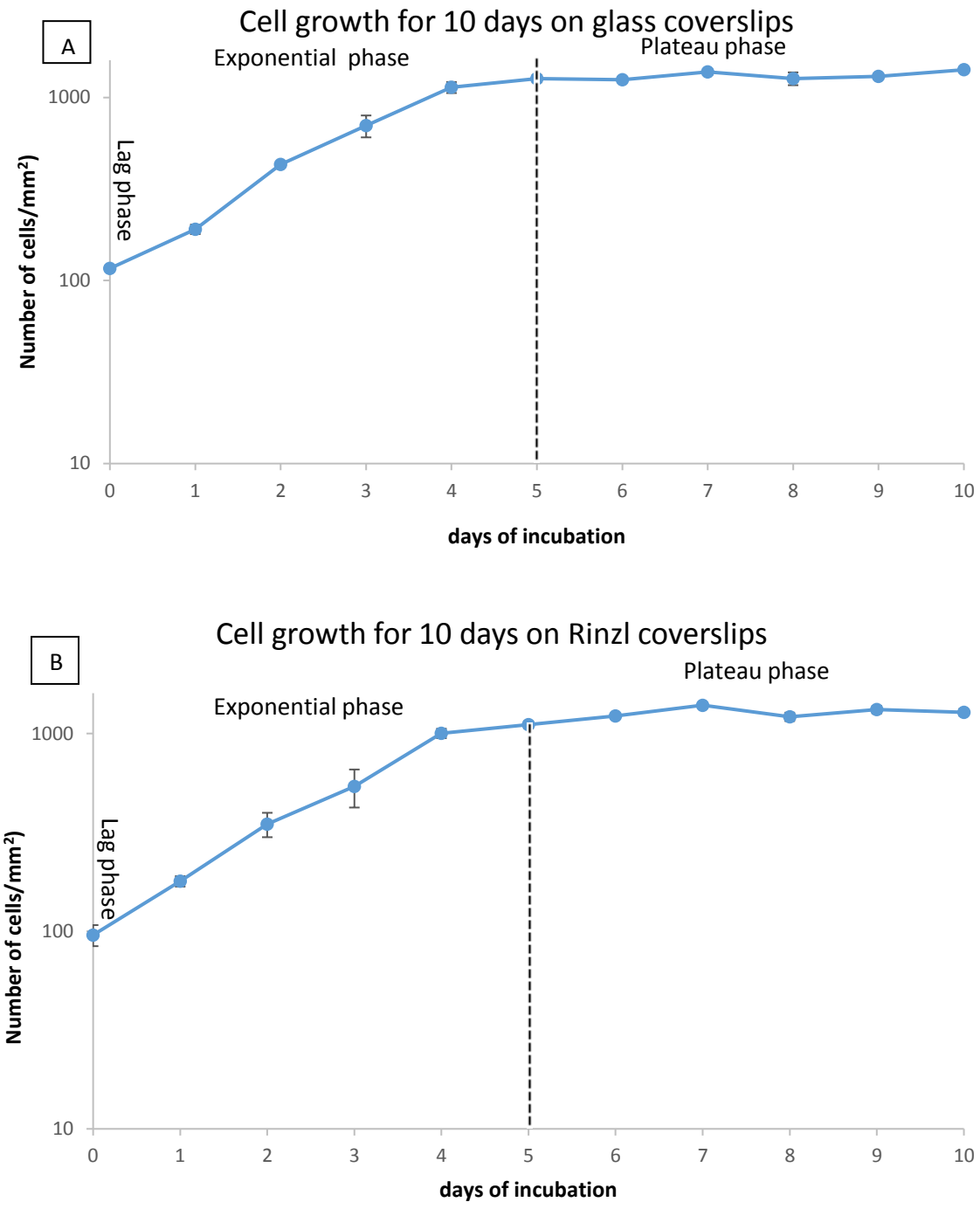

Figure 2.8: Growth curve of human umbilical vein endothelial cells (HUVECs) *in vitro*. Cells were seeded at a density of 10,000/cm^2 on A) glass and B) Rinzl coverslips pre-coated with fibronectin. Every day, cells were stained with SYTO13/GelRed and observed under a fluorescent microscope for cell density/mm^2 assessment (magnification: 10X). Data are the average of three independent experiments ± standard error of the mean (SEM).

Table 2.2: Cell density, cell viability and doubling number for HUVECs seeded on glass coverslips

Day	Cell number/mm^2	Viability (Relative membrane integrity, %)	Doubling number	Cell growth
0	116 ± 4	96.03 ± 3.2		Lag phase
1	191 ± 11	98.81 ± 2.3	0.7	Exponential phase
2	431 ± 13	98.80 ± 0.9	1.9	
3	702 ± 96	98.90 ±1.2	2.6	
4	1137 ± 78	98.2 ± 0.5	3.3	
5	1270 ± 59	98.60 ± 0.2	3.45	Plateau phase
6	1252 ± 13	99.04 ± 0.7	3.43	
7	1379 ± 49	95.16 ± 0.7	3.57	
8	1270 ± 102	97.33 ± 3.9	3.45	
9	1307 ± 15	97.4 ± 0.3	3.49	
10	1418 ± 38	92.9 ± 3.6	3.6	

Table 2.3: Cell density, cell viability and doubling number for HUVECs seeded on Rinzl coverslips

Day	Cell number/mm^2	Viability (Relative membrane integrity, %)	Doubling number	Cell growth
0	96 ± 11	96.0 ± 2.1		Lag phase
1	179 ± 11	98.8 ± 0.3	0.9	Exponential phase
2	348 ± 49	98.8 ± 0.3	1.85	
3	540 ± 117	98.9 ± 0.2	2.5	
4	1002 ± 54	98.2 ± 0.2	3.38	
5	1108 ± 45	98.6 ± 0.1	3.52	Plateau phase
6	1226 ± 29	99.0 ± 0.3	3.67	
7	1390 ± 6	95.1 ± 1.3	3.85	
8	1216 ± 59	97.3 ± 1.4	3.66	
9	1282 ± 57	97.4 ± 1.2	3.73	
10	1322 ± 51	92.9 ± 3.3	3.78	

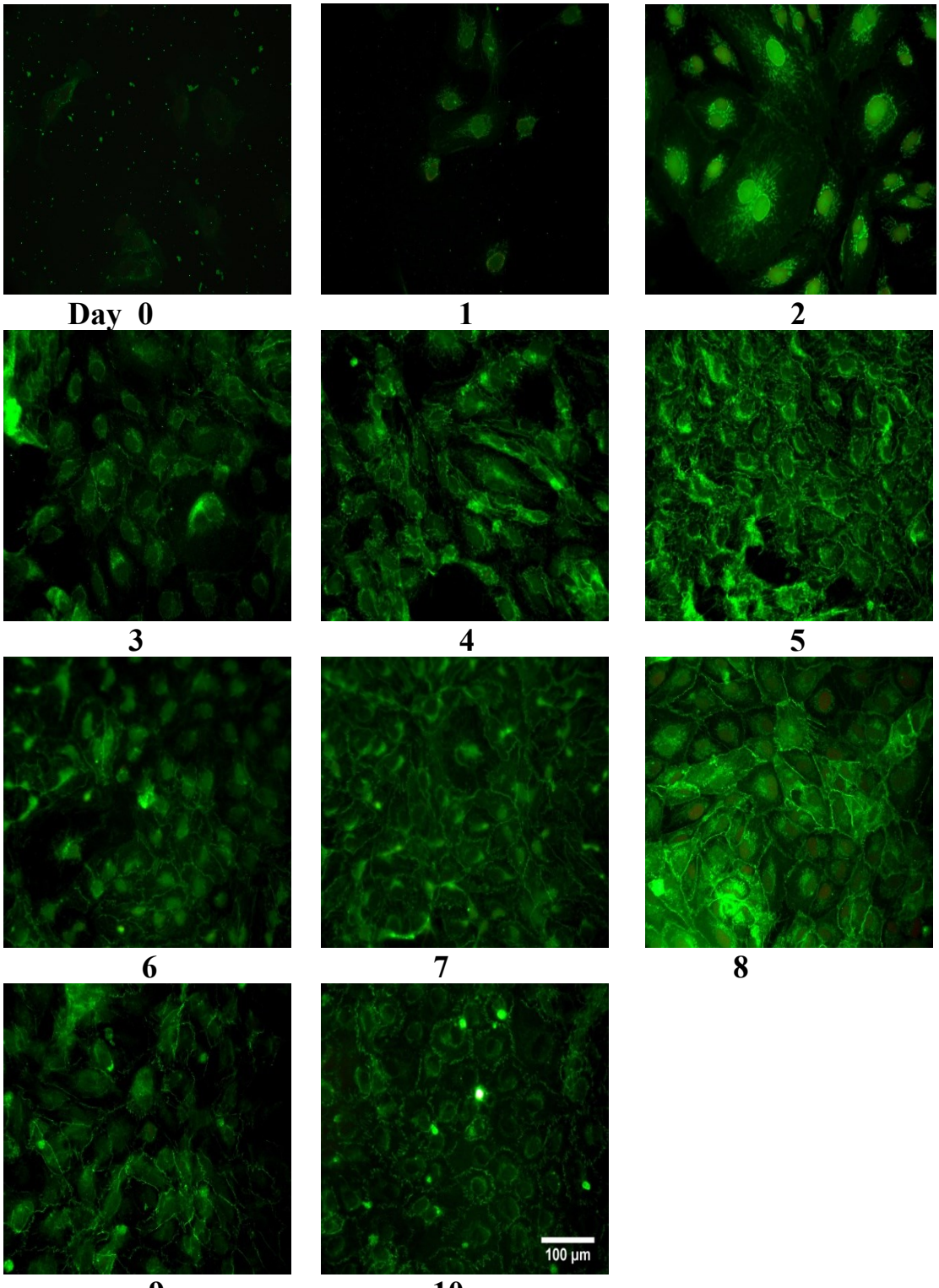

Figure 2.9: Claudin-5 immunolabeling. HUVECs were seeded on glass coverslips (10,000/cm^2). Every day, cells were fixed with methanol. Non-specific staining was blocked with blocking buffer and cells were stained with Claudin-5 antibody, and observed under the fluorescent microscope (magnification: 25X).

2.5 References

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Chapter 3: HUVEC monolayer freezing at –9 °C

3.1 Introduction

While cryopreservation of isolated cells in suspension has been successful for a variety of cell types, attempts at cryopreservation of whole tissues and organs have been largely unsuccessful [1],[2]. There are a diversity of cell types with different hydraulic conductivities (which affects redistribution of biological water across the membrane during freezing and thawing processes), cell–cell, cell–matrix interactions and cell arrangements in tissues which affect their capacity to respond to cryopreservation processes [2]. As a starting point, cryopreservation of cells in a monolayer state (two-dimensional structure) provides valuable insight toward understanding the mechanisms of cryoinjury during freezing and thawing processes in three-dimensional structures (tissues) compared with cells in suspension [3]. In monolayer configuration, cells are in contact with each other through junctions. Moreover, cells are attached to the surface and have cell–surface interactions. Cells in monolayers are in close contact with the surrounding media, which provides a high degree of nutrient penetration and dead cells are not trapped between live cells [4]. While cryopreservation of cells in suspension using established cryopreservation protocols has been successful for a variety of cell types such as human bone marrow mesenchymal cells [5], human umbilical vein endothelial cells (HUVECs) [6], chondrocytes [7], mouse embryonic stem cells [8], and hepatocytes [9], the same protocols have not been successfully extended to cryopreservation of cells in monolayers on substrates or scaffolds for tissue engineered constructs [4],[10],[11],[3],[12],[13]. The aim of this chapter is investigating the effect of freezing at –9 °C on the viability and cell attachment on two different types of substrates (glass and Rinzl plastic).

Although there are several studies regarding cell cryopreservation in suspension, only a few studies investigated cell cryopreservation in monolayers. As explained earlier, during cryopreservation intracellular ice formation is one of the major factors that cause cell injury. The main difference between cell response in monolayers and in suspension during freezing is the incidence of intracellular ice formation [14],[15]. This incidence of ice formation is attributed to morphological states of the cells [14]. A study by Acker *et al.* [15] showed that the presence of cell–cell junctions and cell–surface interactions in a Chinese hamster fibroblast (V-79W) monolayer increased the incidence of intracellular ice formation at a specific temperature compared with single cells [15].

The role of cell–surface interaction is not only to influence intracellular ice formation. One of the main challenges in cryopreservation of cells in monolayers is preserving their attachment to the surface. HUVECs are primary cells that are anchorage-dependent and cell survival and growth are dependent on their attachment to the surface [16]. Therefore, the aim of this study is to investigate how the surface properties affect cell adherence in monolayer cryopreservation. Cell adhesion to the surface is a critical factor for successful monolayer cryopreservation since cell survival and growth in monolayer configuration need cells to be attached to the substrate. Excessive detachment was reported in cryopreservation of human corneal endothelial cells (HCEC) in monolayers by Ebertz *et al* [17]. Surface modification is one of the approaches in order to improve cell attachment. Coating a surface (glass with gelatin) and using Matrigel™ have shown improvement in post-thaw cell attachment for human mesenchymal stem cells in comparison with glass (with no modification) [20]. Very few cells were attached to the bare glass compared with a glass surface coated with gelatin and matrigel [20]. One of the major components of the cytoskeleton, which has a major role in surface movement, is filamentous

actin (F-actin). A study of F-actin after cryopreservation of human mesenchymal stem cells showed that disruption of F-actin organization compared with control cells led to cell detachment for all experimental conditions to variable degrees [22]. Cell–surface interaction is controlled by F-actin in the cytoskeleton. This function of F-actin is associated with an adhesion molecule for the cytoskeleton (α-catenin). Entrapping cells in a collagen sandwich configuration has also been one of the approaches for cryopreservation of hepatocytes [18] but has shown limited success in terms of adhesion efficiency. Freezing affected organization of F-actin in a mouse calvaria-derived osteoblast cell line [19]. This experiment showed that depolymerization of F-actin was related to the dead cells and as temperature decreased, more damage occurred to F-actin, which led to a decrease in the area of surface covered by cells [19].

In cell suspension cryopreservation, when ice forms in the extracellular environment, cells are pushed to the unfrozen fraction. In monolayers, cell movement is limited as cells are fixed and cannot move easily and they experience mechanical stress [20]. It is proposed that mechanical stress due to differences in stretch and contraction between the cell and substrate during the cooling and warming processes of cryopreservation leads to breakage of F-actin in the cytoskeleton [19]. The coefficient of thermal expansion describes changes in the size of an object in response to a change in temperature. The cooling and warming processes in cryopreservation are accompanied by ice expansion and contraction. It was hypothesized by our group that in a monolayer, mismatch between the coefficient of thermal expansion of ice and the substrate affects cell attachment to the substrate during the freeze–thaw process [23]. Previously, this hypothesis was examined by one of our lab members [23]. For this purpose, Chinese hamster fibroblast cell attachment was examined after freezing at –9 °C for cells seeded on two different substrates (glass and Rinzl plastic coverslips). In that study only attachment was assessed, not

viability [23]. The aim of the study reported in this chapter is comparison of HUVEC attachment on two different substrates (glass and Rinzl plastic coverslips) after freezing at –9°C without cryoprotectant. In addition to attachment, cell viability was assessed to investigate the effect of cell–surface interaction on viability of the cells after a cryopreservation process [23].

3.2 Materials and methods

3.2.1 HUVEC preparation

Viability and attachment of HUVECs after freezing at a specific temperature (–9°C) was assessed for cells cultured on both glass and Rinzl coverslips. For this purpose, coverslips were sterilized, washed and placed in a 24-well plate as described in Chapter 2 (section 2.2). HUVECs were seeded at a density of 10,000/cm^2 on fibronectin pre-coated glass and Rinzl coverslips. Freezing was performed on day 7 after cell seeding in a cooling bath (methanol bath). The cooling bath can be adjusted to different temperatures and cooling rates.

3.2.2 Temperature measurement

In this experiment, temperature was measured by T-type thermocouples and a OMB-DAQ-55 data acquisition module (OMEGA Engineering Inc., Stamford, Connecticut, USA). For calibration, all of the thermocouples were placed in a mixture of ice and water and allowed to come to thermal equilibrium. At equilibrium, each thermocouple's readout was recorded as a reference. After determining their reference readout, one of the thermocouples was kept in a mixture of ice and water; another thermocouple was placed directly in the methanol bath; a third thermocouple was placed in a glass vial containing 200 µl EGM-2 (the glass was placed in the methanol bath); and a fourth thermocouple recorded room temperature. To determine each experimental temperature, each thermocouple was adjusted by subtracting its reference readout corresponding to zero degrees.

3.2.3 Freezing experiment

For this experiment, the methanol bath was set at a specific temperature (–9 °C). Coverslips (glass and Rinzl) were transferred to Kimble borosilicate glass shell vials (60965D-12, Kimble Chase, USA) with fine tweezers with the cell side up. Vials contained 190 µl of EGM-2 and were closed with white plastic caps. Samples were labeled as frozen or control for both glass and Rinzl. Experiments for both glass and Rinzl coverslips were repeated 3 times independently (N=3) from different cultures. For each experiment, three coverslips were considered for controls and three coverslips for frozen samples. Frozen samples were placed in the methanol bath preset at –9 °C for 2 minutes to equilibrate at this temperature and then briefly removed from the methanol bath in order to nucleate ice. Ice was nucleated by forceps (pre-cooled in liquid nitrogen) for 3 seconds. Samples were placed back in the methanol bath at –9 °C and were kept for 3 minutes to release the latent heat of fusion. After 3 minutes, the frozen samples were thawed in a water bath at 37 °C and were analyzed for cell viability (membrane integrity assay) and attachment. Control samples were unfrozen samples, which were kept at room temperature beside the methanol bath during the experiment. Coverslips were stained with SYTO13/GelRed as described in Chapter 2 (section 2.2) and 12 images were captured for each coverslip under the fluorescent microscope as explained in section 2.2. Relative viability was assessed as described in the Chapter 2 (section 2.2, Eq.1).

3.2.4 Cell attachment assessment

Since cell loss due to detachment occurs in monolayer freezing, cell attachment after freezing was measured based on:

$$cell\ attachment = \frac{total\ number\ of\ cells\ on\ a\ coverslip}{total\ number\ of\ cells\ on\ a\ control\ coverslip} \times 100 \qquad \text{(Eq.1)}$$

3.2.5 Statistical analysis

The aim of this study was to compare the effect of two different substrates on HUVEC viability after freezing at –9 °C. The dependent variable in this study was viability and the independent variable was the type of substrate (glass or Rinzl). Three independent experiments were performed for both glass and Rinzl coverslips. For this purpose, the mean difference between HUVEC viability on glass and Rinzl coverslips (two populations) after freezing at (–9 °C) was evaluated by an independent samples t-test. These are the assumptions for this t-test:

1) independent observations,

2) normal distribution, and

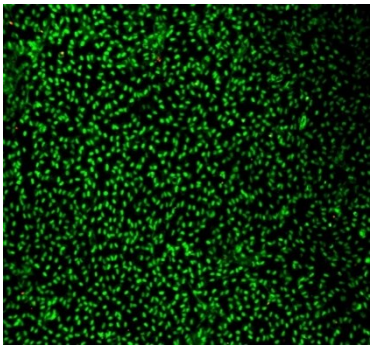
3) homogeneity of variance (equal variance between two populations).

Three independent experiments were conducted for this study. Statistical analysis was performed by SPSS statistics version 24. Significance was accepted with $p < 0.05$.

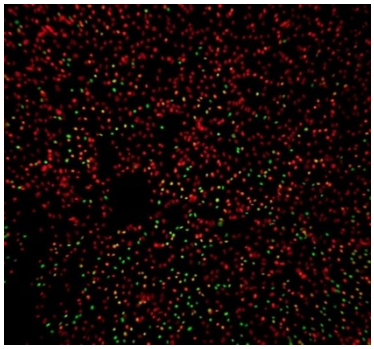
3.3 Results

To assess the effect of the substrate on HUVEC viability and attachment, HUVECs were frozen at –9 °C on glass and Rinzl plastic coverslips on day 7 of culture. The coverslips were coated with fibronectin prior to cell seeding. Viability and attachment in this study are the average of three independent experiments and data are presented as mean ± standard error of the mean

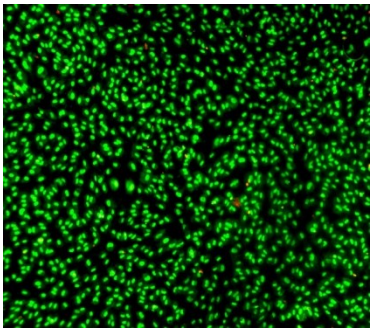
(SEM). Figure 3.1 shows viability of controls and frozen cells on both glass and Rinzl coverslips. Comparison of HUVEC viability on glass and Rinzl coverslips showed that in the absence of freezing (unfrozen sample), there was no significant difference between cell viability on glass (98.7 ± 0.04%) and Rinzl (98.8 ± 0.06%) coverslips (Figure 3.2). Controls were confluent monolayers and cells were completely attached to the surface. However, HUVEC viability was greatly affected after thawing on both glass and Rinzl coverslips and there was a dramatic drop in cell viability for cells on glass (viability was 17.5 ± 7.6%) (Figure 3.2). HUVEC response to freezing on Rinzl coverslips was accompanied by a reduction in viability to 34.8 ± 11.3% (Figure 3.2). The difference between viability on glass and Rinzl was not statistically significant ($p = 0.09$). In addition to viability, cell attachment was also affected by freezing (Figure 3). Attachment is the ratio of total cells (red and green) on frozen coverslips to total cells in control (before freezing). HUVECs were detached from both frozen glass and Rinzl coverslips compared with control. Comparison of HUVEC attachment on frozen glass (94 ± 8%) and Rinzl (80 ± 11%) coverslips showed no significant difference ($p = 0.18$) (Figure 3.3). Although the viability dramatically decreased, a cell adhesion assessment showed that in fact most of the cells remained attached on both types of coverslips after freezing.



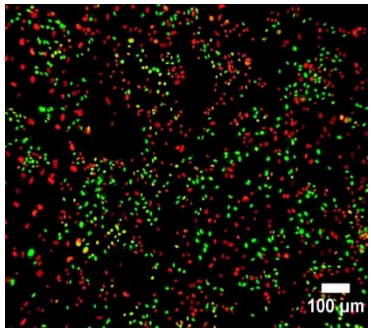
A) glass (control)



B) glass (frozen at –9 °C)



C) Rinzl (control)



D) Rinzl (frozen at –9 °C)

Figure 3.1: Fluorescent microscope images of HUVECs (magnification: 10X) on glass (A and B) and Rinzl coverslips (C and D). Cells were frozen at –9 °C and after thawing cells were stained with SYTO13/GelRed. Control cells (A and C) were a confluent monolayer (live cells) and frozen samples (B and D) contained live (green) and dead (red) cells. Viability was assessed using the Viability3 program. Scale bar represents 100µm.

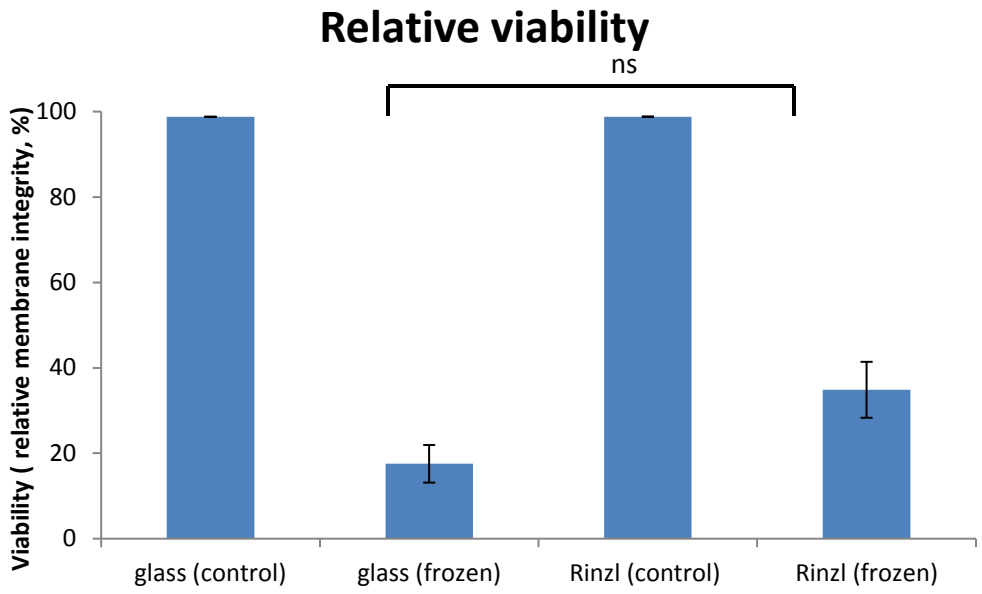


Figure 3.2: HUVEC viability after freezing at –9 °C. Coverslips were stained with SYTO13/GelRed and viability assessed under a fluorescent microscope using the Viability3 program. Each experiment was performed in triplicate and results are the average of three independent experiments. Data are mean ± standard error of the mean (SEM). Statistical analysis was performed using the independent samples t-test. The level of significance was considered to be $p < 0.05$. ns = no significant difference.

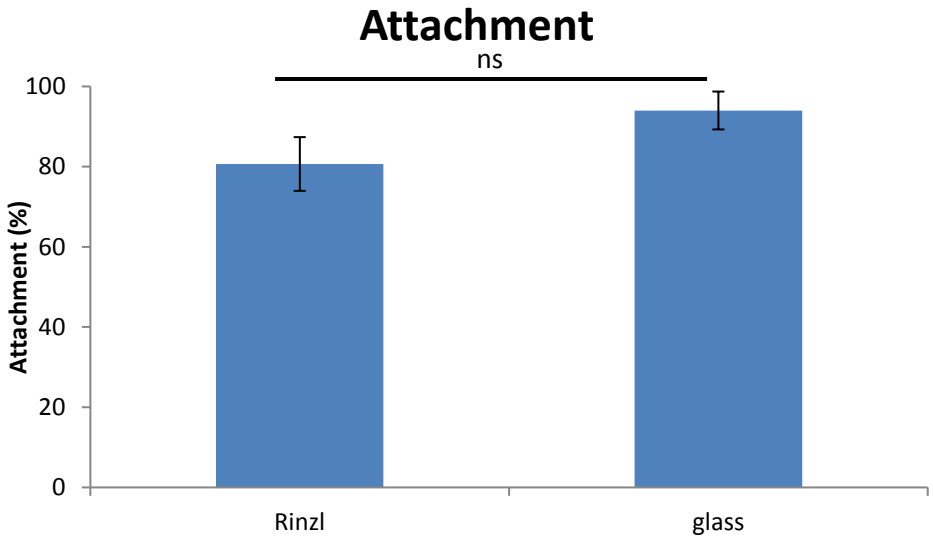


Figure 3.3: HUVEC attachment after freezing at –9 °C on glass and Rinzl coverslips. Attachment was calculated based on the ratio of the total number of cells in each image to the total number of cells on a control coverslip. Data are mean ± standard error of the mean (SEM). Data are the average of three independent experiments and in each experiment samples were triplicate. Statistical analysis was performed by the independent samples t-test. The level of significance was considered $p < 0.05$. ns = no significant difference.

3.4 Discussion

Primary cells such as HUVECs (derived from umbilical veins) are anchorage dependent and need to be attached in order to grow and remain viable. One of the main challenges in monolayer cryopreservation is cell loss after the freeze–thaw process. Cell detachment limits the usefulness of monolayers. Still, it is not clear when cell detachment occurs (during the freezing or warming process). Over the last few decades, one of the alternative approaches to prevent cell loss was coating the surface of the substrate with a specific extracellular matrix protein, such as collagen [11]. This approach has been used for primary hepatocyte and embryonic stem cell cryopreservation in monolayers [18]. Microencapsulation [21] and sandwich configuration (arresting cells between two layers of collagen gel) [18] has also been studied for monolayer cryopreservation. It is proposed that the presence of extracellular matrix stabilizes membranes against injury in the freeze–thaw process. In this study cells were exposed to –9 °C at first for 2 minutes to equilibrate with this temperature and ice was nucleated using forceps pre-cooled in liquid nitrogen and samples were placed again at –9 °C to release the latent heat of fusion. For this study, HUVEC confluent monolayers were frozen at –9 °C, since V-79W hamster fibroblast cells showed 100% intracellular ice formation in a confluent monolayer [14]. Although in this study the time of exposure to –9 °C was only 5 minutes, this temperature is high in comparison with cryogenic temperature (the temperature of liquid nitrogen –196 °C, which is used for cryopreservation of cells). Comparison of cell attachment after the freeze–thaw process on glass and Rinzl plastic coverslips showed that the mismatch in the coefficient of thermal expansion between glass and ice did not lead to cell detachment at –9 °C in the case of HUVECs, as there is no significant difference between cell loss for cells on glass and Rinzl coverslips. A previous experiment for Chinese hamster fibroblasts (V-79-4) showed that after freezing at –9 °C, cell

detachment from Rinzl coverslips was less than for glass coverslips [23]. It needs to be noted that the results of this study cannot completely be extended to cell detachment during cryopreservation processes. During commonly used protocols for cryopreservation, temperature decreases based on a specific cooling rate (usually slow cooling) and finally cells are plunged into liquid nitrogen (rapid cooling). Therefore, cells are exposed to the cooling environment for a longer period of time compared with this experiment. In addition, temperature and size and shape of ice (affected by cooling rate) are other factors that need to be considered to explain the damage mechanism for attached cells in monolayers. In this experiment, cells were exposed to one specific temperature for 5 minutes. In addition, cryopreservation temperature (liquid nitrogen: –196 °C) is one of the factors that is proposed to affect the structural properties and response of F-actin. Moreover, monolayer freezing was conducted on a different cell type in this experiment than in the previous experiment, which makes it difficult to compare. In this study, dead cells remained attached to the surface. Future studies for F-actin organization will help to explain this phenomenon. While cells are attached to the surface, the distribution of F-action may be affected by the freezing process, which can have an effect on cell signaling and cell viability.

3.5 References

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Chapter 4: Graded freezing on glass and Rinzl coverslips in the absence and presence of cryoprotectants

4.1 Introduction

While applying commonly used cryopreservation protocols for a variety of cell types in suspension has been successful [1],[2],[3], developing a cryopreservation protocol for cells in a monolayer has not been straightforward. This challenge has been attributed to the difference in the morphological status of the cells in monolayers compared with single cells in suspension [4]. The primary goal for developing a monolayer cryopreservation protocol is obtaining high cell recovery after the thawing process. In particular, for cells in monolayers, this high recovery needs to be achieved by minimizing both cell detachment and membrane damage during the cryopreservation process [5].

In order to develop a cryopreservation protocol, several factors need to be considered such as cooling rate [5], warming rate [6], substrate, and cryoprotectant solutions [7]. Conventional cryopreservation protocols usually consist of adding the cryoprotectants and reducing the temperature at the rate of 1°C/min (slow cooling) to a specific temperature such as –80 °C using a controlled rate freezer [8], and plunging into liquid nitrogen for a period of time which is followed by rapid thawing. While getting a high rate of recovery is vital for successful cryopreservation, in order to minimize the damage it is also essential to understand different sources of cryoinjury, which occurs to the cells during cooling to different subzero temperatures.

One of the important factors in developing an effective protocol is cooling rate [9]. As explained in Chapter 1, based on cooling rate, cells may experience two different types of damage [10]. When cells are cooled slowly, solution effects lead to dehydration (cell shrinkage), which affects cell survival [10]. On the other hand, exposing cells to rapid cooling increases the likelihood of

intracellular ice formation, which is lethal to cells [11]. The optimum cooling rate that minimizes both sources of cell damage is specific for each cell type. Using the optimum cooling rate has improved cell survival after thawing processes.

Two main interrupted cooling procedures have been studied in order to explore where in the cryopreservation protocol cryoinjury occurs. Two-step freezing or interrupted rapid cooling with hold time was studied by Farrant *et al* [12]. In this protocol, cells were cooled rapidly to different intermediate temperatures and held for a period of time before rapid thawing or plunge into liquid nitrogen before thawing [12]. McGann used a graded freezing procedure (interrupted slow cooling without hold time) in order to explore progressive damage during slow cooling to the storage temperature for Chinese hamster fibroblast cells [13]. In this procedure, cells are cooled slowly to various intermediate temperatures and either rapidly thawed at 37 °C or plunged into liquid nitrogen before thawing [14],[15]. This procedure helps to distinguish the damage that occurs during slow cooling to different intermediate temperatures and the damage that occurs during rapid cooling when cells are plunged into liquid nitrogen for storage.

Another important factor in developing a successful cryopreservation protocol is the presence of cryoprotectants in a cryopreservation solution. Low molecular weight penetrating cryoprotectants reduce the damage in slow cooling processes. DMSO has been an important penetrating cryoprotectant in commonly used cryopreservation protocols, which has been often accompanied by the presence of other cryoprotectants. A combination of DMSO and HES has been studied for cryopreservation of a variety of cell types in suspension [16],[17]. HUVECs have shown high viability after cryopreservation in suspension in the presence of 5% DMSO and 6% HES [3].

The aim of this chapter is developing a cryopreservation protocol for HUVECs in monolayers, based on understanding the role of various factors during graded freezing. Cells were cooled at

the rate of 1°C/min to various subzero temperatures and thawed rapidly or plunged into liquid nitrogen (–196°C) before thawing. The CPA solution consisted of penetrating and non-penetrating cryoprotectants. DSMO was used in this experiment in order to minimize damage during the slow cooling process by reducing the amount of ice formed. Hydroxyethyl starch (HES) was used as a non-penetrating CPA in order to dehydrate the cells earlier in the freezing process to reduce the risk of intracellular ice formation.

4.2 Materials and methods

4.2.1 Graded freezing of HUVEC monolayers attached to glass and Rinzl coverslips

HUVECs to be frozen in monolayers were cultured for a period of 7 days to confluency at a density of 1.0×10^4 /cm^2 on Rinzl and glass coverslips in a 24-well plate as described for growth curve experiments (Chapter 2, Section 2.2). On day 7, plates were transferred to room temperature from the incubator and Rinzl or glass coverslips (with the cell side up) were transferred to glass vials (45 x 15mm, 60965D-1, Kimble Inc., USA) containing 190 µl of EGM-2 using fine tweezers. All of the vials were kept on ice for 15 minutes before the freezing experiment.

In this experiment, controls consisted of positive controls (pre-experiment and post-experiment) and a negative control. A pre-experiment coverslip was assessed for viability after 15 minutes incubation on ice. The negative control consisted of a vial plunged directly into liquid nitrogen after 15 minutes incubation on ice (Figure 4.1). The experimental temperatures for graded freezing in the absence of CPAs were –3, –10, –15, –25, –35, and –45 °C. A post-experiment coverslip was incubated on ice for the duration of the experiment and it was analyzed at the end of experiment after the sample directly thawed for –45 °C.

This experiment was performed for glass and Rinzl coverslips separately. For each intermediate temperature, a pair of glass vials (each containing a coverslip) was considered. After 15 minutes incubation on ice, vials were placed in a stirred methanol bath pre-set at –3 °C for 2 minutes to equilibrate with this temperature. In the absence of CPA, ice nucleation temperature was –3 °C. After two minutes, vials were removed from the bath and ice was nucleated by touching the vials using forceps pre-cooled in liquid nitrogen (touching with forceps for 3 seconds just a little above the coverslip). After ice nucleation, vials were placed back into the stirred methanol bath at –3 °C for 3 minutes to release the latent heat of fusion. The methanol bath was then cooled at the rate of 1 °C/min (Figure 4.2).

During the experiment, for each experimental temperature, pairs of vials were considered. One vial was placed in liquid nitrogen (plunge sample) and one vial transferred to a water bath at 37 °C and thawed rapidly (direct thaw sample) for viability and attachment analysis. Plunge samples were kept in liquid nitrogen for 1 hour. A post-experiment sample was analyzed after the direct-thaw sample from –45 °C. After analyzing the post-experiment sample, the negative control sample was assessed and then samples from liquid nitrogen were thawed rapidly in the water bath at 37 °C based on the order placed in liquid nitrogen and analyzed for viability and attachment (Figure 4.2).

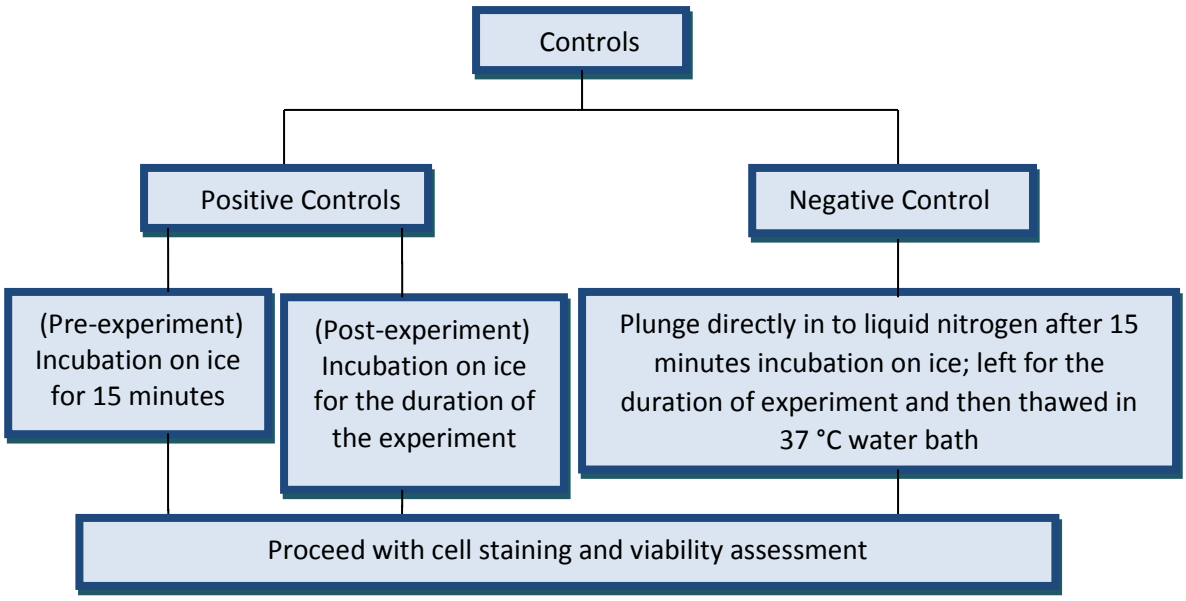


Figure 4.1: Controls in graded freezing experiments. Positive controls were HUVECs incubated on ice for 15 minutes (pre-experiment) and analyzed for viability and attachment. Post-experiment controls were incubated on ice for the duration of the experiment and assessed for viability and attachment after analyzing the sample that was directly thawed from –45 °C. Negative controls were plunged into liquid nitrogen after 15 minutes incubation on ice, held in liquid nitrogen for the duration of the experiment, and then thawed in a water bath at 37 °C and assessed for cell viability at the end of the experiment.

Overview of graded freezing experiments in the absence of cryoprotectants on glass and Rinzl coverslips

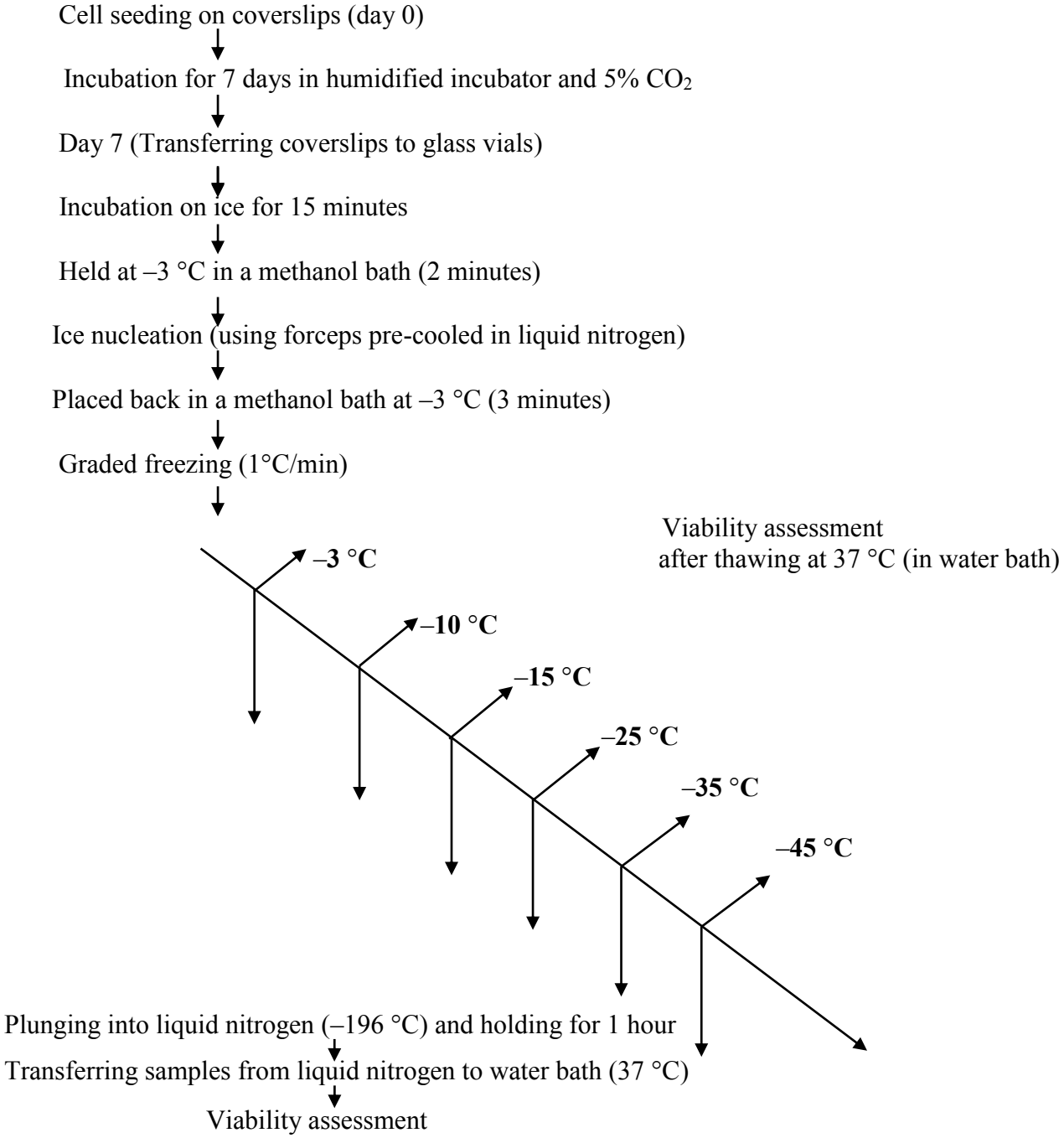


Figure 4.2: Overview of the graded freezing experiment process in the absence of CPAs. On day 7, HUVECs were subjected to graded freezing (1 ºC/min) and for each experimental temperature a pair of vials were considered (direct thaw and plunge thaw). After thawing, cells were stained with SYTO13/GelRed and assessed for viability and attachment by fluorescent microscopy using the Viability3 program.

4.2.2 Graded freezing in the presence of CPAs on glass and Rinzl coverslips

In order to perform graded freezing in the presence of cryoprotectants, cryoprotectant solutions were prepared and added before the freezing process as described below.

4.2.2.1 Preparation and addition of cryoprotectant solutions

HUVECs were also frozen in the presence of cryoprotectant solutions including different concentrations of DMSO (10% w/w and 20% w/w DMSO) and a combination of 5% DMSO and 6% hydroxyethyl starch (HES). All of these cryoprotectant solutions were prepared in EGM-2. In order to decrease cryodamage due to osmotic stress during addition of cryoprotectants, all of these cryoprotectant solutions were prepared in double concentration as a stock solution and then added 1:1 to each glass vial. In the first step, 95 µl of pre-warmed EGM-2 growth medium was added to each vial. Coverslips were transferred to each vial (cell side up) with fine tweezers and 95 µl of a cryoprotectant solution was added to each vial and mixed with EGM-2. After mixing, all vials were kept on ice for 15 minutes. The total volume of solution in each vial was 190 µl (Figure 4.3). The procedure for graded freezing was the same as the procedure described in section 4.2.1 for graded freezing in the absence of CPA except the nucleation temperature, which is explained below.

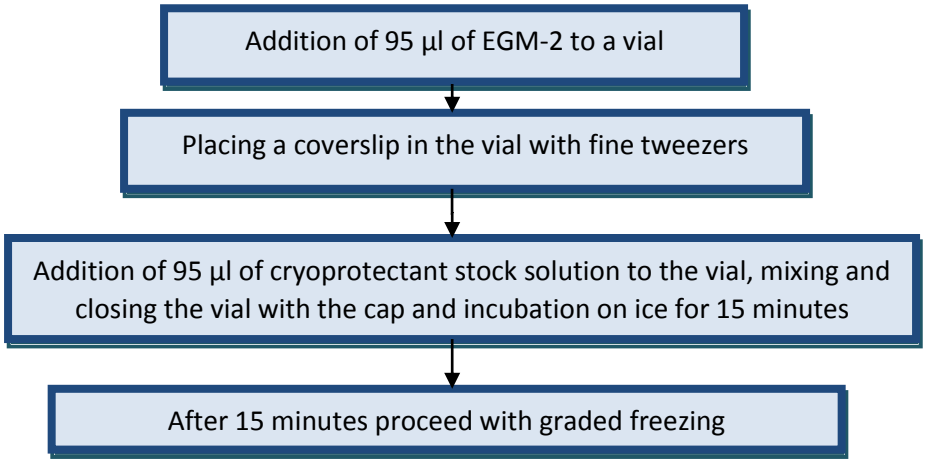


Figure 4.3: Cryoprotectant addition steps for graded freezing in the presence of CPAs

4.2.2.2 Experimental temperature in the presence of CPAs

For the experiment in the presence of 10% DMSO the nucleation temperature was –5 °C and the experimental temperatures were: –10, –15, –25, –35 and –45 °C.

For the experiment in the presence of 20% DMSO, the nucleation temperature was –10°C and the experimental temperatures were –10, –15, –25, –35 and –45 °C.

In the presence of 5% DMSO and 6% HES, ice was nucleated at –5 °C and the experimental temperatures were –5, –10, –15, –25, –35 and –45 °C. After ice nucleation, the experiment was performed in the same way as graded freezing in the absence of CPA (section 4.2.1).

4.2.3 Viability assessment

For positive controls, and after thawing for negative controls and samples for each experimental temperature, HUVECs were stained with SYTO13/GelRed as described in Chapter 2 (Section 2.2) and observed under the fluorescent microscope. Images were captured and were analyzed with the Viability3 program. For each direct-thaw or plunge-thaw sample at a specific temperature there was one coverslip. 12 images were captured per each coverslip as described in

Section 2.2. Results are the average of three independent experiments. Absolute viability was calculated based on Eq. 4.1:

$$\text{Absolute viability} = \frac{Average\ \ number\ of\ \ green\ cells\ \ on\ a\ frozen\ coverslip}{Average\ number\ of\ total\ cells\ on\ a\ pre{-}experiment\ coverslip} \times 100 \quad \text{Eq. 4.1}$$

Relative viability and attachment were assessed as described in Chapter 2 (Section 2.2, Eq. 2.1) and Chapter 3 (Section 3.2, Eq. 3.1) respectively.

4.2.3.1 Statistical analysis

For each experiment, in order to determine whether there is any statistically significant difference for viability among the means for these six groups for direct-thaw or plunge-thaw samples, statistical analysis was performed by one-way analysis of variance (ANOVA) using SPSS statistics Version 24.

1) In this experiment, the dependent variable is viability (continuous; 0–100%).

2) The independent variable is temperature, which consists of six independent groups (in the absence of CPA: –3, –10, –15, –25, –35 and –45 °C), (–5, –10, –15, –25, –35 and –45 °C in the presence of 10% DMSO or in the presence of 5% DMSO and 6% HES). The experiment in the presence of 20% DMSO had five independent groups (–10, –15, –25, –35 and –45 °C).

3) Observations were independent. For each experimental temperature, there was one coverslip and three independent experiments were performed for each experimental condition.

4) Levene's test was used in SPSS statistics to check homogeneity of variances.

In order to determine which specific group was statistically significantly different from the other, Tukey's post-hoc test was performed. The significance level was set at 0.05. The results are the

average of three independent experiments (N = 3). Each experiment consists of 12 images for each experimental temperature for direct or plunge-thaw.

4.3 Results for glass coverslips

4.3.1 Graded freezing in the absence of CPAs on glass coverslips

HUVECs on glass coverslips were subjected to graded freezing (cooling rate: 1 °C/min) in the absence of cryoprotectants. Data are presented as mean ± standard error of the mean (SEM), which is for three independent experiments.

Controls: After 15 minutes incubation on ice, a pre-experiment sample showed very high viability (98.6 ± 0.3%). A negative control showed dramatically low cell viability (0.4 ± 0.3%). A post-experiment sample showed 99.4 ± 0.2% viability.

Direct-thaw (Figure 4.4): In the absence of cryoprotectants, ice was nucleated at –3 °C. The post-thaw relative viability assessment for direct-thaw samples revealed a significant reduction in the number of the cells with intact membranes, cooled from –3 °C (86.2 ± 0.3%) to –15 °C (24.6 ± 6.9 %) ($p = 0.001$). Further cooling was accompanied by an increase in HUVEC viability at –25 °C. The relative viabilities for coverslips thawed from –25 °C and –35 °C were 52.1 ± 5.7% and 50.5 ± 9.7% respectively. The sample thawed from –45 °C showed a 20% reduction in viability compared with sample thawed directly from –35 °C.

Plunge-thaw (Figure 4.4): In contrast, plunging into liquid nitrogen from each experimental temperature resulted in much lower viability compared with the corresponding direct-thaw sample. The maximum viability was 3.4 ± 1.2% for the sample plunged into liquid nitrogen from –15 °C.

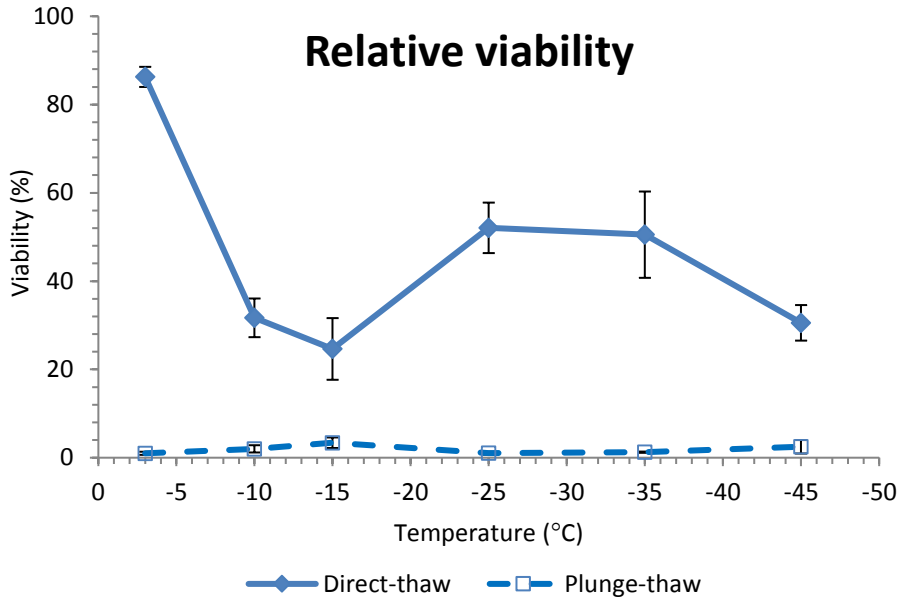


Figure 4.4: Relative cell viability for graded freezing on glass coverslips in the absence of cryoprotectants (direct-thaw and plunge-thaw samples). Results are the average of three independent experiments. Data are presented as mean ± standard error of the mean (SEM).

Cell attachment assessment (Figure 4.5 A): Direct-thaw samples experienced variable degrees of detachment for all experimental temperatures except for HUVECs thawed from –25 °C and –35°C (attachment =100%, there was no detachment for cells after thaw). The maximum detachment was observed for samples thawed directly from –10 °C. HUVECs also showed detachment from the surface after plunge- thaw at all experimental temperatures. Coverslips plunged from –5°C showed that only 58.6 ± 12.8% were attached. No significant difference was observed for cell detachment between HUVECs plunged from –5 °C and –10 °C. At –15 °C, both direct-thaw and plunge-thaw samples showed the same level of attachment. The maximum detachment was observed for samples plunged into liquid nitrogen from –10 °C and the minimum cell detachment occurred for coverslips plunged into liquid nitrogen from –35 °C. Therefore, plunging into liquid nitrogen at all experimental temperatures led to cell detachment

from the surface. Figure 4.5 B shows absolute viability for all experimental temperatures for both direct and plunge-thaw.

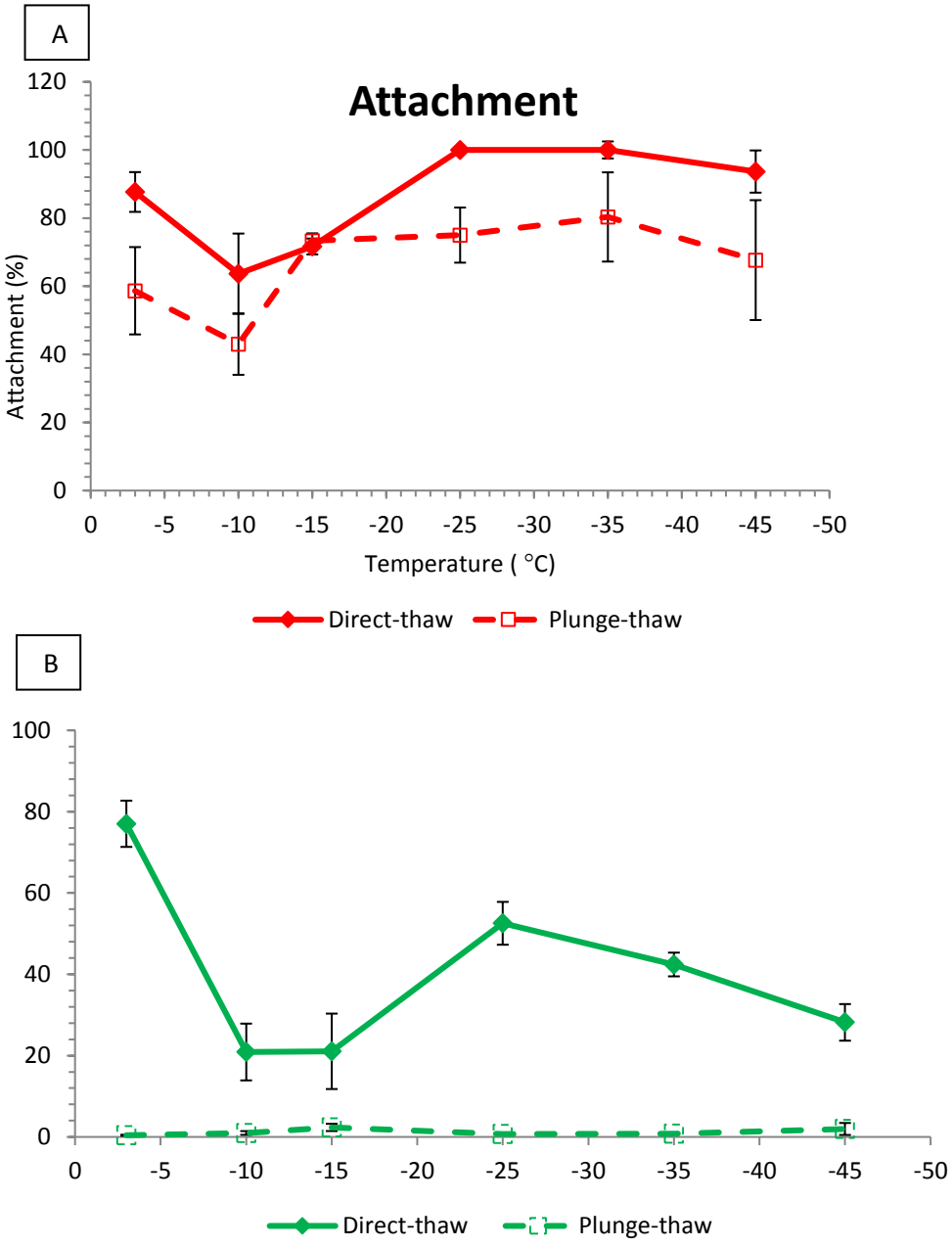


Figure 4.5: A) Cell attachment after graded freezing on glass coverslips for direct-thaw and plunge-thaw. B) Absolute cell viability in the absence of cryoprotectants (direct-thaw and plunge-thaw samples). Results are the average of three independent experiments. Data are presented as mean ± standard error of the mean (SEM).

4.3.2 Graded freezing in the presence of 10% DMSO on glass coverslips

In order to evaluate the effect of cryoprotectants on HUVEC viability, graded freezing was performed in the presence of 10% w/w DMSO in EGM-2 solution.

Controls: After 15 minutes incubation with the cryoprotectant solution, HUVECs were subjected to graded freezing (1°C/min). Viability of pre-experiments in the presence and absence of DMSO showed no significant difference (99.4 ± 0.2% and 99.4 ± 0.3% respectively). Post-experiment samples at the end of the experiment showed 99.0 ± 0.3% and 97.2 ± 1.0% viability in the absence and presence of 10% DMSO respectively. As expected, negative controls showed very low viability in the absence and presence of 10% DSMO, 0.4 ± 0.3% and 6.3 ± 2.1% respectively. However, viability in the presence of DMSO was higher ($p = 0.001$).

Direct-thaw (Figure 4.6): HUVECs showed maximum viability (87.4 ± 6.0%) after direct-thaw from –5 °C, and the viability reduced as temperature decreased to –10 °C (75.6 ± 3.2%) $p = 0.007$. No significant difference was observed between viability of HUVECs directly thawed from –10 °C and –15 °C. HUVECs showed higher viability (85.1 ± 2%) at –25 °C compared with –15 °C ($p = 0.04$). Further cooling to –45 °C led to loss of viability (63.3 ± 7.1%) compared with other temperatures ($p = 0.001$).

Plunge-thaw (Figure 4.6): Compared with direct– thaw samples, HUVEC viability dramatically reduced after plunge thaw at all intermediate temperatures. HUVECs showed maximum viability for the coverslips plunged into liquid nitrogen from –15 °C (11.4 ± 7.6%). HUVECs plunged from –45°C showed the lowest level of cell viability (4.1 ± 0.6%).

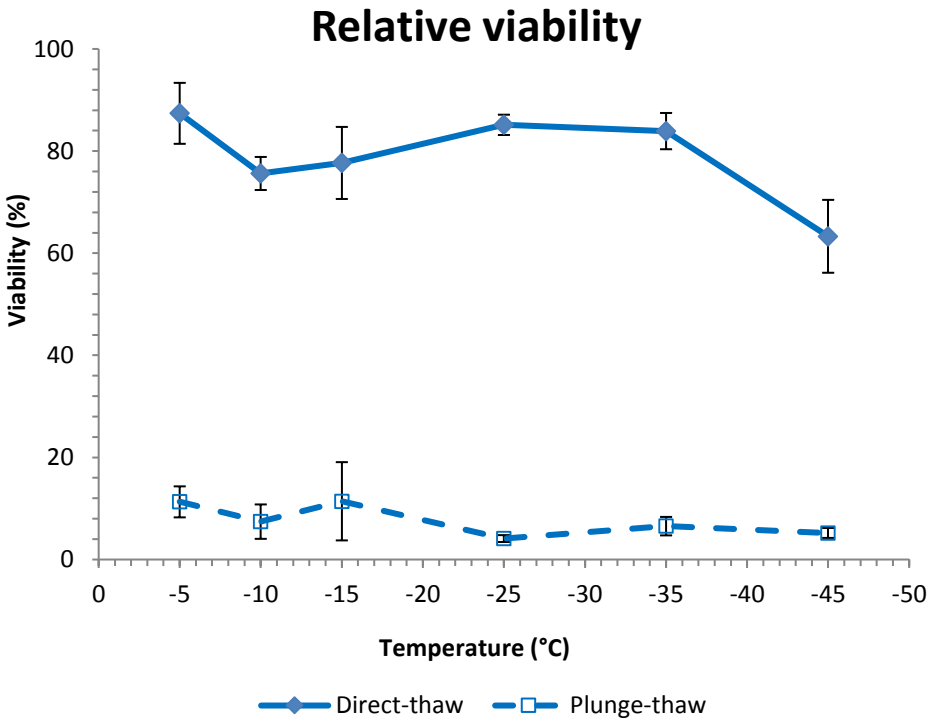


Figure 4.6: Relative cell viability in the presence of 10% DMSO on glass coverslips after graded freezing on glass coverslips (direct-thaw and plunge-thaw samples). Results are the average of three independent experiments. Data are presented as mean ± standard error of the mean (SEM).

Cell attachment assessment (Figure 4.7A): There were no significant differences for cell detachment from the coverslips thawed directly from all experimental temperatures. The same result was observed for plunge-thaw samples.

Figure 4.7 B shows absolute viability for all experimental temperatures for both direct and plunge thaw.

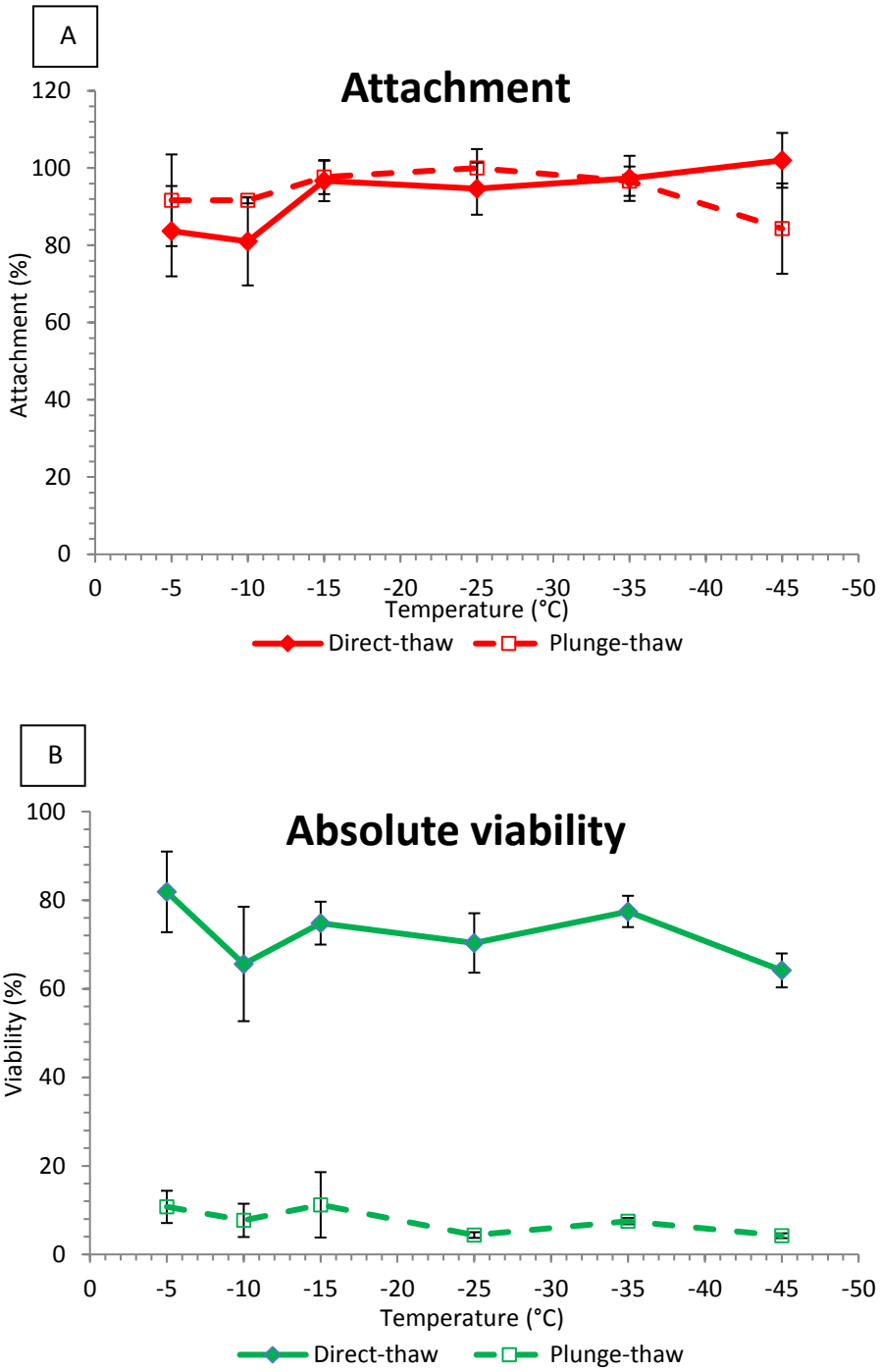


Figure 4.7: A) Cell attachment after graded freezing in the presence of 10% DMSO on glass coverslips (direct-thaw and plunge-thaw samples). B) Absolute viability after direct– thaw and plunge– thaw in the presence of 10% DMSO on glass coverslips. Results are the average of three independent experiments. Data are presented as mean ± standard error of the mean (SEM).

4.3.3 Graded freezing in the presence of 20% DMSO on glass coverslips

Controls: HUVECs on glass coverslips were exposed to 20% DMSO for 15 minutes. After 15 minutes incubation, pre-experiments in the absence and presence of 20% DMSO showed very high cell viability (99.1 ± 0.4% and 97.2 ± 0.7%, respectively). There was no statistically significant difference between viability of post-experiments compared with pre-experiments. Cells plunged into liquid nitrogen after 15 minutes incubation with DMSO showed 10.7% viability while in the absence of DMSO only 1.4% of cells were viable.

Direct-thaw (Figure 4.8): In the presence of 20% DMSO, ice was nucleated at –10 °C. HUVEC viability after direct thaw increased from –10 °C to –15 °C, 75.4 ± 7.2% to 84.8 ± 2.8% ($p = 0.005$) and it reached its maximum level for the coverslips thawed directly from –15 °C (84.8 ± 5.9%). No significant difference was observed between cell viability for HUVECs thawed directly from –25 °C and –35 °C. As temperature decreased to –45 °C, viability also decreased (59.6 ± 2.7%) compared with –35 °C ($p = 0.0001$) and viability was at the lowest after direct-thaw from –45 °C (59.6 ± 2.7%).

Plunge-thaw (Figure 4.8): Compared with direct thaw, HUVECs showed very low viability after plunge-thaw from each experimental temperature. No significant difference was observed after plunge-thaw except for cells plunged from –45 °C into liquid nitrogen, which showed the maximum viability (11.1 ± 3.7%) compared with the results for the rest of the experimental temperatures ($p = 0.001$).

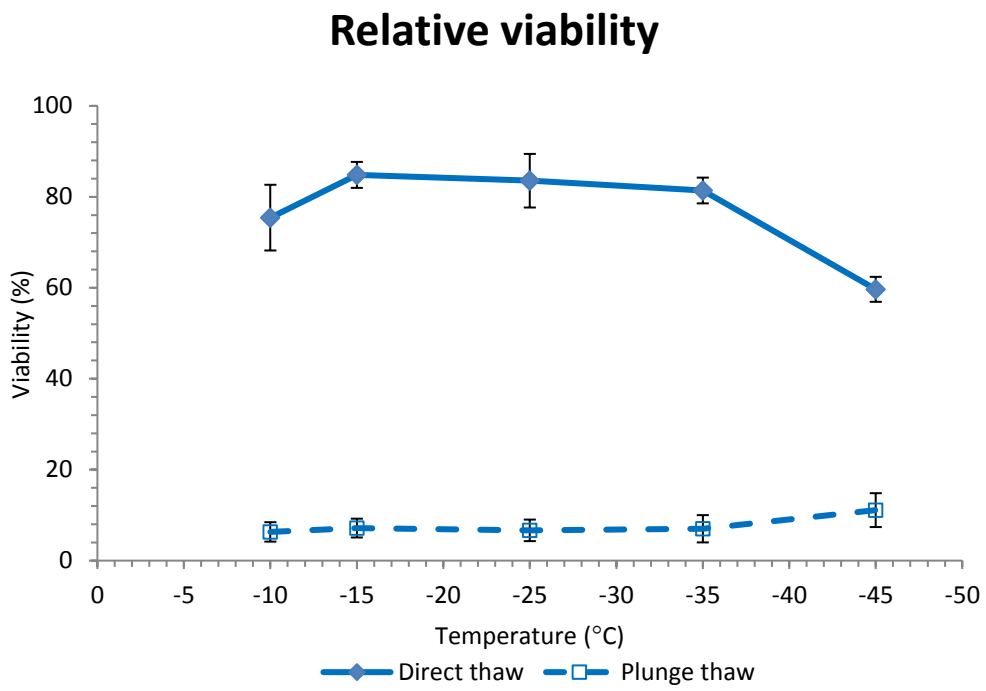


Figure 4.8: Relative viability after graded freezing in the presence of 20% DMSO on glass coverslips after direct-thaw and plunge-thaw. Results are the average of three independent experiments. Data are presented as mean ± standard error of the mean (SEM).

Cell attachment assessment (Figure 4.9 A): Graded freezing in the presence of 20% DMSO showed that after direct thaw and plunge thaw, there was very little cell detachment at all experimental temperatures. Both direct-thaw and plunge-thaw samples showed the same behavior at all the intermediate temperatures.

Absolute viability for both direct-thaw and plunge-thaw samples are presented in Figure 4.9 B.

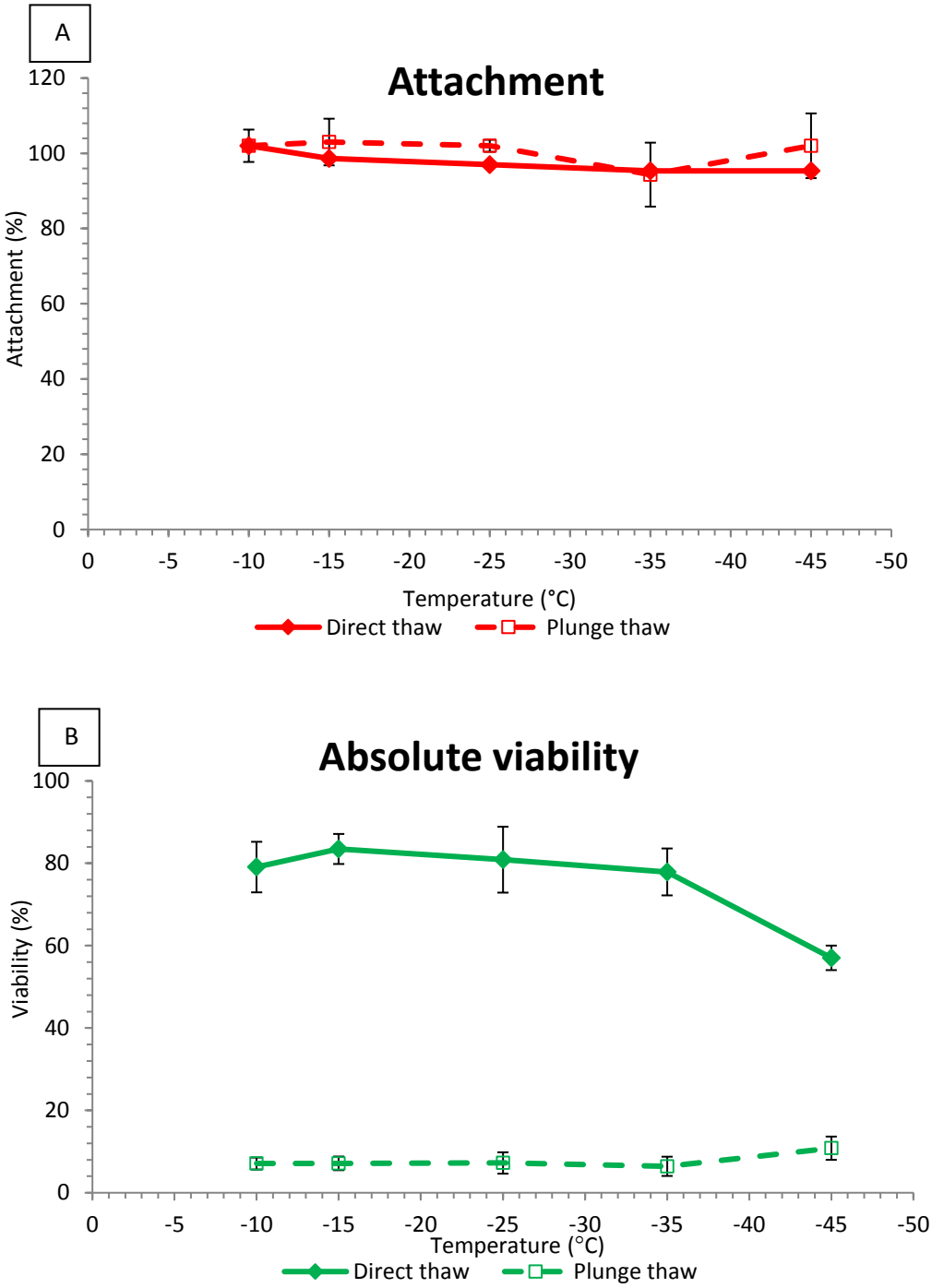


Figure 4.9: A) Cell attachment after graded freezing on glass coverslips in the presence of 20% DMSO (direct-thaw and plunge-thaw samples). B) Absolute viability in the presence of 20% DMSO after direct thaw and plunge thaw. Results are the average of three independent experiments. Data are presented as mean ± standard error of the mean (SEM).

4.3.4 Graded freezing in the presence of 5% DMSO and 6% HES on glass coverslips

In this experiment, ice was nucleated at –5 °C and cells were subjected to graded freezing (cooling rate: 1 °C/min).

Controls: Pre-experiments in the absence and presence of 5% DMSO and 6% HES showed very high viability: 99.2 ± 0.3% and 99.1 ± 0.1% respectively. A negative control sample showed loss of HUVEC viability in the absence and presence of CPA (0.9 ± 0.3% and 1.0 ± 0.3% respectively. Viability of post-experiments showed no difference compared with pre-experiments (98.6 ± 0.2% in the absence and 99.1 ± 0.3% in the presence of CPA).

Direct-thaw (Figure 4.10): Cells thawed directly showed very high viability at the higher subzero temperatures. There was no significant difference between viability of the cells after direct thaw from –5 °C to –25 °C. Further cooling to –35 °C and –45 °C was accompanied by loss of cell viability (90.6 ± 1.4% and 76.6 ± 5.0% respectively) compared with the higher experimental temperatures.

Plunge-thaw (Figure 4.10): Compared with direct-thaw samples, plunge-thaw samples showed very low cell recovery. The highest cell recovery was observed after plunge-thaw from –35 °C, (12.7 ± 2.7%) (Figure 4.10).

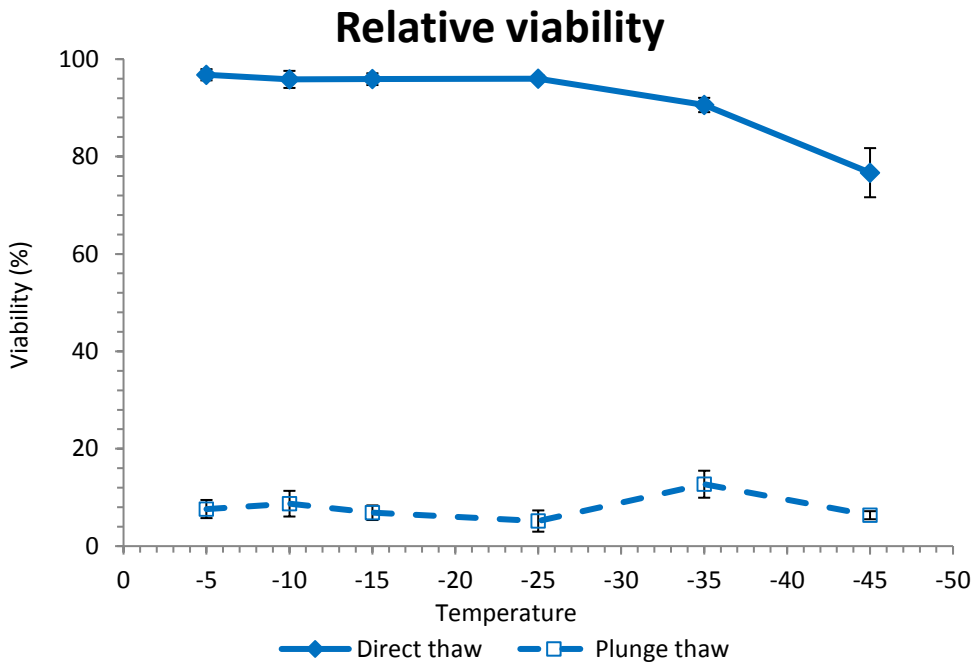


Figure 4.10: Relative viability after graded freezing in the presence of 5% DMSO and 6% HES on glass coverslips after direct thaw and plunge thaw. Results are the average of three independent experiments. Data are presented as mean ± standard error of the mean (SEM).

Cell attachment assessment (Figure 4.11 A): Plunge-thaw samples showed that the majority of cells were attached at all experimental temperatures. No significant difference was observed in viability of the cells for plunge-thaw samples. Direct-thaw samples showed high cell attachment, with no statistically significant difference for different experimental temperatures.

Absolute viabilities for both direct-thaw and plunge-thaw samples are presented in Figure 4.11 B.

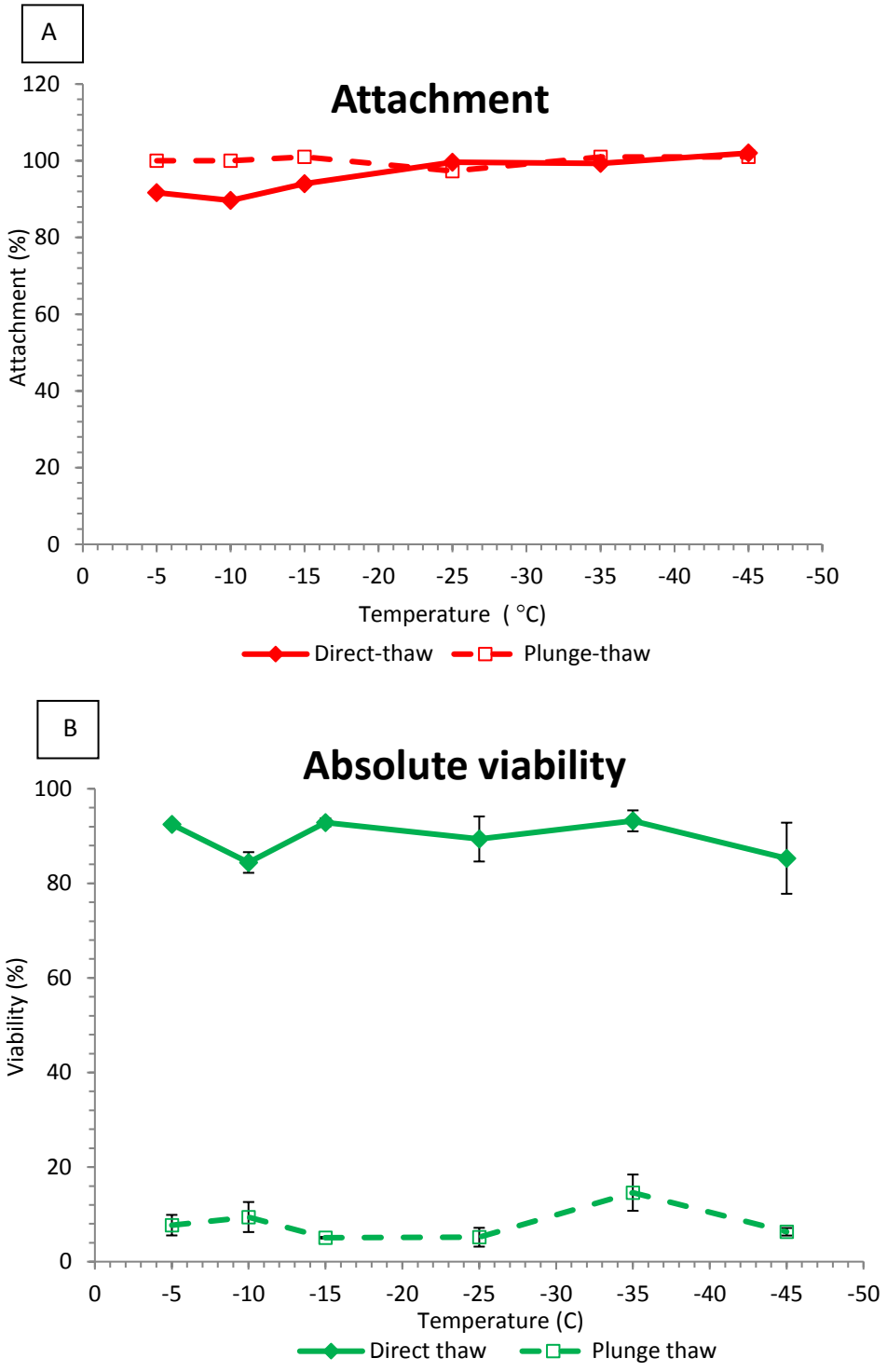


Figure 4.11: A) Cell attachment after graded freezing on glass coverslips in the presence of 5% DMSO and 6% HES for direct-thaw and plunge-thaw. B) Absolute viability after graded freezing in the presence of 5% DMSO and 6% HES (direct-thaw and plunge-thaw samples). Results are the average of three independent experiments. Data are presented as mean ± standard error of the mean (SEM).

4.4 Discussion—graded freezing on glass coverslips

4.4.1 Comparison of HUVEC absolute viability after graded freezing in the absence and presence of CPAs on glass coverslips

Comparison of direct-thaw absolute viability for HUVECs seeded on glass coverslips (Figure 4.12) showed that the presence of CPAs significantly increases cell viability compared with results in the absence of CPAs ($p = 0.001$). Directly thawed cells showed very high viability in the presence of 5% DMSO and 6% HES for all experimental temperatures. While the presence of DMSO increased cell viability compared to the results in the absence of CPAs, there was no statistically significant difference between cell viability for cells in the presence of 10% and 20% DMSO. It is therefore proposed that 20% DMSO is not more effective than 10% DMSO in preserving cell viability in directly thawed samples.

In contrast with direct-thaw samples, in the absence of cryoprotectants cell viability was very low after plunge-thaw for all experimental temperatures (Figure 4.13). The presence of 10% DMSO led to an increase in cell viability after plunge- thaw for all experimental temperatures compared with results in the absence of DMSO ($p = 0.001$) except at –45 °C. Incubation with 20% DMSO did not increase cell viability after plunge- thaw compared with 10% DMSO for all temperatures except at –45 °C ($p = 0.001$). The combination of DMSO and HES did not affect cell viability compared with 20% DMSO except for samples plunged into liquid nitrogen from –35 °C which showed higher viability compared to cells exposed to 20% DMSO ($p = 0.000$).

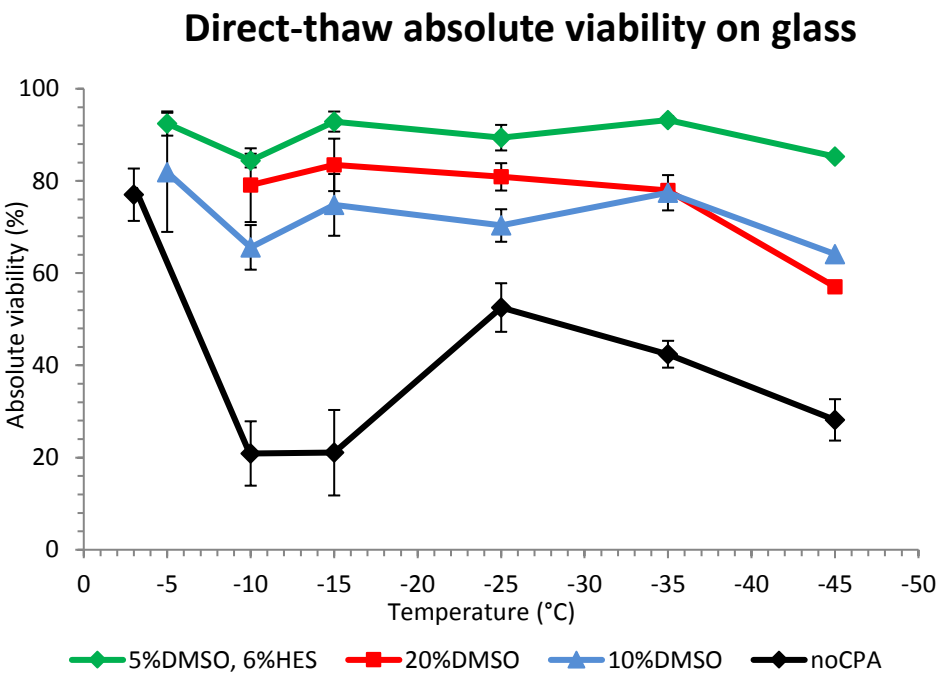


Figure 4.12: Comparison of absolute viability of monolayers on glass coverslips after direct-thaw in the absence or presence of cryoprotectants. Results are the average of three independent experiments. Data are presented as mean ± standard error of the mean (SEM).

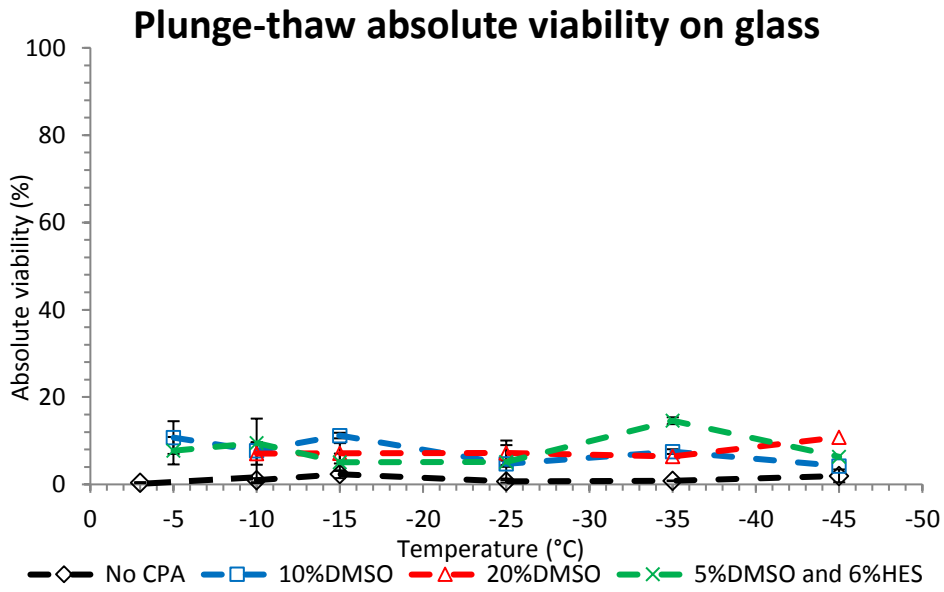


Figure 4.13: Comparison of absolute viability of monolayers on glass coverslips after plunge-thaw in the absence or presence of cryoprotectants. Results are the average of three independent experiments. Data are presented as mean ± standard error of the mean (SEM).

4.4.2 Analysis of response of HUVECs during direct- thaw and plunge- thaw on glass coverslips

Graded freezing on glass in the absence of CPA for direct-thaw samples showed that decreasing temperature from –3 °C to –15 °C led to a decrease in absolute cell viability (Figure 4.5 B). An increase in absolute cell viability was observed as temperature decreased to –25 °C. Further decreasing the temperature to –45 °C was accompanied by loss of absolute cell viability (Figure 4.5 B).

Direct- thaw from –3 °C showed minimal loss of absolute cell viability (Figure 4.5 B). In this study, ice was nucleated at –3 °C. During the freezing process, as temperature decreases, ice forms initially in the extracellular environment [18]. As temperature decreases from –3 °C to –15 °C, more ice forms in the extracellular environment. This ice formation results in increase of the solute concentration in the extracellular environment. The increase in solute concentration causes osmotic efflux of water from the cells. This efflux of water leads to cell dehydration and decrease in cell volume [10]. Therefore, cell volume decreases and a lower amount of water is available in cells at –15 °C compared with –3 °C. In comparison with the study by Acker *et al.* which showed 100% formation of intracellular ice in a MDCK monolayer at temperatures lower than –3 °C (cooling rate 25 °C/min, which produces a high degree of super cooling), in these experiments during cooling from –3 °C to –15 °C, intracellular ice is less likely to be formed due to the low cooling rate [19]. Cooling rate is a primary factor that affects intracellular ice formation [20]. In this study, the cooling rate was 1 °C/min, which is considered to be slow cooling.

One of the main challenges in monolayer cryopreservation is preserving cell attachment to the surface. As described earlier, the absolute viability is the ratio of the average total number of cells on a coverslip to the average total number of cells on a pre-experiment coverslip. One

possible explanation for the decrease in the absolute viability of direct-thaw samples from –3 °C to –15 °C is partly due to cell detachment (87.6 ± 5.8 % at –3 °C vs 71.6 ± 2.3 % at –15 °C) (Figure 4.5A). A study by Ebertz *et al.* also showed cell detachment in monolayer cryopreservation of human corneal endothelial cells [21].

In the second section of Figure 4.5 B, from –15 °C to –25 °C, an increase in absolute cell viability was observed. This higher absolute viability compared with the first section, can be due to lower detachment from the coverslips.

In the third section, as temperature decreased from –25 °C to –45 °C, cells showed a reduction in absolute cell viability (Figure 4.5 B). The high concentration of solutes in both the intra- and extra-cellular environments can be a factor [25], and mechanical stress due to the presence of extracellular ice may be another factor, which affects cell viability [24].

The difference between responses of the cells in monolayer and suspension may be due to cell–cell and cell–surface interactions [4]. A study by Acker *et al.* showed that on cooling at a specific rate, the incidence of intracellular ice formation in hamster fibroblasts was higher in a colony attached to the substrate compared with cells in suspension, due to the presence of cell–cell and cell–surface interactions [4]. Cell–cell interaction is one of the factors that can lead to ice propagation during a freezing process. As discussed earlier, two theories are present for ice propagation in adjacent cells: Surface-catalyzed nucleation [23] or the presence of gap junctions, which facilitate ice propagation to adjacent cells in a monolayer [19].

For all experimental temperatures, the maximum damage occurred when cells were plunged into liquid nitrogen, which showed itself as a dramatic loss of absolute cell viability. Plunging into liquid nitrogen is detrimental for all experimental temperatures. This result is due to rapid

cooling, which at every step is accompanied by intracellular ice formation. A study by Acker *et al.* showed that intracellular ice formation in a monolayer can confer protection [22]. However, in this project plunging into liquid nitrogen results in rapid cooling (high cooling rate). A likely explanation for low viability is that damaging intracellular ice formed during the plunge step. Moreover, mechanical stress can be another factor for this excessive damage. As cells are attached, their movement is limited. Ice propagation in the extracellular environment also causes mechanical stress and cell deformation [24].

In this thesis and an earlier report [24], it is proposed that this mechanical stress arises from the difference in the coefficient of thermal expansion between the surface and extracellular ice. Extracellular ice and the substrate both contract and expand during cooling and warming respectively, which may cause structural stresses to the cells leading to cell damage and consequently cell death. The presence of a cryoprotectant (10% DMSO) led to an increase in absolute cell viability for all experimental temperatures for direct-thaw samples. This protection can be explained by replacing part of the intracellular water and reducing the freezing point of the cytoplasm progressively as ice forms outside the cell and solutes (including DMSO) concentrate. Although, absolute viability was higher for all experimental temperatures compared with cells in the absence of cryoprotectants ($p = 0.001$), this protection was not enough for cells plunged into liquid nitrogen and still cells showed very low viability.

Increasing the concentration of DMSO to 20% w/w did not have a beneficial effect in terms of HUVEC viability after plunge-thaw. The presence of penetrating and non-penetrating cryoprotectants compared with other experimental conditions increased cell viability for all direct– thaw samples. However, the combination of DMSO and HES was not successful for monolayer cryopreservation of HUVECs on glass coverslips. In conclusion, plunge-thaw

samples showed very low viability on glass coverslips in the absence and presence of cryoprotectants. The next study is the result of HUVEC graded freezing on Rinzl plastic coverslips in the absence and presence of cryoprotectants.

4.5 Results for Rinzl coverslips

4.5.1 Graded freezing in the absence of CPAs on Rinzl coverslips

In order to assess the effect of substrate on viability and attachment of HUVECs, graded freezing (cooling rate: 1°C/min) was also performed for HUVECs seeded on Rinzl coverslips as described for glass coverslips. Results are the average of three independent experiments and data are presented as mean ± standard error of the mean (SEM).

Controls: Pre-experiment controls showed that HUVECs were viable after 15 minutes incubation on ice (98.31 ± 0.6%). Plunging into liquid nitrogen led to low cell viability (1.1 ± 0.6 %). Post-experiment controls showed the same viability as pre-experiment controls (98.3 ± 0.2 %). There was no significant difference in HUVEC viability between pre and post-experiments controls.

Direct-thaw (Figure 4.14): In the absence of cryoprotectants, decreasing temperature from –3°C to –15 °C led to loss of viability, 83.6 ± 5.3% and 36.8 ± 7.8% respectively ($p = 0.02$).

This dramatic decrease was accompanied by an increase in viability when cells were directly thawed from –25 °C (60.5 ± 6.8%) ($p = 0.001$) compared with –15 °C and –10 °C. Further cooling to –35 °C (48.1 ± 7.6%) and –45°C (23.8 ± 11.2%) resulted in decrease of cell viability compared with samples thawed directly from –25 °C (60.5 ± 6.8%), $p = 0.001$ (Figure 4.14).

Plunge-thaw (Figure 4.14): Comparison of viability for plunge-thaw samples showed that there were significant differences in the viability of the cells between different groups (experimental

temperatures). Decreasing temperature from –10 °C to –15 °C led to a considerable increase in cell viability (p = 0.00). HUVECs showed no significant difference for viability after plunging into liquid nitrogen from –25°C compared with –15 °C (p = 0.95). Further cooling resulted in loss of cell viability for samples plunged into liquid nitrogen from –35 °C (31.8 ± 13.0%) and –45 °C (14.8 ± 0.6%).

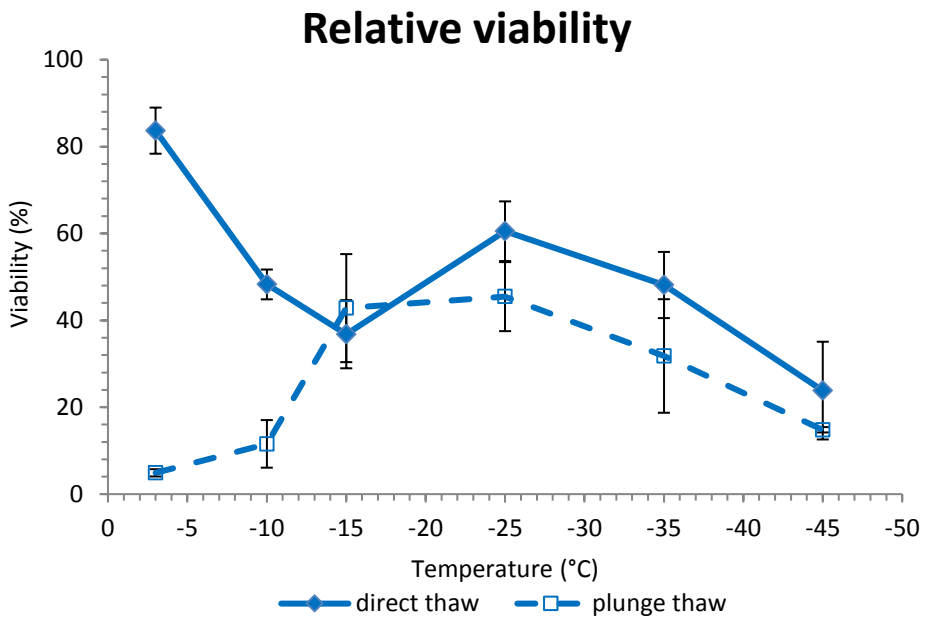


Figure 4.14: Relative viability after graded freezing in the presence of 5% DMSO and 6% HES on Rinzl coverslips after direct-thaw and plunge-thaw. Results are the average of three independent experiments. Data are presented as mean ± standard error of the mean (SEM).

Cell attachment assessment (Figure 4.15 A): The attachment assessment showed cell detachment for plunge–thaw and direct–thaw samples was not significantly different for all experimental temperatures, except at –10 °C for which direct-thaw samples showed less cell detachment compared with plunge-thaw samples. Absolute viability for both direct-thaw and plunge-thaw samples are presented in Figure 4.15 B.

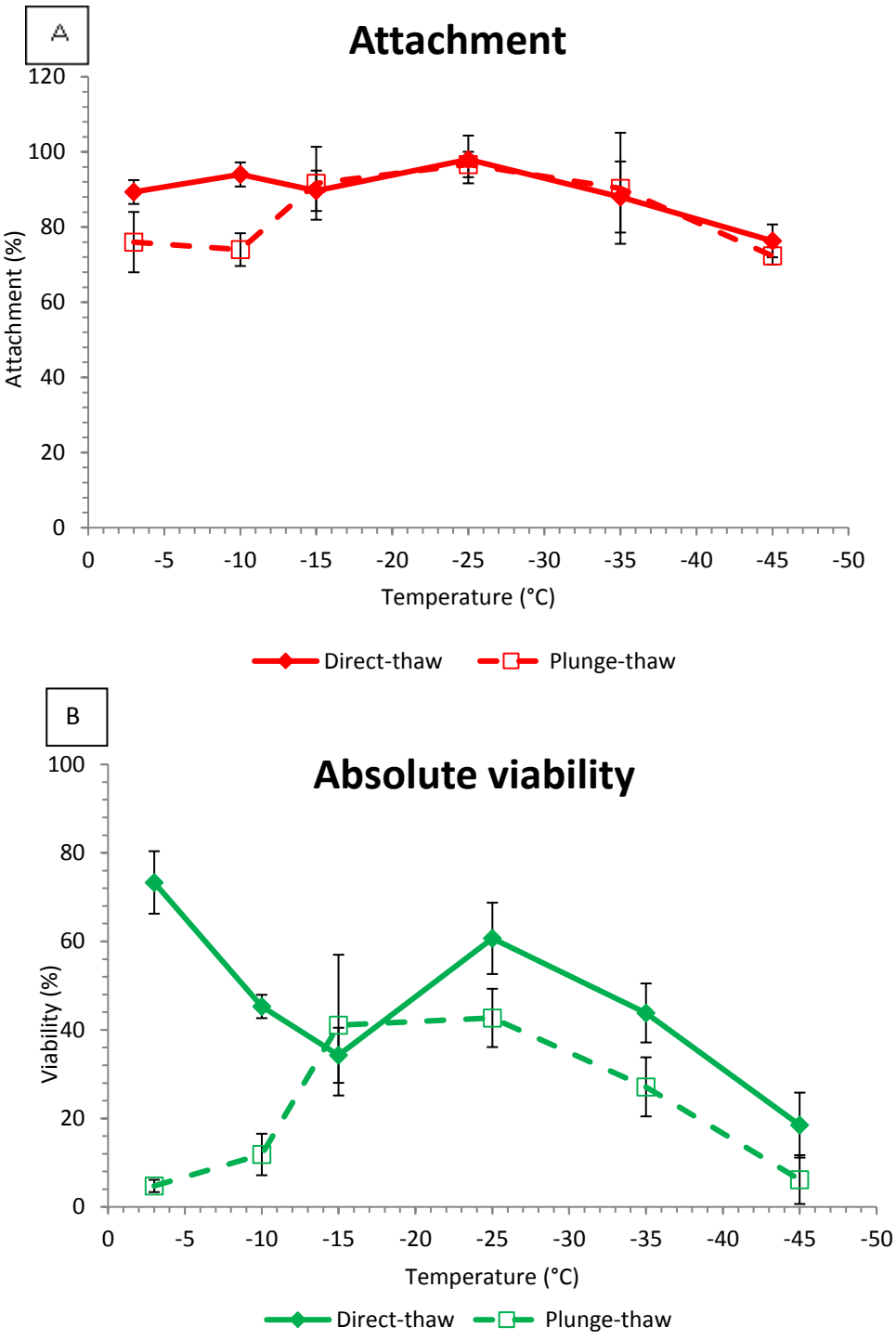


Figure 4.15: A) Cell attachment after direct- thaw and plunge- thaw in the absence of CPA on Rinzl coverslips. B) Absolute viability after graded freezing in the absence of CPA on Rinzl coverslips (direct- thaw and plunge- thaw samples). Results are the average of three independent experiments. Data are presented as mean ± standard error of the mean (SEM).

4.5.2 Graded freezing in the presence of 10% DMSO on Rinzl coverslips

In order to assess the effect of cryoprotectants on HUVEC viability on Rinzl coverslips, cells were subjected to graded freezing in the presence of cryoprotectants (cooling rate: 1 °C/min).

Controls: Incubation of pre-experiment controls on ice in the absence and presence of 10% DMSO for 15 minutes showed high viability of HUVECs (99.1 ± 0.3% and 97.7 ± 0.6% respectively). Post-experiment controls also showed high viability at the end of the experiments.

Direct-thaw (Figure 4.16): There was no statistically significant difference between cell viability after direct thaw from different experimental temperatures.

Plunge-thaw (Figure 4.16): Viability assessment after plunge thaw showed an increase in viability of the cells as temperature decreased from –5 °C (25.3 ± 5.7%) to –15 °C (46.0 ± 1%) ($p = 0.001$). The maximum level of viability was observed for a sample directly thawed from –15 °C (46.0 ± 15%). There was no statistically significant difference between cell viability after plunge thaw from –15 °C to –45 °C.

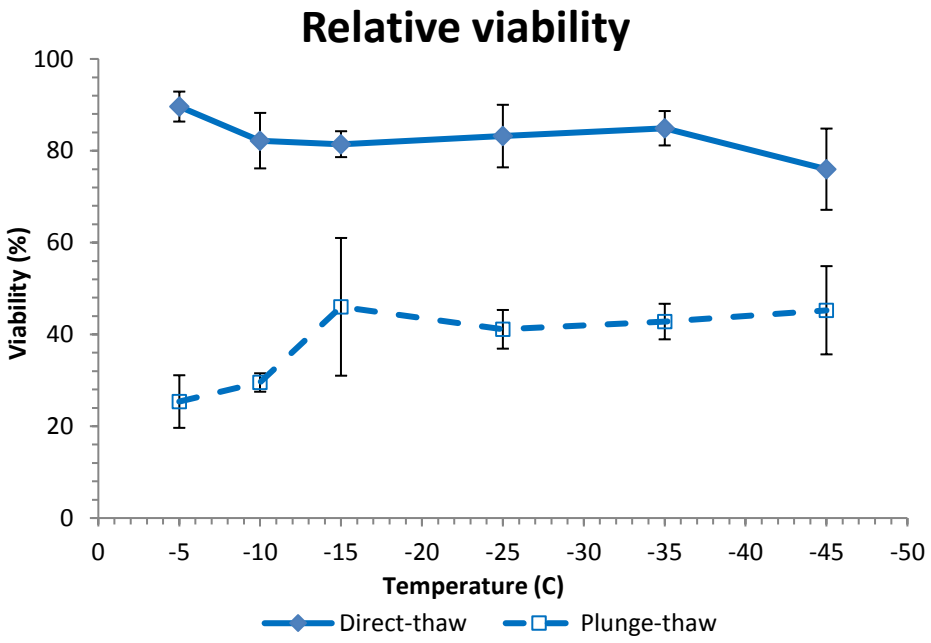
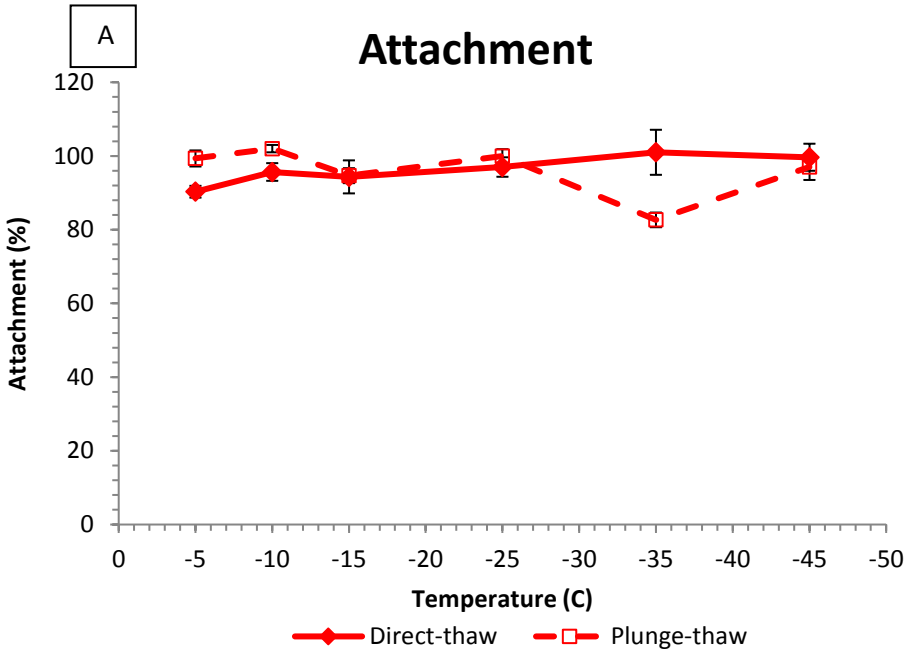


Figure 4.16: Relative viability after graded freezing in the presence of 10% DMSO on Rinzl coverslips (direct-thaw and plunge-thaw samples). Results are the average of three independent experiments. Data are presented as mean ± standard error of the mean (SEM).

Cell attachment assessment (Figure 4.17 A): No significant difference was observed between cell detachment for direct-thaw and plunge-thaw samples for all experimental temperatures except for –35 °C for which the sample plunged into liquid nitrogen showed less attachment compared with the corresponding direct-thaw sample. Absolute viabilities for both direct-thaw and plunge-thaw samples are presented in Figure 4.17 B.



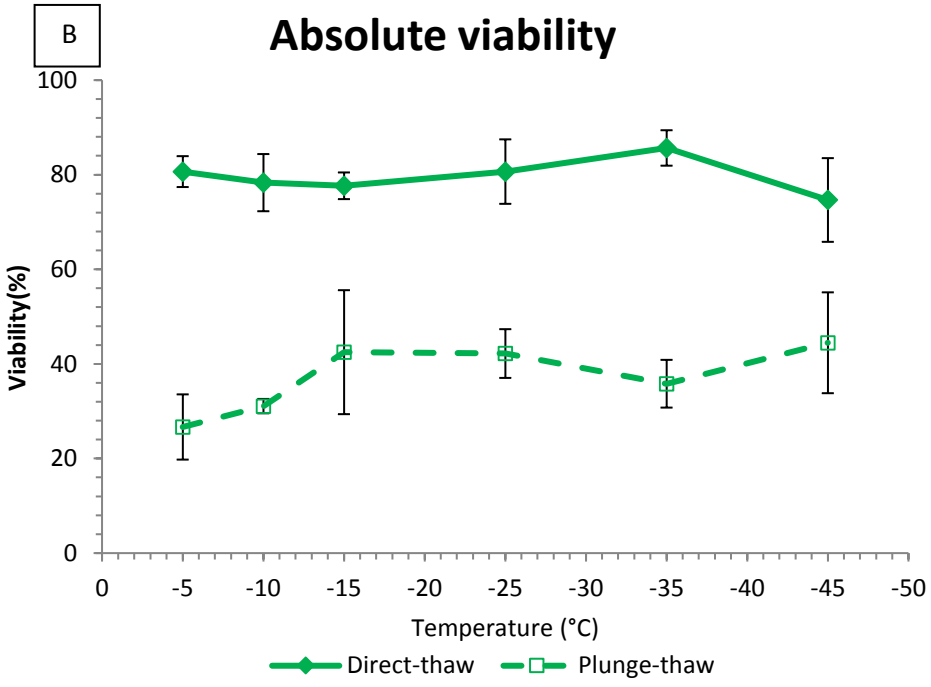


Figure 4.17: A) Cell attachment after direct thaw and plunge thaw in the presence of 10% DMSO on Rinzl coverslips. B) Absolute viability after graded freezing in the presence of 10% DMSO on Rinzl coverslips (direct-thaw and plunge-thaw samples). Results are the average of three independent experiments. Data are present as mean ± standard error of the mean (SEM).

4.5.3 Graded freezing in the presence of 20% DMSO on Rinzl copverslips

Controls: The viability of pre-experiment controls in the presence and absence of 20% DMSO was not significantly different and both samples showed high cell viability. Post-experiment controls also showed very high viability at the end of the experiment (98.9 ± 0.2% in the absence and 96.8 ± 1.4% in the presence of CPA).

Direct-thaw (Figure 4.18): For direct thaw samples, graded freezing experiments showed that there was no significant difference between cell viability as temperature decreased from –10 °C to –25 °C. However, further cooling resulted in decreasing cell viability for samples directly thawed from –35 °C (67.5 ± 6.6%) ($p = 0.001$) and –45 °C (57.4 ± 6.2%) ($p = 0.009$) compared with the rest of the temperatures

Plunge-thaw (Figure 4.18): Samples showed an increase in cell viability from –10 °C (10.9 ± 2.9%) to –45 °C (49.7 ± 11.2%). Negative controls showed different responses. In the presence of 20% DMSO, viability was higher (19.4 ± 4.6%) compared with cells in the absence of DMSO (3.1 ± 2.9%)

Cell attachment assessment (Figure 4.19 A): No significant difference was observed for cell attachment for all of the experimental temperatures for both direct thaw and plunge thaw samples (Figure 4.19 A). Absolute viabilities for both direct-thaw and plunge-thaw samples are presented in Figure 4.19 B.

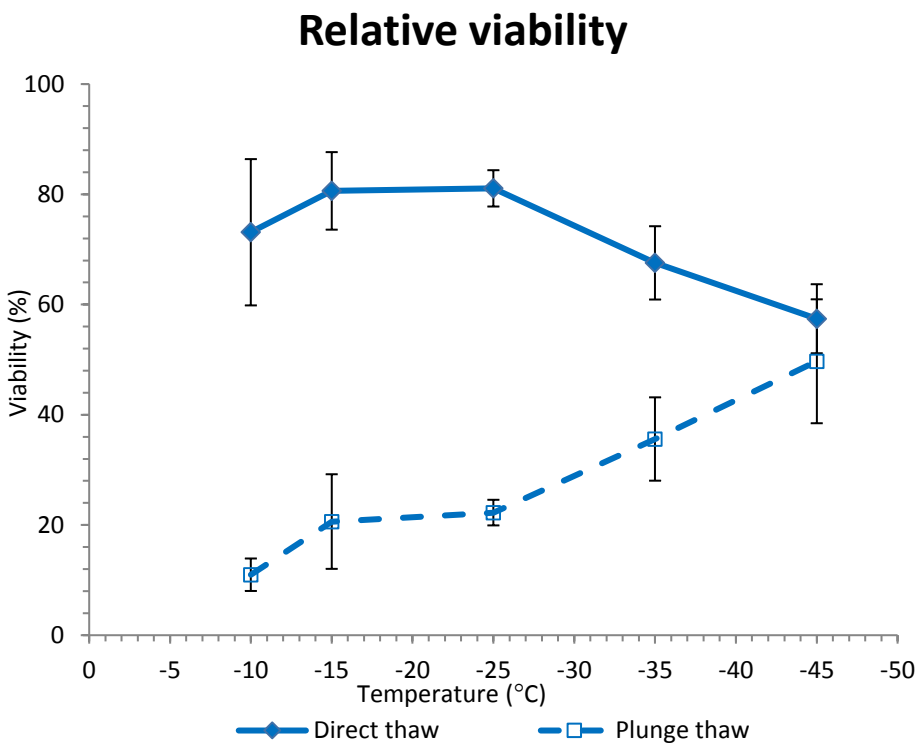


Figure 4.18: Relative viability after graded freezing in the presence of 20% DMSO on Rinzl coverslips (direct-thaw and plunge-thaw samples). Results are the average of three independent experiments. Data are presented as mean ± standard error of the mean (SEM).

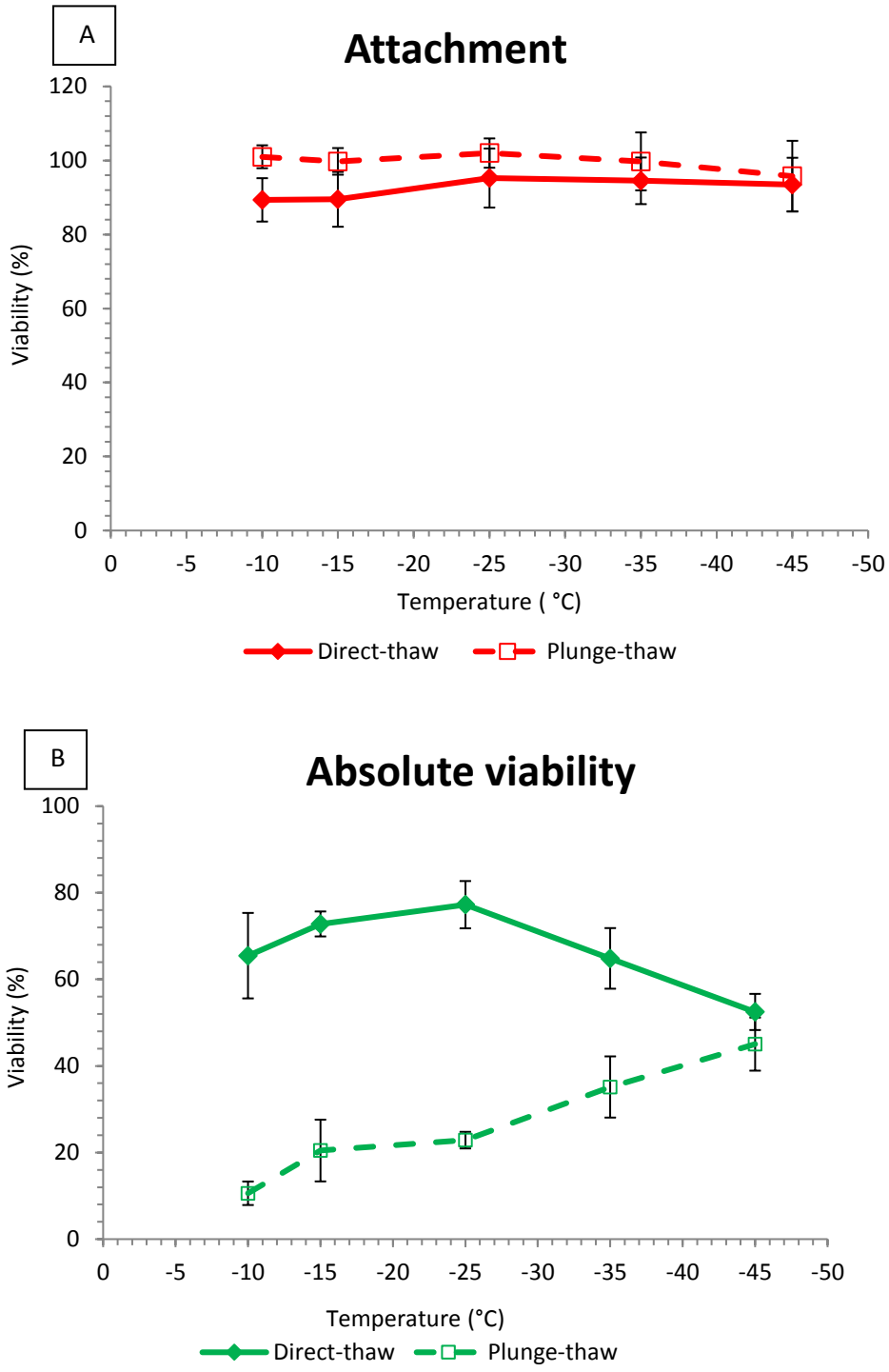


Figure 4.19: A) Cell attachment after direct thaw and plunge thaw in the presence of 20% DMSO on Rinzl coverslips. B) Absolute viability after graded freezing in the presence of 20% DMSO on Rinzl coverslips (direct-thaw and plunge-thaw samples). Results are the average of three independent experiments. Data are presented as mean ± standard error of the mean (SEM).

4.5.4 Graded freezing in the presence of 5% DMSO and 6% HES on Rinzl coverslips

The response of cells to graded freezing in the presence of 5% DMSO and 6% HES was different from other experiments both for direct- thaw and plunge- thaw.

Controls: No significant difference was observed for viability of the cells between pre- and post-experiment controls in the absence or presence of 5% DMSO and 6% HES.

Direct-thaw (Figure 4.20): For all experimental temperatures, cells showed high recovery after direct thaw (maximum viability: 95.4 ± 0.9% and minimum viability: 87.7 ± 5.1%). No significant difference was observed for cell viability among experimental temperatures.

Plunge-thaw (Figure 4.20): After plunging into liquid nitrogen from –5 °C only 60 ± 19.3% of cells were viable. Further cooling to –10 °C led to loss of viability (44.3 ± 13.9%) compared with the cells plunged from –5 °C. There was a loss of cell viability from –5 °C to –10 °C after plunge-thaw followed by an increase in cell viability for cells plunged into liquid nitrogen from –15°C ($p = 0.001$) and –25 °C ($p = 0.004$). Maximum recovery was observed after plunging into liquid nitrogen from –45 °C (88.6 ± 9.9%).

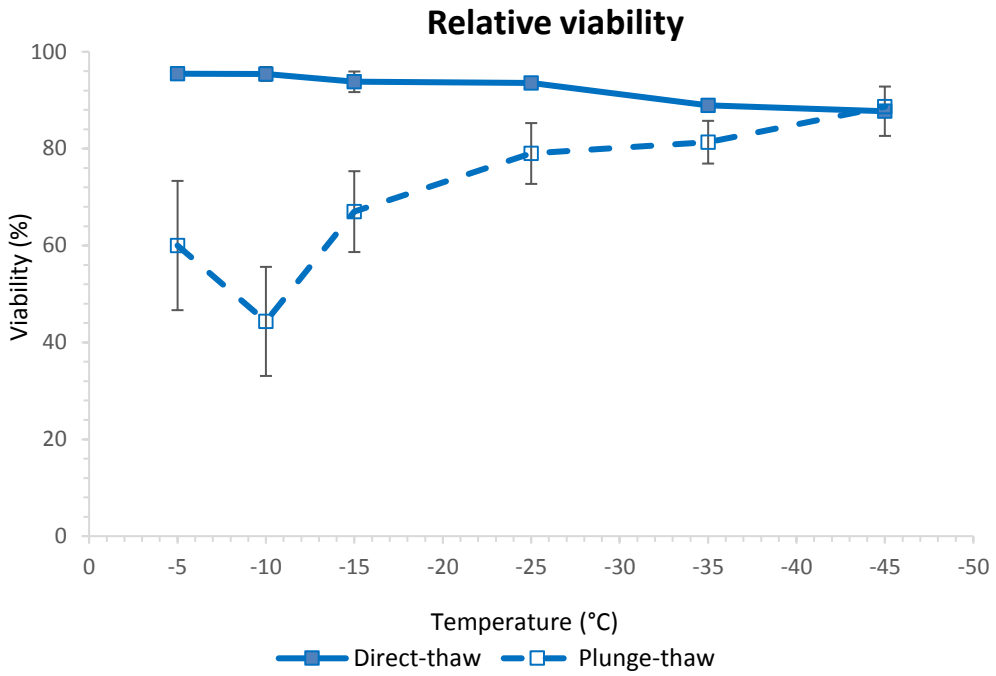


Figure 4.20: Relative viability after graded freezing in the presence of 5% DMSO and 6% HES on Rinzl coverslips (direct-thaw and plunge-thaw samples). Results are the average of three independent experiments. Data are present as mean ± standard error of the mean (SEM).

Cell attachment assessment (Figure 4.21 A): For direct-thaw samples, cells showed the same level of attachment except for direct- thaw from –10°C for which cells showed less attachment compared with the rest of the experimental temperatures. In addition to the rest of the direct-thaw samples, which could preserve their attachment to the surface, plunge-thaw samples showed that freezing did not affect cell attachment and all of the cells were attached to the surface (Figure 4.21 A).

Absolute viabilities for both direct-thaw and plunge-thaw samples are presented in Figure 4.21 B.

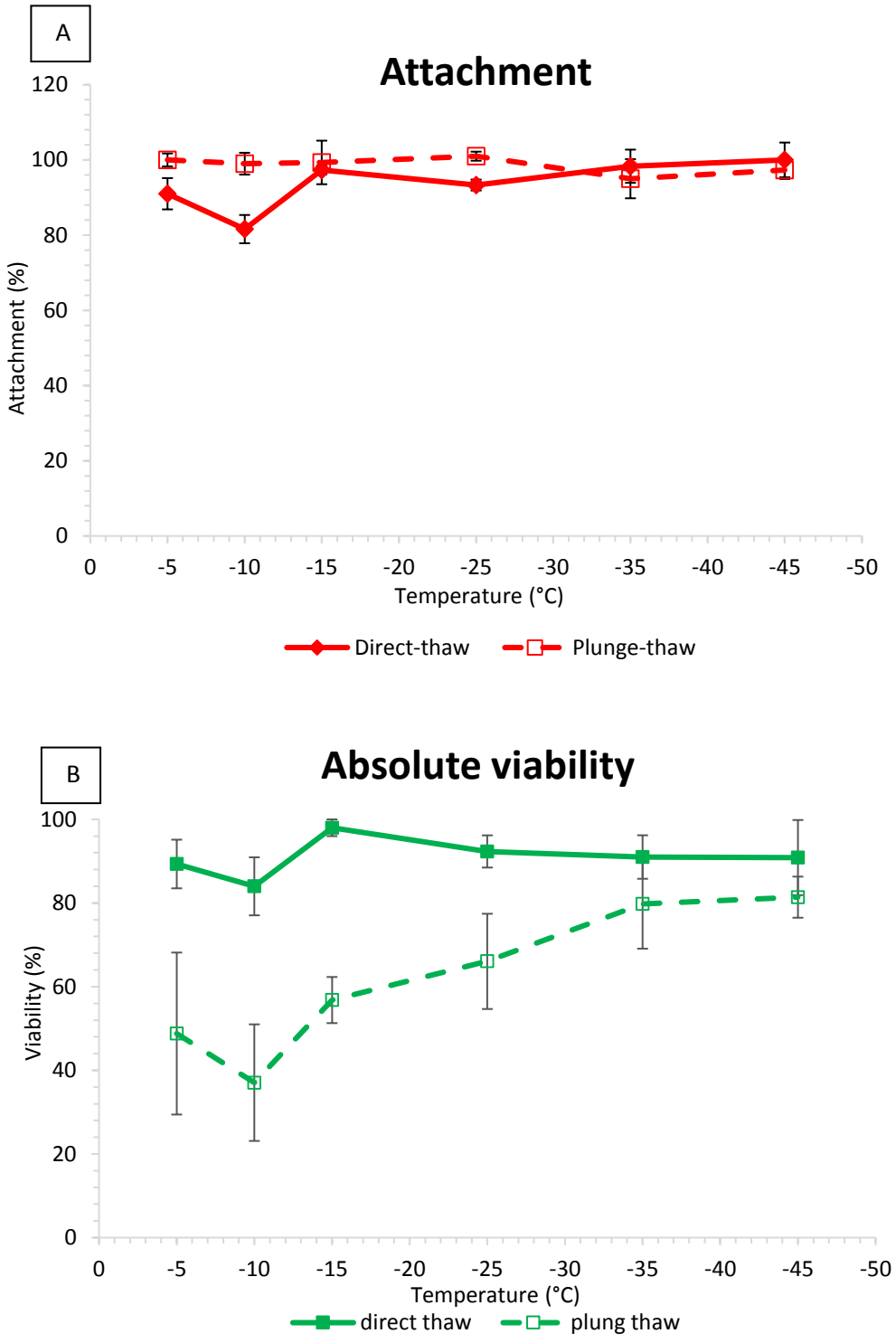


Figure 4.21: A) Cell attachment after direct thaw and plunge thaw in the presence of 5% DMSO and 6% HES on Rinzl coverslips. B) Absolute viability after graded freezing in the presence of 5% DMSO and 6% HES on Rinzl coverslips (direct-thaw and plunge-thaw samples). Results are the average of three independent experiments. Data are presented as mean ± standard error of the mean (SEM).

4.5.5 Discussion —graded freezing on Rinzl coverslips

4.5.5.1 Comparison of absolute viability for HUVECs on Rinzl cover slips in the absence and presence of CPAs after direct- thaw

Comparison of absolute viabilities for direct-thaw samples showed that the presence of cryoprotectants does affect cell viability compared with the cells in the absence of CPA. This difference was particularly noticeable in the presence of 5% DMSO and 6% HES with the highest viability (90.8 ± 10.2%).

The presence of 10% DMSO increased cell viability at all experimental temperatures compared with results in the absence of DMSO ($p = 0.001$). Increasing the concentration of DMSO led to an increase in the viability of the cells compared with the results in the absence of DMSO. However, increasing the DMSO concentration to 20% did not affect cell viability compared with the results for cells exposed to 10% DMSO except at –35 °C ($p = 0.001$) and –45 °C ($p = 0.001$) (Figure 4.22 A).

For all experimental temperatures, the presence of 5% DMSO and 6% HES in a cryoprotectant solution led to an increase in cell viability compared with the cells in the absence of CPA. In addition to direct- thaw, the presence of DMSO and HES led to an increase in cell viability for plunge-thaw samples with the highest absolute viability at –45 °C (81.4 ± 0.3%). The results of the graded freezing experiments showed that a substrate is one of the factors which affect cell viability for monolayer freezing (Figure 4.22 B).

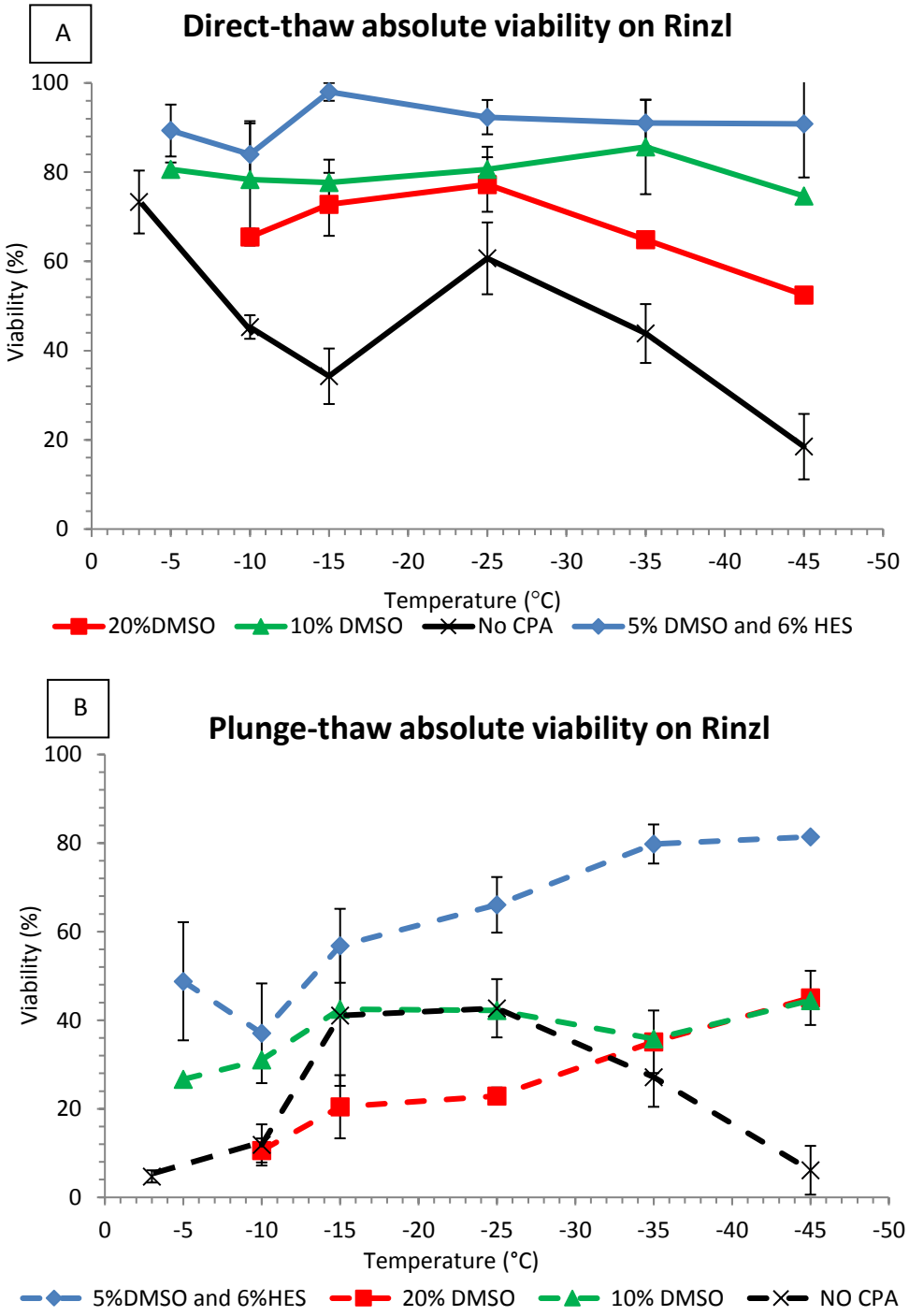


Figure 4.22: A) Comparison of absolute viability of monolayers on Rinzl after direct-thaw in the absence and presence of cryoprotectants. B) Comparison of absolute viability of monolayers on Rinzl after plunge- thaw in the absence and presence of cryoprotectants on Rinzl coverslips. Results are the average of three independent experiments. Data are presented as mean ± standard error of the mean (SEM).

4.5.5.2 Comparison of the maximum absolute viability for HUVECs on glass and Rinzl coverslips after plunge- thaw

The maximum absolute viability on Rinzl coverslips was achieved in the presence of 5% DMSO and 6% HES. Rinzl increased cell viability to 81.4 ± 0.3% for the cells plunged into liquid nitrogen from –45 °C (Figure 4.23). On glass coverslip the maximum absolute viability was 14.5 ± 3.8% for cells plunged from –35 °C into liquid nitrogen in the presence of 5% DMSO and 6% HES (Figure 4.23). In the presence of 5% DMSO and 6% HES, HUVECs showed a high level of attachment on both glass and Rinzl coverslips.

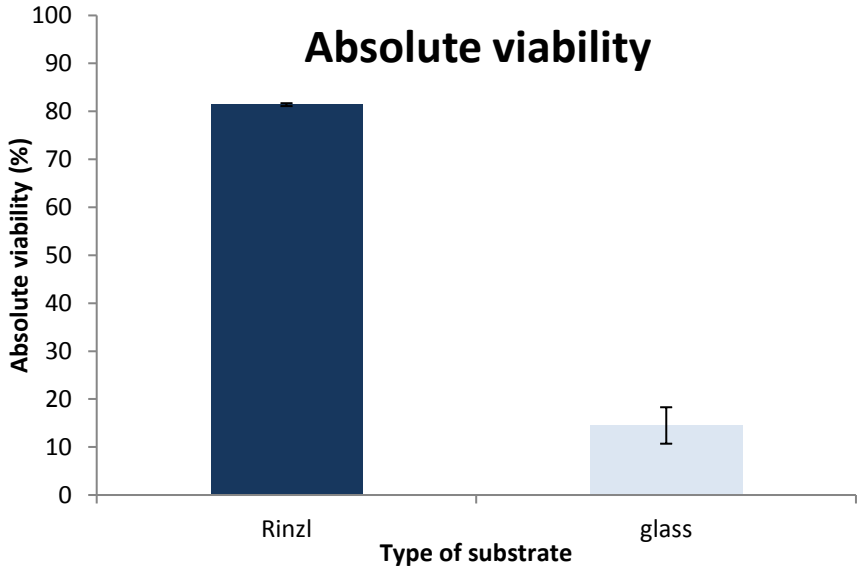


Figure 4.23: Comparison of the maximum absolute viability for HUVECs after plunge- thaw in the presence of 5% DMSO and 6% HES on Rinzl and glass coverslips

4.5.5.3 Analysis of graded freezing for HUVECs on Rinzl coverslips

Graded freezing for HUVECs on Rinzl coverslips showed that Rinzl coverslips could provide better protection to cells during freezing and thawing processes compared with glass. In the absence of CPA, HUVECs on Rinzl coverslips showed the same behavior for direct- thaw as they did on glass coverslips for direct- thaw. However, HUVEC behavior after plunge- thaw was distinguishable from cells on glass coverslips.

In contrast with cells on glass coverslips, which showed very low viability as temperature decreased, with Rinzl coverslips the absolute viability after plunge thaw increased to a maximum HUVEC viability of 42.7 ± 6.6% at –25 °C. It is noticeable that this level of viability was achieved in the absence of cryoprotectants, which is much higher compared with glass coverslips (maximum HUVEC viability 2.3 ± 0.9% at –15 °C). One explanation can be the lower difference between the coefficient of thermal expansion of ice and Rinzl. This may contribute to the increase in cell viability as temperature decreased from –3 to –25 °C (this increase in viability did not occur for glass coverslips). This is the first report for assessment of the effect of Rinzl plastic coverslip on cell viability and attachment of HUVECs in monolayers. The exact mechanism is unclear. Based on these results, it is proposed that this substrate may reduce the tension due to mismatch of contraction of ice and substrate during freezing as temperature decreases to –35 °C. This property of Rinzl arises from its coefficient of thermal expansion, which is close to that of ice (discussed in Chapter 3).

One of the critical factors for cell survival in monolayers is the cell's interaction with the extracellular matrix, which is facilitated by their cytoskeleton. Anchorage-dependent cells such as HUVECs need attachment to a surface for survival and growth. As discussed in Chapter 3, F-actin is an essential component in the cytoskeleton and it is located just beneath the plasma

membrane. Damage due to ice formation, stretch or contraction of substrate and extracellular ice formation affects F-actin organization and consequently cell viability [18],[21]. Further studies will help to explain how interactions between cells and the substrate can affect intracellular ice formation, propagation, shape and size of the ice crystals. Future studies for assessment of F-actin organization and depolymerization will help to understand how Rinzl can minimize the damage to the plasma membrane and cytoskeleton. The best results for cryopreservation of HUVEC monolayers was achieved in the presence of 5% DMSO and 6% HES after plunge from –45°C with the absolute viability of 81.4 ± 0.3 %. However, it needs to be noted that this chapter reported viabilities immediately after thaw before cryoprotectant removal. The next chapter will discuss the viability after cryoprotectant removal.

4.6 References

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Chapter 5: Further optimization (cryoprotectant removal, cryoprotectant solution and cooling rate)

5.1 Introduction

In the previous chapter, HUVEC monolayers showed the highest viability after cooling at 1 °C/min to –45 °C and then plunging into liquid nitrogen in the presence of 5% DMSO and 6% HES on Rinzl coverslips. The viability was assessed immediately after thaw which was before removal of the cryoprotectants. In this chapter, HUVECs were subjected to slow cooling (1 °C/min) on Rinzl coverslips and viability was assessed for samples plunged into liquid nitrogen from –45 °C as in the previous chapter, however here viability was assessed after CPA removal. Since removal of CPA can cause osmotic damage to cells, CPA was removed by serial dilution. In addition, the effect on viability of other factors was also assessed. Four factors hypothesized to improve post-CPA-removal viability were investigated: *i*) preparing the CPA solution in CryoStor instead of standard growth medium, *ii*) including chondroitin sulfate in the CPA solution, *iii*) reducing the cooling rate, and *iv*) removing the CPA 24 hours post-thaw.

5.2 Viability after CPA removal by serial dilution

Although the presence of CPAs is essential to increase cell survival, their addition before freezing and removal after thawing respectively cause potential osmotic stress and associated volume excursion on cells [1],[2].

When cells are exposed to CPA solutions (penetrating), the concentration of CPA is initially higher in the extracellular environment [1]. A relatively hyperosmotic extracellular environment produces an osmotic stress, which leads to water efflux and cell shrinkage. After equilibrium, the chemical potential of this type of CPA is equal in both the intra and extracellular environment. After the thawing process, due to removal of the CPA, cells experience another osmotic damage

as the cells will first swell due to water penetration to the cells and slowly return to their isotonic condition [3].

Each cell can withstand this change in volume as long as it does not exceed its osmotic tolerance limit which is a biological property and unique for each cell type [4]. If the change in cell volume exceeds its limit, it may provoke cell injury, which may lead to irreversible loss of cell function. Therefore, the processes of addition and removal of CPAs are very critical in cryopreservation processes [5].

The cryopreservation protocol needs to be carefully designed in order to preserve cell viability not only immediately after thaw but also when cells are cultured after the thawing process. One of the factors that may affect cell viability after thawing is CPA removal.

The minimum number of CPA addition and removal steps accompanied by the least volume excursion are two characteristics of optimal CPA addition and removal procedures [6]. The most common approach for addition and removal is in a step-wise manner [7], since abrupt addition and removal may lead to cell injury and significant loss of functional integrity. A study by Gao *et al.* showed that the damage due to removal is more than the damage due to addition of CPA. One step addition and removal of 1M glycerol for human sperm led to significant loss of cell motility [4]. Therefore, in order to decrease damage due to CPA removal, this step can be performed by serial dilution [8].

In this study, both penetrating and non-penetrating CPAs are present. As described in Chapter 4, all of the CPAs were prepared in double concentration as stock solutions and were added 1:1 in a glass vial that had an equal amount of growth medium in order to minimize damage due to osmotic stress. In order to remove CPA, serial dilution was performed.

5.2.1 Materials and methods for CPA removal by serial dilution

In order to assess the effect of CPA removal on viability, experiments were performed on Rinzl coverslips. Similarly to previous experiments (Chapter 4), controls were pre-experiments and post-experiments in the absence and presence of CPA. On day 7, HUVECs were frozen on Rinzl coverslips (cooling rate of 1 °C/min) in the presence of 5% DMSO and 6% HES as described in Chapter 4 (a sample was plunged into liquid nitrogen from –45°C) and thawed in a 37 °C water bath to assess viability immediately after thaw. The same procedure was repeated for another frozen coverslip; however, after thawing instead of directly assessing viability, CPA serial dilution was performed in 2 steps. In the first step 200 µl of 20% fetal calf serum (FCS) solution (FCS in EGM-2 solution) was added to the glass vial and incubated for 2 minutes at room temperature. In the second step, 200 µl of 10% fetal calf serum (FCS in EGM-2) was added to the present solution (incubation time: 2 minutes) and this step was repeated 3 times (Figure. 5.1).

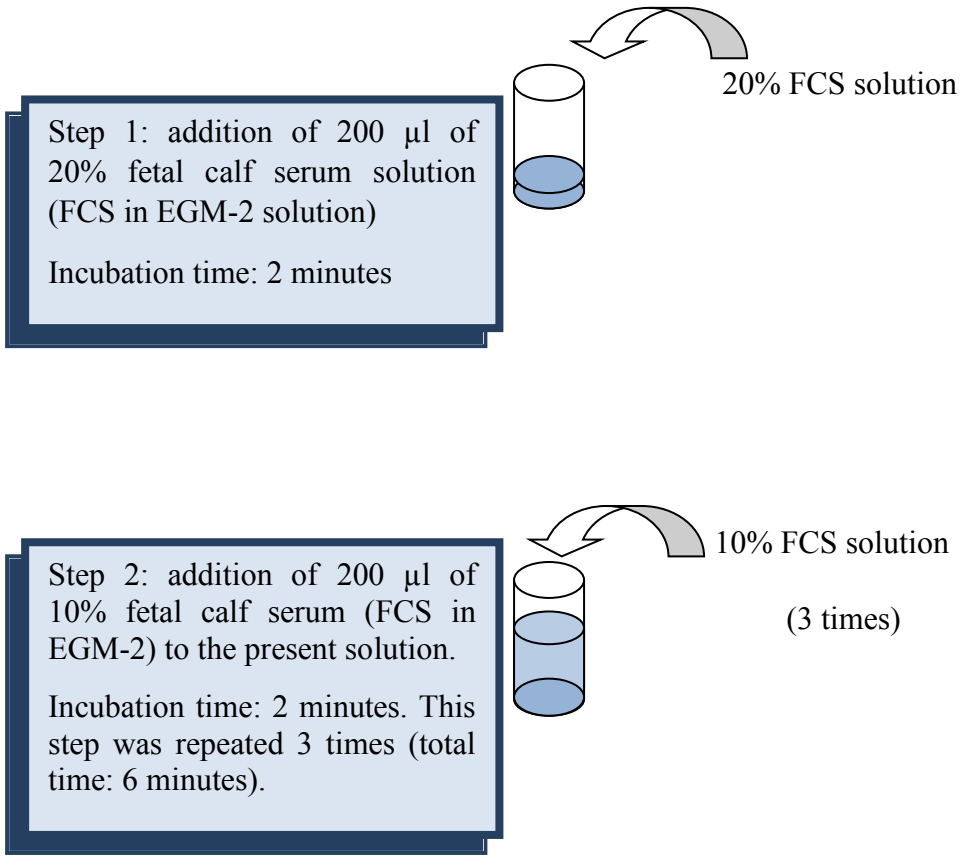

Figure 5.1: CPA serial dilution. CPA was removed from Rinzl coverslips in a step-wise manner. After thawing, in the first step 200 µl of 20% fetal calf serum (FCS) solution (FCS in EGM-2 solution) was added to the glass vial and incubated for 2 minutes at room temperature. In the second step, 200 µl of 10% fetal calf serum (FCS in EGM-2) was added to the present solution (incubation time: 2 minutes) and this step was repeated 3 times. After 8 minutes (total time of incubation), 810 µl of this solution was removed and cells were stained as described in Chapter 2 (Section 2.2) for viability assessment.

After 8 minutes (total incubation time for step 1 and 2), the total volume was almost 1000 µl. After discarding 810 µl of this solution, cells were incubated in the dark with 10 µl of SYTO13 ®/GelRed for 4 minutes and viability was assessed under fluorescent microscopy using the Viability3 program as described in Chapter 2 (section 2.2). For each coverslip, 12 images were taken and the results are presented as mean ± standard error of the mean SEM. The results are the average of three independent experiments (N = 3).

5.2.2 Results for CPA removal by serial dilution

Pre-experiment controls showed very high viability in the absence and presence of cryoprotectants (99.4 ± 0.1% vs 99.7 ± 0.1%) (Samples 1 and 2, Figure 5.2). Relative viability for the coverslip plunged into liquid nitrogen from –45°C was 79.6 ± 1.5%, which is the viability immediately after thawing (Sample 3, Figure 5.2). However, a considerable decrease in viability of the cells was observed after cryoprotectants were removed (18.9 ± 9.4%) (Sample 4, Figure 5.2). Comparison of viability showed that cells had high viability for both post-experiments in the absence (98.6 ± 0.3%) and presence of CPA before removal (99.3 ± 0.2%) (Samples 5 and 6, Figure 5.2). This procedure of cryoprotectant removal was also examined for a post-experiment coverslip containing cells that had not undergone freezing. This removal procedure did not have any effect on cell viability (98.7 ± 0.4%) (Sample 7, Figure 5.2).

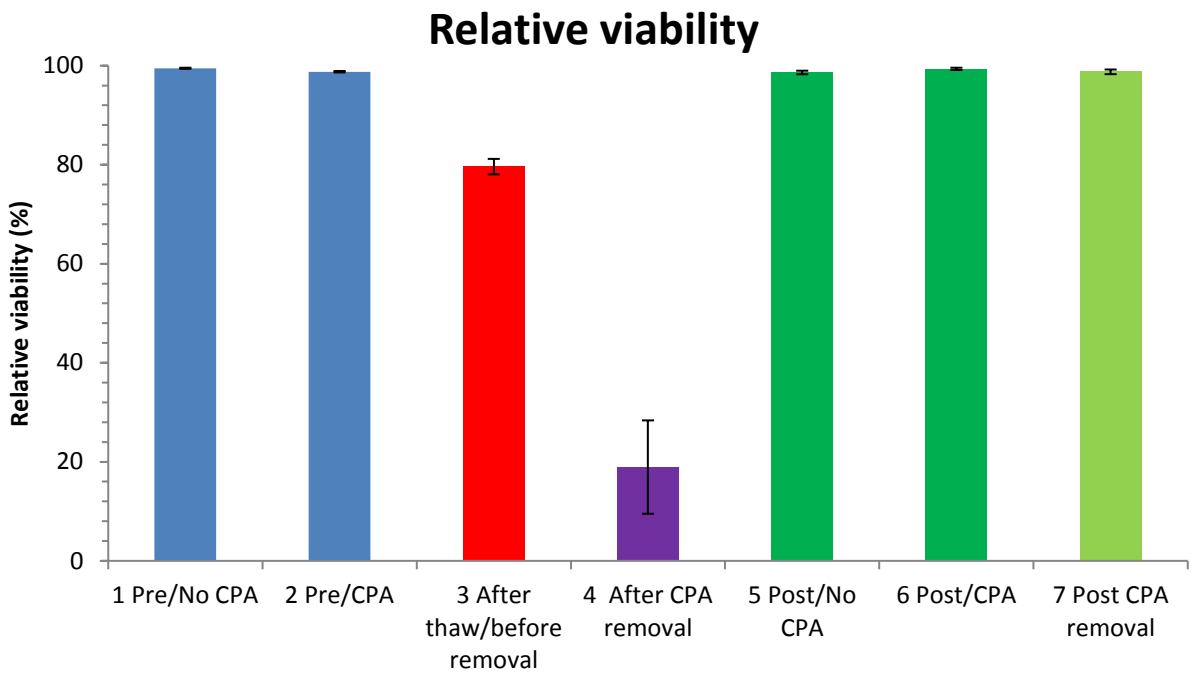

Figure 5.2: CPA removal from HUVECs in monolayers on Rinzl coverslips after freeze-thaw processes (cryoprotectant solution was prepared in EGM-2 and consisted of 5% DMSO and 6% HES). Pre-experiment viability assessed the samples in the absence and the presence of cryoprotectants (Samples 1 and 2) after 15 minutes incubation on ice. Post-experiments were incubated on ice for the duration of the experiment in the absence and presence of CPA (Samples 5 and 6) and Sample 7 was a post-experiment coverslip which was assessed for viability after CPA removal. For frozen samples plunged into liquid nitrogen from –45°C, one sample was assessed directly after thaw before CPA removal (Sample 3) and the other sample after CPA removal (Sample 4). Data are the average of three independent experiments and are presented as mean ± standard error of the mean (SEM).

5.3 Cryopreservation in the presence of CryoStor®

Typical viability assessment after cryopreservation is performed immediately after thawing (1–6 hours) which is usually based on quantitative assays such as membrane integrity by dye exclusion assays [9][10]. These assays only show live and dead cells. However, multiple mechanisms of cell death (cell rupture, necrosis and apoptosis) are present, any of which, or a combination of any, may lead to cell death after cryopreservation. Understanding the role of each

mechanism in cryopreservation processes can help to recognize an effective approach to improve cell survival after post-thaw in culture. One of the factors that affect post-thaw cell viability in culture is apoptosis [11].

A study by Borderie *et al.* revealed a high percentage of apoptotic cells in post-thaw culture (24 hours after the thawing process) for human corneal keratocytes based on viability assessment using trypan blue and flow cytometry compared with cells immediately after thawing [9]. This phenomenon is referred to as cryopreservation induced delayed-onset cell death (CIDOCD), which is defined as cell death not immediately post-thaw but manifested after cell recovery in culture [11].

One of the strategies to improve post-thaw viability has been modification of the CPA vehicle solution. In comparison with standard extracellular culture media with DMSO, these intracellular-like solutions preserve cell homeostasis by maintaining ionic balance of cells at low temperatures [12]. A study by Baust *et al.* showed that cryopreservation of a renal cell line (Madin–Darby Canine Kidney cells) in CryoStor C5 (containing 5% DMSO) showed 72% viability 24 hour post-thaw. In contrast, viability in the presence of 5% DMSO in culture medium was 30% 24 hours post-thaw [13].

To assess the effect of vehicle solution on post-thaw viability, cryoprotectant was prepared in CryoStor®. This solution is protein and serum free, reduces the level of cell death induced by cryopreservation, and improves post-thaw cell viability [12].

5.3.1 Materials and methods for cryopreservation in the presence of CryoStor

In order to assess the impact of CryoStor (CryoStor® cell cryopreservation media, CS10, C2874, Sigma-Aldrich) on cell viability after graded freezing, cryoprotectant solution was

prepared in CryoStor (CS10) as a stock solution. The stock solution consisted of 10% w/w DMSO, 12% w/w HES and was added 1:1 to each glass vial. As described before (Chapter 2, Section 2.2), HUVECs were seeded on Rinzl coverslips at a density of 10,000/cm^2. Experiments were performed for HUVECs which were in culture for 7 days. On day 7, 95 µl of growth medium (EGM-2) was added to each glass vial. Rinzl coverslips were transferred to glass vials with fine tweezers and 95 µl of cryoprotectant solution (prepared in CryoStor) was added to each vial (final concentration of CPA: 5% DMSO and 6% HES). Vials were incubated on ice for 15 minutes and subjected to graded freezing (1°C/min). A sample from –45 °C was plunged into liquid nitrogen, held for 1 hour and then thawed in a water bath at 37 °C. Samples in this experiment consisted of pre-experiments in the absence of CPA (cells were incubated with 190 µl of growth medium, EGM-2) and in the presence of CPA solution (combination of EGM-2 and CPA solution), samples plunged into liquid nitrogen from –45 °C, and post-experiment samples in the absence and presence of CPA but for which no freezing had occurred. 12 images were taken for each coverslip. Results are the average of three independent experiments (N = 3).

5.3.2 Results for cryopreservation in the presence of CryoStor

In this study, the viabilities of HUVECs after 15 minutes incubation on ice in the absence and presence of CPA were 99.7 ± 0.7% and 99.3 ± 0.2%, respectively. Post-experiment samples in the absence and presence of CPA also showed very high viability (99.8 ± 0.3% and 99.4 ± 0.08%, respectively). Preparation of CPA solution in CryoStor did not improve viability of the cells after plunge- thaw. Viability immediately reduced after thaw (before CPA removal) for the cells plunged into liquid nitrogen from –45 °C (4.2 ± 2.2%) (Figure 5.3). However, in the previous experiment (Section 5.2.2) the viability of HUVECs that were cryopreserved in the presence of 5% DMSO and 6% HES in EGM-2 immediately after thaw was 79.6 ± 1.5%.

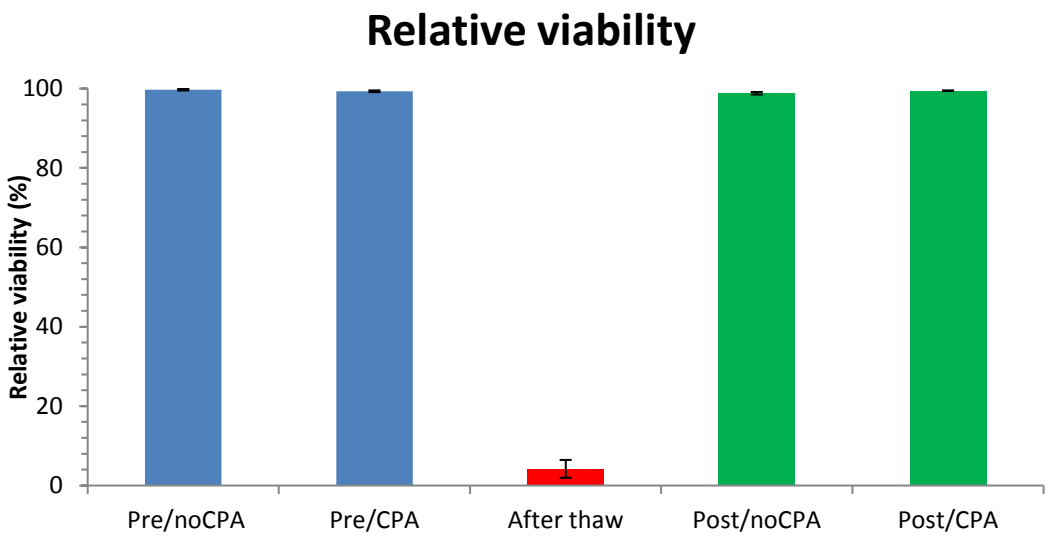

Figure 5.3: CPA removal for HUVECs on Rinzl coverslips (cryoprotectant solution was prepared in CryoStor and consisted of 5% DMSO and 6% HES in CryoStor). Pre-experiment controls were incubated in the absence and presence of CPA for 15 minutes and assessed for viability. HUVECs were plunged into liquid nitrogen from –45 °C thawed rapidly in a 37 °C water bath and assessed for viability before CPA removal. Post-experiment controls in the absence and presence of CPA were assessed for viability. Results are the average of three independent experiments. Data are presented as mean ± standard error of the mean (SEM).

5.4 Addition of chondroitin sulfate (CS) to the cryoprotectant solution

Chondroitin sulfate (CS) is one the major components of extracellular matrix and has been extensively used for tissue engineering as a scaffold [14]. The presence of chondroitin sulfate A has been reported on the surface of HUVECs, and it is proposed that cell adhesion *in vivo* is mediated by chondroitin sulfate chains, which link to thrombomodulin (TD). TD is an integral protein, which is expressed on the surface of endothelial cells [15].

Chondroitin sulfate has been used in cryopreservation solutions. A study by Proulx *et al.* showed that the presence of 0.08% CS in a culture medium for pig cornea endothelium helped cells to be tightly packed and had a beneficial effect on overall morphology in culture [16]. Viability assessment after cryopreservation of porcine corneal endothelium in the presence of different

concentrations of chondroitin sulfate (0, 2, 5, 10, and 20 %) in fetal calf serum with a cooling rate of 1 °C/min showed higher cell survival in the presence of 2% CS compared with the controls, which was accompanied by high morphological integrity [17]. This result was not restricted to immediately after the thawing process. Another study by Hagenah *et al.* confirmed that endothelium cryopreserved in the presence of 2% CS also had higher integrity in post-thaw culture after cryopreservation. Cells preserved in the presence of CS only showed 33% loss of viability while cells cryopreserved in the absence of CS showed 73% loss of viability [18]. The exact mechanism of CS function on endothelial cells is unknown; however, several studies have mentioned its beneficial effect on endothelial cell growth. CS has shown a growth stimulating effect on human corneal endothelial cells [19]. The addition of chondroitin sulfate has also shown a beneficial effect for vitrification of intact human articular cartilage; addition of chondroitin sulfate to the vitrification solution led to an increase in cell recovery from 52.4% to 75.4% [20].

In this experiment, HUVECs were subjected to graded freezing in the presence of 2% CS, 5% DMSO and 6% HES. Chondroitin sulfate A (CHS-A) (C8529, Sigma-Aldrich) used in this experiment was extracted from bovine trachea and it is in the form of sodium salt (storage temperature 2–8 °C).

5.4.1 Materials and methods for addition of CS to the CPA solution

In order to assess the effect of chondroitin sulfate on cell viability during cryopreservation, chondroitin sulfate was added to the cryopreservation solution. In this experiment, HUVECs on Rinzl coverslip were frozen in the presence of 2% w/w chondroitin sulfate, 5% w/w DMSO, and 6% w/w HES in EGM-2 medium (cryoprotectant solution was prepared in double concentration as a stock solution) and added 1:1 to each glass vial as described in Chapter 4 Section 4.2.2.1. On

day 7, HUVEC monolayers were frozen according to the graded freezing protocol (1 °C/min) as described in Chapter 4 (Section 4.2.2). For this experiment, two coverslips were considered for pre-experiments (sample 1 was in the absence of CPA, and sample 2 was in the presence of CPA) and were assessed after 15 minutes incubation on ice. Three coverslips were subjected to graded freezing (Figure 5.4). These coverslips were plunged into liquid nitrogen from –45 °C for one hour and then thawed in a water bath at 37 °C. One coverslip was assessed immediately after thaw. (Since it was not possible to capture an image of a Rinzl coverslip in the presence of chondroitin sulfate, 500µl of PBS was added to the vial after thaw and after 1 minute, the solution was removed. 190µl of PBS was added to the vial and HUVECs were stained with Syto13/GelRed and observed under a fluorescent microscope for viability (Sample 3)). For the second coverslip, cryoprotectants were removed by serial dilution as described before and cells were stained with Syto13/GelRed (Sample 4). Viability was assessed using the Viability3 program. For the third coverslip, the same procedure was repeated and after serial dilution, the coverslip was transferred with fine tweezers from the glass vial to a well in a 24-well plate containing 500 µl of growth medium supplemented with penicillin and incubated in a humidified incubator at 37 °C and 5% CO_2. After 24 hours, the coverslip was stained with Syto13/GelRed and assessed for viability as described in Chapter 2 Section 2.2 (Sample 5). Samples 6 and 7 were coverslips in the presence on CPA incubated on ice for the duration of experiment for which no freezing had occurred. Sample 6 was assessed for viability before CPA removal. Sample 7 was assessed after CPA removal by serial dilution. Sample 8 was a coverslip, which after CPA serial dilution was incubated in incubator with 500 µl of EGM-2 and assessed for viability 24 hours after CPA removal. Data are presented as mean ± standard error of the mean (SEM). Results are the average of three independent experiments.

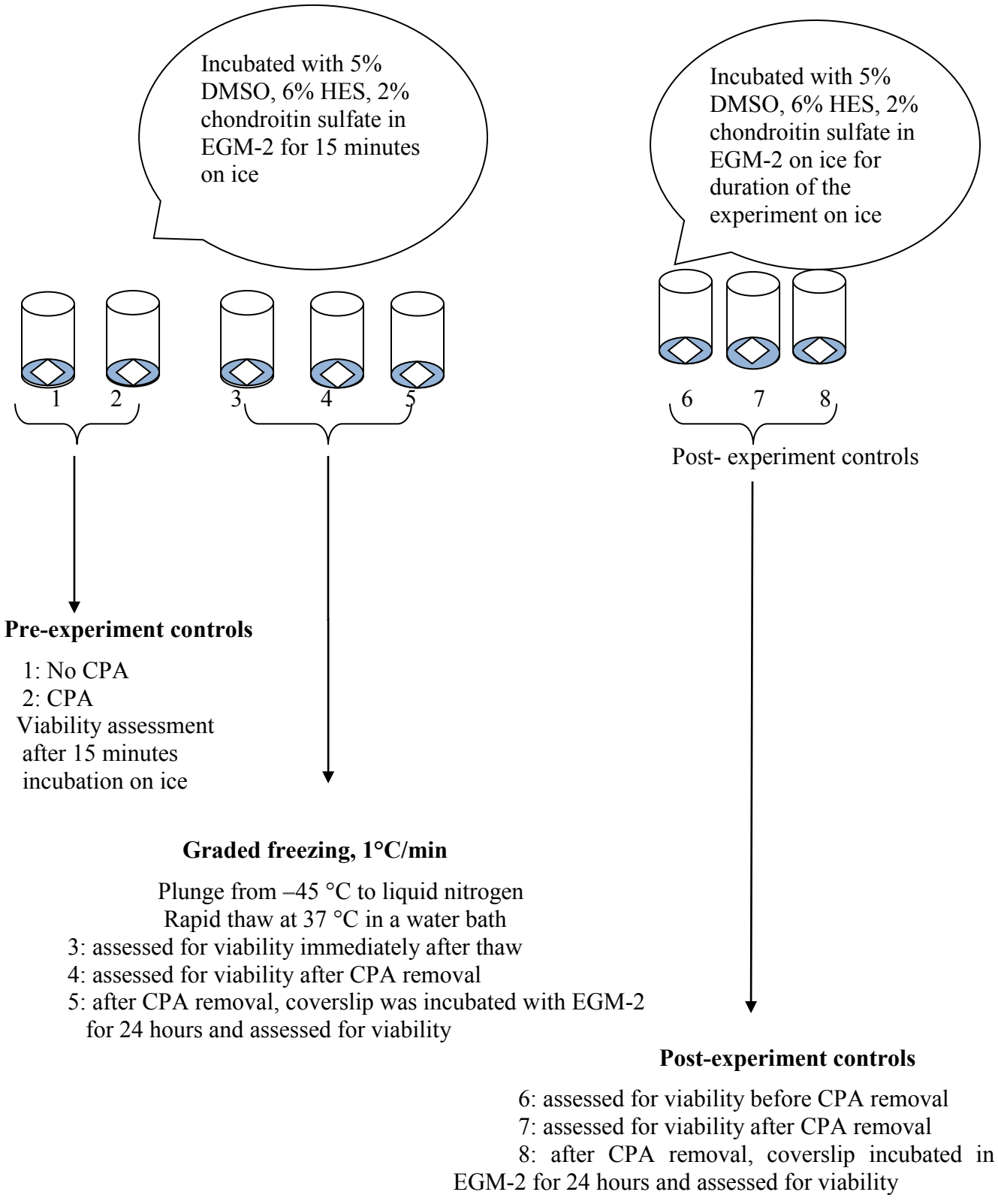

Figure 5.4: HUVEC graded freezing in the presence of 2% chondroitin sulfate, 5% DMSO and 6% HES

5.4.2 Results for addition of CS to the CPA solution

HUVECs in the absence and presence of 5% DMSO, 6% HES and 2% CS showed very high viability after 15 minutes incubation on ice. HUVECs were subjected to graded freezing (1 °C/min) and plunged into liquid nitrogen from –45 °C (Figure 5.5).

Viability immediately after thaw (before CPA removal) showed that 99.6 ± 0.08% of HUVECs were alive. In contrast with HUVECs frozen in the absence of CS, HUVECs frozen in the presence of CS showed very high viability after CPA removal (96.6 ± 2%). However, 24 hours after CPA removal and incubation in EGM-2, HUVEC viability dramatically reduced (0.6 ± 0.1%). Post-experiment controls involving no freezing showed high viability after CPA removal and after 24 hours (Figure 5.5).

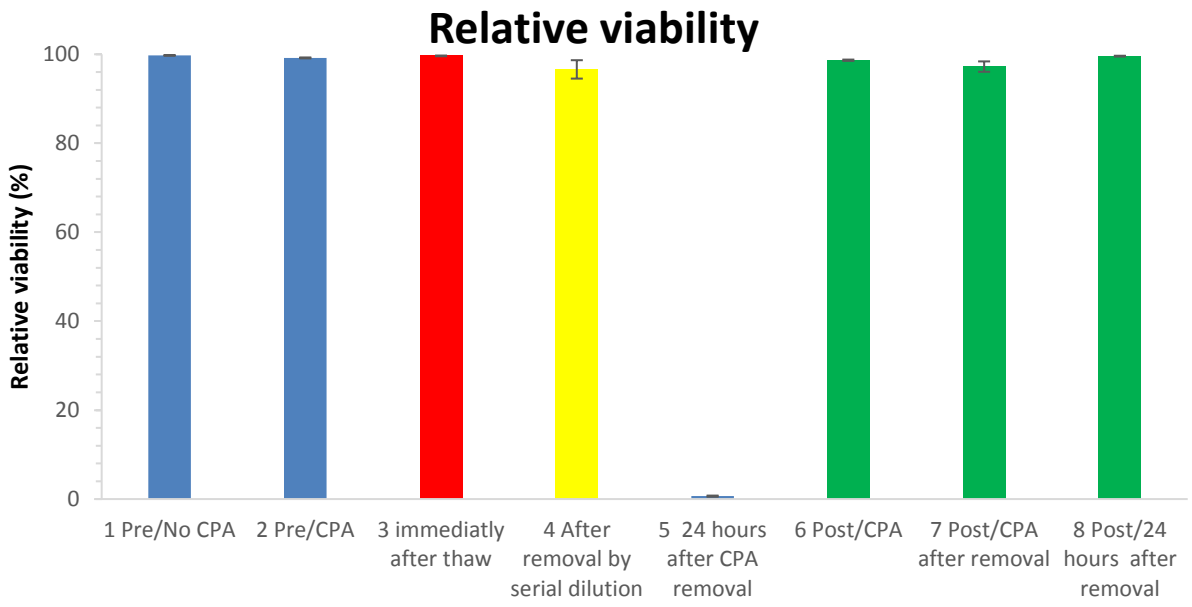

Figure 5.5: CPA removal from HUVECs frozen in the presence of 2% CS, 5% DMSO and 6% HES. Pre-experiments (samples 1 and 2) were in the absence and presence of CPA. Frozen HUVECs were thawed and assessed for viability immediately after thaw (Sample 3). Sample 4 was HUVECs, which were assessed for viability after CPA serial dilution. Sample 5 was a coverslip for which after thawing, CPA was removed by serial dilution and the coverslip was transferred with fine tweezers to a 24-well plate containing 500 µl of EGM-2 incubated for 24 hours and assessed for viability. Samples 6 and 7 were post-experiment coverslips that had not undergone freezing. Sample 6 assessed before removal. Sample 7 was assessed after CPAS removal by serial dilution. Sample 8 was a post-experiment coverslip, assessed 24 hours after CPA removal. Data are presented as mean ± standard error of the mean (SEM). Results are the average of three independent experiments.

5.5 Modification of cooling rate and CPA removal procedure

5.5.1 Cooling rate and CPA removal procedure

One of the factors which improve cell survival in cryopreservation is avoidance of intracellular ice formation. Intracellular ice is formed when cells are frozen rapidly (high cooling rate). When cells are cooled rapidly, the degree of supercooling increases and the reduced amount of water movement across the membrane increases the likelihood of intracellular ice formation [21]. Since cell survival in monolayers is lower compared with cells in suspension, a study by Routledge *et*

al. showed that cooling rate can affect this response [22]. Rabbit cornea endothelium exposed to propane-1,2-diol was cooled at two different cooling rates (0.2 and 1°C/min) followed by rapid thawing [22]. A comparison of cells cooled at the two different cooling rates showed that better cornea thickness, endothelial cell morphology and function were achieved with the lower cooling rate (0.2 °C/min) compared with cells cooled at the higher cooling rate; it is proposed that the lower cooling rate increases cell tolerance to cryoinjury in slow cooling in monolayers and that the optimum cooling rate is lower compared to that for cells in suspension [22]. In addition, in this study, another approach was considered for CPA removal, CPA was removed 24 hours after thaw rather than immediately after thaw. Based on the results of the three previous experiments in this chapter, which showed that viability was low immediately after CPA removal or 24 hours after CPA removal (in case of CS), it is hypothesized that cell membranes after freezing/thawing processes are more susceptible to osmotic injury caused by removal of CPA. Therefore, to test this hypothesis, CPA was removed 24 hours after the thawing process.

5.5.2 Materials and methods

5.5.2.1 Graded freezing (cooling rate: 0.2 °C/min)

In order to assess the effect of a lower cooling rate on viability of HUVECs, cells were subjected to graded freezing (cooling rate: 0.2 °C/min). Previous experiments in this chapter showed that addition of chondroitin sulfate to the cryopreservation solution (5% DMSO and 6% HES) led to higher viability after cryoprotectant removal compared with the cells in the presence of 5% DMSO and 6% HES without CS. In this experiment, chondroitin sulfate was also added to the cryopreservation solution. Therefore, cells were incubated on ice for 15 minutes in the presence of 5% DMSO, 6% HES and 2% CS and graded freezing (cooling rate: 0.2 °C/min) was performed. The experimental temperatures were –5, –10, –15, –25, –35 and –45 °C. For each

experimental temperature, a pair of coverslips was considered and after thawing, viability was assessed as described in Chapter 2 (Section 2.2). In this experiment, viability was assessed immediately after thaw (In order to remove CS, coverslips were washed with PBS as explained in section 5.4.1).

5.5.2.2 Modification of CPA removal procedure

In this experiment, controls were pre-experiment and post-experiment in the presence and absence of CPA without freezing. Since the effects of other optimizations in this chapter were assessed for cells plunged from –45 °C to liquid nitrogen, the CPA removal procedure also was performed for HUVECs cooled to –45 °C at a cooling rate of 0.2 °C/min and plunged into liquid nitrogen.

Four coverslips were plunged into liquid nitrogen from –45 °C. The first coverslip was assessed for viability immediately after thaw. (Since it was not possible to capture an image of a Rinzl coverslip in the presence of chondroitin sulfate, 500µl of PBS was added to the vial after thaw and after 1 minute, the solution was removed. 190µl of PBS was added to the vial and HUVECs were stained with Syto13/GelRed and observed under a fluorescent microscope for viability, Sample 5, Figure 5.7). For the second coverslip after thawing, CPA was removed by serial dilution and viability was assessed as described in Section 5.2.1 (sample 6, Figure 5.7). For the third coverslip, CPA was removed after thawing by serial dilution and the coverslip was transferred with fine tweezers from the glass vial to a well in a 24-well plate containing 500 µl of growth medium supplemented with penicillin and incubated in a humidified incubator at 37 °C and 5% CO2 (sample 7, Figure 5.7). For the fourth coverslip, after thawing, CPA was not removed; instead 200 µl of EGM-2 containing 1% penicillin (LONZA, CC-3162) was added to the same vial and the vial was placed in an incubator for 24 hours. After 24 hours, CPA serial

dilution was performed and cells were stained with SYTO13/GelRed (10 µl) for 4 minutes in a dark place and then viability was assessed with the Viability3 program as described in Chapter 2 (Section 2.2) (Sample 8, Figure 5.7).

5.5.3 Results

5.5.3.1 Viability immediately after thaw (cooling rate of 0.2 °C/min)

HUVECs were frozen in the presence of 5% DMSO, 6% HES and 2% CS at a cooling rate of 0.2 °C/min. HUVECs showed very high viability at every intermediate temperature both for direct-thaw and plunge-thaw samples. No significant difference for cell viability was observed between direct-thaw samples and plunge-thaw samples (Figure 5.6).

5.5.3.2 Viability after CPA removal

In Figure 5.7, pre-experiment controls (sample 1 and 2) in the absence and presence of CPA showed very high viability (99.7±0.03% and 99.2±0.1%). Post-experiment controls (samples 3 and 4 were without freezing) also showed very high viability (98.1±0.1% and 97.2±1.2%). In this experiment, Sample 5 was a coverslip that was assessed immediately after thawing and relative viability was 98.54 ± 1.01%. Sample 6 was a coverslip that showed very high viability after CPA removal. Sample 7 was a coverslip that was incubated in EGM-2 after CPA removal; viability dramatically reduced 24 hours after CPA removal. Sample 8 showed very high viability because CPA was not removed after thaw but rather after 24 hours (relative viability: 81.7 ± 9.1% and absolute viability: 70.7 ± 8.9%). Therefore, the combination of lower cooling rate and modification of the CPA removal procedure could dramatically increase cell viability.

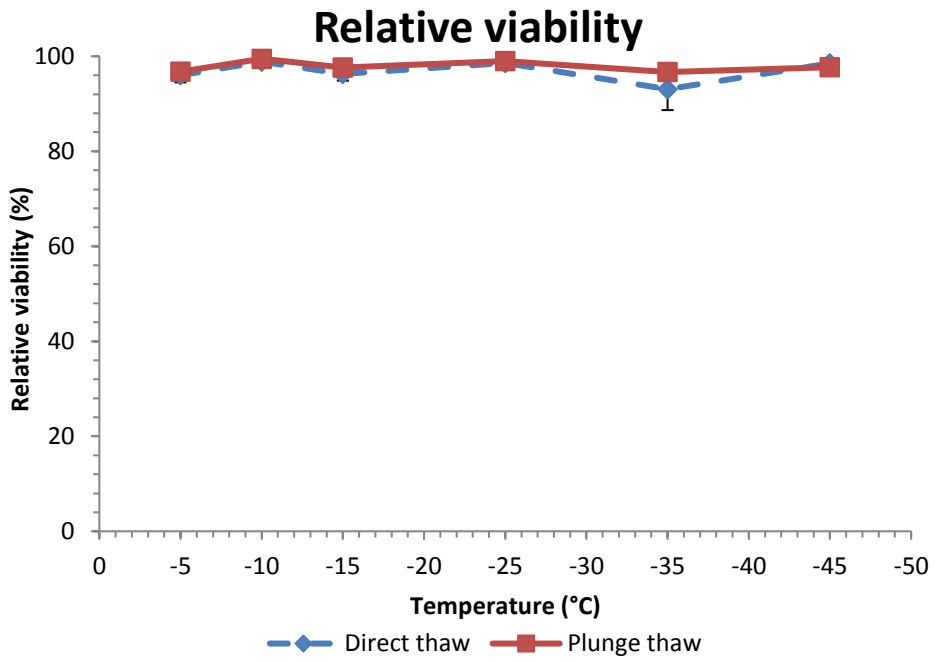

Figure 5.6: HUVEC graded freezing on Rinzl coverslips at a cooling rate of 0.2 °C/min in the presence of 5% DMSO, 6% HES and 2% chondroitin sulfate (CS). HUVECs were seeded on Rinzl coverslips and on day 7 graded freezing was performed in the presence of 5% DMSO, 6% HES and 2% CS (for each experimental temperature, a pair of samples was considered for direct-thaw and plunge-thaw). At each experimental temperature, after thawing cells were stained and assessed for viability as described in Chapter 2 (Section 2.2). Data are presented as mean ± standard error of the mean (SEM). Results are the average of three independent experiments.

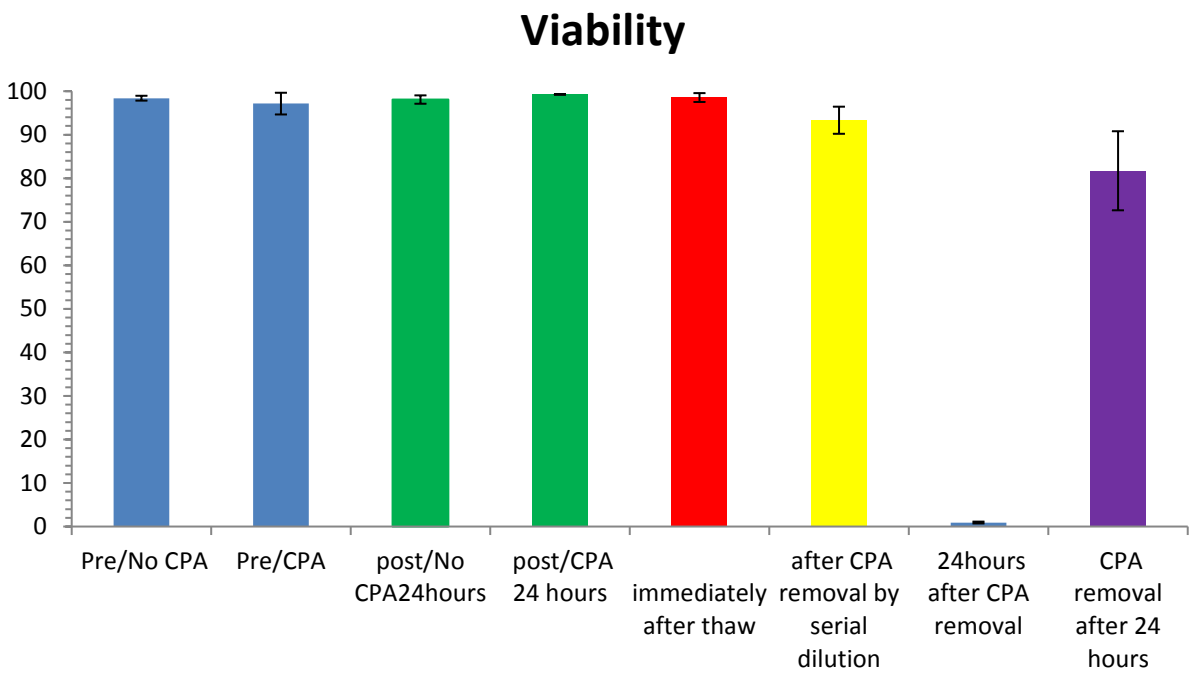

Figure 5.7: Modification of CPA removal procedure. Samples 1 and 2 are pre-experiment controls in the absence and presence of cryoprotectants. Samples 3 and 4 are post-experiment controls in the absence and presence of CPA without freezing. Sample 5 was a coverslip that was plunged into liquid nitrogen from –45 °C and cells were assessed for viability immediately after thaw. For sample 6, after thawing, CPA was removed by serial dilution and viability was assessed. For sample 7, CPA was removed as for sample 6 and the sample was placed in EGM-2 in a 24-well plate for 24 hours in an incubator. For sample 8, CPA was not immediately removed; instead 200 µl of EGM-2 containing 1% penicillin (LONZA, CC-3162) was added to the same vial and the vial was placed in an incubator for 24 hours. CPA serial dilution was performed after 24 hours and cells were stained and assessed for viability.

5.6 Discussion

In order to prevent osmotic shock during CPA removal, after graded freezing at 1 °C/min for HUVECs on Rinzl coverslips, CPA was removed by serial dilution. However, this procedure was not successful in terms of preserving cell viability after CPA removal and caused dramatic reduction in cell viability. In order to assess the efficiency of this procedure for CPA removal, apart from the frozen coverslip, CPA also was removed from the post-experiment coverslip control that had not undergone freezing. Removal of CPA from the post-experiment control did not show any reduction in cell viability compared with post-experiment viability before CPA removal. Therefore, this CPA removal procedure itself does not produce the cell damage for unfrozen cells. However, frozen HUVECs have experienced stress in the freeze–thaw process (shrinkage and swelling) and also these cells' membranes have been exposed to extracellular ice. Moreover, cells in a monolayer are fixed in place and they are influenced by contraction and expansion of ice and substrate during the freeze–thaw process which affects the cytoskeleton of cells. All of these processes can produce stress on the cell membrane; therefore, the cell membrane of frozen cells may not be in the same condition as that of unfrozen cells, which can be an explanation for the difference in cell response to CPA removal.

Replacement of the CPA vehicle solution by CryoStor was expected to improve cell survival after graded freezing. Application of this solution in other studies has led to improvement of cell survival 24-hours post-thaw [13], it has been reported that CryoStor CS5 (containing 5% DMSO) could increase 24-hour post-thaw cell viability for a renal cell line (Madin–Darby Canine Kidney cells) (viability was 72%) compared with cells cryopreserved in standard growth medium and 5% DMSO (viability was 28%) [13]. In this project application of this reagent was completely

unsuccessful (relative viability: 4.2 ± 2.2%) even immediately after thawing for graded freezing of HUVECs in monolayers. The difference of this study compared with other studies in the presence of CryoStor is that in the previous studies no other cryoprotectant was added to CryoStor C5 (CryoStor already contained 5% DMSO as a cryoprotectant). In the case of this study, HES was also added to CryoStor. It is not clear why cell recovery dramatically reduced after thawing, it is assumed that addition of HES affected CryoStor's function in terms of preserving cell viability.

The exact mechanism of cryopreservation-induced apoptosis is not known but several studies have reported the incorporation of multiple pathways to cryopreservation-induced apoptosis which occurs in post-thaw culture. Apoptosis does not occur immediately post-thaw as reported by Frim *et a;* it showed itself in post-thaw culture [10].

Although addition of chondroitin sulfate to the cryoprotectant solution improved cell survival immediately after thaw and after CPA removal compared with the first experiment (serial dilution of CPA which only consisted of HES and DMSO in EGM-2), relative cell viability dramatically reduced 24 hours after CPA removal. Chondroitin sulfate has been used in cryoprotectant solutions for cryopreservation of intact human articular cartilage [20], porcine cornea (2% w/v) [17], and even bovine corneal endothelial cells (2.5% w/v) [23]. The cryoprotective mechanism of chondroitin sulfate is unknown.

The other factor that affected relative cell viability was cooling rate. Graded freezing in the presence of CS and a cooling rate of 0.2 °C/min resulted in very high viability at each experimental temperature. A comparison of relative cell viability after further modification showed that combination of a lower cooling rate 0.2 °C/min and the presence of chondroitin sulfate in a cryoprotectant solution was successful in increasing relative cell viability after CPA

removal (Figure 5.7). It seems that a lower cooling rate provides better protection for cells plunged into liquid nitrogen. One possible explanation is that the lower cooling rate allows cells to have enough time to dehydrate, which prevents intracellular ice formation in the next step which is plunging into liquid nitrogen. Apart from that it is assumed that the presence of chondroitin sulfate can be another factor which helps to increase cell survival. Moreover, in this experiment comparison of CPA removal after thawing and after 24 hours showed that CPA removal immediately after thaw affects cell viability in post-thaw culture. It is assumed that it is necessary to give time to cells to recover from the stress of the freeze–thaw process. This study showed that it is possible to achieve 81.7 ± 9.1% relative cell viability by modification of the graded freezing procedure (cooling rate, CPA removal procedure, and addition of chondroitin sulfate).

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Chapter 6: Conclusion

The aim of this study was developing a cryopreservation protocol for HUVECs in monolayers. This aim was achieved by considering different factors: *i*) HUVEC culture on two different substrates over a period of 10 days, assessing cell morphology, viability and achieving a growth curve which resulted in considering a confluent monolayer of viable HUVECs on day 7 for cryopreservation studies; *ii*) comparison of the effect of substrates on cell viability and attachment; *iii*) comparison of the effect of absence or presence and composition of cryoprotectants in the cryopreservation solution for cell viability and *iv*) further optimization in terms of CPA removal, cooling rate and presence of chondroitin sulfate in the cryopreservation solution. As hypothesized, HUVEC viability was affected by the type of substrate both in the presence or absence of cryoprotectants during cryopreservation. A comparison of viability in the absence of cryoprotectants showed that Rinzl could provide protection to cells in plunge-thaw samples compared with glass (maximum viability in the absence of cryoprotectants was (45.4 ± 7.9% and 2.33 ± 0.89% on Rinzl and glass coverslips respectively). This protection was higher in the presence of cryoprotectants (10% and 20% DMSO) compared with HUVECs on glass in the presence of 10% and 20% DMSO. However, attachment was not affected by the type of the substrate during cryopreservation of HUVEC monolayers.

This study showed that the cryoprotective effect of DMSO was better in the presence of HES and that a combination of 5% DMSO and 6% HES led to higher viability on Rinzl coverslips compared with 10% and 20% DMSO for HUVECs in monolayers at a cooling rate of 1 °C/min. While achieving high cell recovery immediately after thaw is essential, it does not necessarily guarantee viability of the cells after 24 hours of post-thaw culture. Cryopreservation at a lower cooling rate (0.2 °C/min) and in the presence of 2% chondroitin sulfate in a cryopreservation

solution in addition to 5% DMSO and 6% HES led to very high viability at all experimental temperatures. Cryoprotectant removal 24 hours after thaw dramatically increased viability of HUVECs in monolayers compared with other methods that were used in this study (Table 6.1). Therefore, cryopreservation in the presence of 5% DMSO, 6% HES and 2% chondroitin sulfate at a cooling rate of 0.2 °C/min on Rinzl coverslips with CPA removed by serial dilution 24 hours after thaw was the best protocol in this study for cryopreservation of HUVECs in monolayers resulting in a relative viability of 81.7 ± 9.1% and an absolute viability of 70.7 ± 8.9% after CPA removal. In future studies, it will be beneficial to investigate the cell functionality after CPA removal as well.

Table 6.1: Summary of experiments on Rinzl coverslips for developing a cryopreservation protocol for HUVECs in monolayers

	Maximum relative viability % (Maximum absolute viability, %)			
Rinzle coverslip (1°C/min)	Immediately after thaw	After CPA removal	24 hours after CPA removal	CPA removal after 24 hours
No CPA	45.4 ± 7.9 (42.7 ± 6.5)	N/A	N/A	N/A
10% DMSO	46 ± 15 (42.48 ± 13.1)	N/A	N/A	N/A
20% DMSO	49.7 ± 11.2 (45.0 ± 6.1)	N/A	N/A	N/A
5% DMSO + 6% HES	79.6 ± 1.5 (79.7 ± 4.8)	18.9 ± 9.4 (10.4±8.3)	N/A	N/A
5% DMSO + 6% HES + CryoStor	4.2 ± 2.2 (3.86 ± 3.67)	N/A	N/A	N/A
5% DMSO + 6% HES + 2% chondroitin sulfate	*99.6 ± 0.1 (80.0 ± 12.5)	96.6 ± 2.0 (95.0 ± 1.2)	0.65 ± 0.12 (0.6 ± 0.2)	N/A
5% DMSO + 6% HES +2% chondroitin sulfate (0.2 °C/min)	***98.5 ± 1.0 (95.6 ± 21.1)**	**93.3 ± 3.1 (92.1 ± 3.2)**	**0.92 ± 0.37 (0.8 ± 0.5)**	**81.7 ± 9.1 (70.71 ± 8.90)**

*Since it was not possible to capture an image of a Rinzl coverslip in the presence of chondroitin sulfate after thaw, 500µl of PBS was added to the vial after thaw and after 1 minute, the solution was removed. 190µl of PBS was added to the vial and HUVECs were stained with Syto13/GelRed and observed under a fluorescent microscope for viability.

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Appendix

Graded freezing in the absence of CPA on glass coverslips

A) Control

Controls	Pre-experiment No cooling	Post-experiment No cooling	Plunge into liquid nitrogen
Viability (%)	98.6 ± 0.3	99.4 ± 0.2	0.4 ± 0.3

B) Relative and absolute viability (Direct-thaw samples)

Temperature °C / Viability (%)	– 3	– 10	– 15	– 25	– 35	– 45
Relative viability	86.3 ± 2.2	31.7 ± 4.4	24.6 ± 6.9	52.1 ± 5.7	50.5 ± 9.7	30.5 ± 4.0
Absolute viability	77.0 ± 5.6	20.8 ± 6.9	21.0 ± 9.2	52.5 ± 5.2	42.4 ± 2.9	28.17 ± 4.5

C) Relative and absolute viability (Plunge-thaw samples)

Temperature °C / Viability (%)	– 3	– 10	– 15	– 25	– 35	– 45
Relative viability	0.99 ± 0.35	2.01 ± 0.82	3.39 ± 1.2	1.02 ± 0.32	1.24 ± 0.14	2.47 ± 1.52
Absolute viability	0.41 ± 0.12	0.97 ± 0.46	2.33 ± 0.89	0.7 ± 0.29	0.82 ± 0.08	1.96 ± 1.47

Graded freezing in the presence of 10% DMSO on glass coverslips

A) Controls

Controls	Pre-exp No CPA	Pre-exp CPA	Post-exp No CPA	Post-exp CPA	Plunge No CPA	Plunge CPA
Viability (%)	99.45 ± 0.17	99.39 ± 0.33	99.01 ± 0.28	97.21 ± 1.05	0.43 ± 0.32	6.35 ± 2.11

B) Relative and absolute viability (Direct-thaw samples)

Temperature °C / Viability (%)	– 5	– 10	– 15	– 25	– 35	– 45
Relative viability	87.36 ± 5.97	75.61 ± 3.23	77.67 ± 7.05	85.13 ± 1.98	83.91 ± 3.55	63.30 ± 7.13
Absolute viability	81.86 ± 9.10	65.59 ± 12.92	74.80 ± 4.84	70.33 ± 6.70	77.44 ± 3.53	64.13 ± 3.83

C) Relative and absolute viability (Plunge-thaw samples)

Temperature °C / Viability	– 5	– 10	– 15	– 25	– 35	– 45
Relative viability	11.30 ± 3.03	7.42 ± 3.36	11.41 ± 7.66	4.10 ± 0.67	6.54 ± 1.81	5.2 ± 0.97
Absolute viability	10.73 ± 3.64	7.69 ± 3.75	11.19 ± 7.38	4.73 ± 0.64	7.51 ± 0.70	4.22 ± 0.54

Graded freezing in the presence of 20% DMSO on glass coverslips

A) Control

Controls	Pre-exp No CPA	Pre-exp CPA	Post-exp No CPA	Post-exp CPA	Plunge No CPA	Plunge CPA
Viability (%)	99.1 ± 0.4	97.2 ± 0.7	99.2 ± 0.1	97.9 ± 0.4	1.4± 1.2	10.6 ± 3.6

B) Relative and absolute viability (Direct-thaw samples)

Temperature °C / Viability (%)	–10	–15	–25	–35	–45
Relative viability	75.42 ± 7.22	84.82 ± 2.85	83.54 ± 5.89	81.39 ± 2.83	59.65 ± 2.74
Absolute viability	79.08 ± 6.13	83.48 ± 3.64	80.88 ± 8.00	77.90 ± 5.69	57.04 ± 2.97

C) Relative and absolute viability (Plunge-thaw samples)

Temperature °C / Viability (%)	–10	–15	–25	–35	–45
Relative viability	6.31 ± 2.13	7.15 ± 2.06	6.65 ± 2.36	7.00 ± 2.99	11.10 ± 3.72
Absolute viability	7.09 ± 1.45	7.13 ± 1.65	7.23 ± 2.58	6.41 ± 2.33	10.81 ± 2.82

Graded freezing in the presence of 5%DMSO and 6% HES on glass coverslips

A) Control

controls	Pre-exp No CPA	Pre-exp CPA	Post-exp No CPA	Post-exp CPA	Plunge No CPA	Plunge CPA
Viability (%)	99.2 ± 0.3	99.1 ± 0.1	98.67 ± 0.26	99.08 ± 0.33	0.9 ± 0.3	1.0 ± 0.3

B) Relative and absolute viability (Direct-thaw samples)

Temperature °C / Viability (%)	–5	–10	–15	–25	–35	–45
Relative viability	96.79 ± 1.14	95.84 ± 1.75	95.88 ± 1.20	96.01 ± 0.68	90.58 ± 1.46	76.68 ± 5.04
Absolute viability	92.45 ± 1.86	84.41 ± 2.63	92.87 ± 1.49	89.38 ± 2.18	93.21 ± 2.77	85.31 ± 0.82

C) Relative and absolute viability (Plunge-thaw samples)

Temperature °C / Viability (%)	–5	–10	–15	–25	–35	–45
Relative viability	7.62 ± 1.86	8.71 ± 2.63	6.86 ± 1.49	5.15 ± 2.18	12.71 ± 2.77	6.35 ± 0.82
Absolute viability	7.72 ± 2.18	9.44 ± 3.14	5.07 ± 0.14	5.17 ± 1.99	14.59 ± 3.83	6.32 ± 0.79

Graded freezing in the absence of CPAs on Rinzl coverslips

A) Control

Controls	Pre-exp No CPA	Post-exp No CPA	Plunge No CPA
Viability (%)	98.3 ± 0.6	98.3 ± 0.2	1.10 ± 0.6

B) Relative and absolute viability (Direct-thaw samples)

Temperature °C / Viability (%)	–3	–10	–15	–25	–35	–45
Relative viability	83.6 ± 5.3	48.2 ± 3.4	36.8 ± 7.8	60.5 ± 6.8	48.13 ± 7.6	23.83 ± 11.2
Absolute viability	73.3 ± 7.0	45.3 ± 2.6	34.2 ± 6.2	60.6 ± 8.0	43.83 ± 6.6	18.47 ± 7.3

C) Relative and absolute viability (Plunge-thaw samples)

Temperature °C / Viability	–3	–10	–15	–25	–35	–45
Relative viability (%)	4.9 ± 0.8	11.5 ± 5.4	42.8 ± 12.4	45.4 ± 7.9	31.7 ± 13.00	14.8 ± 0.6
Absolute viability (%)	4.7 ± 1.3	11.8 ± 4.6	41.0 ± 15.9	42.7 ± 6.5	27.1 ± 6.6	6.12 ± 5.5

Graded freezing in the presence of 10% DMSO on Rinzl coverslips

A) Control

Controls	Pre-exp No CPA	Pre-exp CPA	Post-exp No CPA	Post-exp CPA	Plunge No CPA	Plunge CPA
Viability (%)	99.1 ± 0.3	97.7 ± 0.6	98.1 ± 0.4	92.7 ± 1.2	1.2 ± 0.6	15.4 ± 6.5

B) Relative and absolute viability (Direct–thaw samples)

Temperature °C / Viability (%)	–5	–10	–15	–25	–35	–45
Relative viability	89.63 ± 3.2	82.20 ± 6.0	81.44 ± 2.8	83.20 ± 6.8	84.91 ± 3.7	75.98 ± 8.8
Absolute viability	80.66 ± 2.7	78.33 ± 3.8	77.66 ± 5.7	80.66 ± 7.3	85.66 ± 3.1	74.66 ± 8.6

C) Relative and absolute viability (Plunge–thaw samples)

Temperature °C / Viability (%)	–5	–10	–15	–25	–35	–45
Relative viability	25.3 ± 5.7	29.52 ± 2	46 ± 15	41.1 ± 4.2	42.8 ± 3.8	45.2 ± 9.6
Absolute viability	26.6 ± 11.9	31.08 ± 1.5	42.48 ± 13.1	42.19 ± 5.1	35.8 ± 5.04	44.4 ± 10.6

Graded freezing in the presence of 20% DMSO on Rinzl coverslips

A) Controls

Controls	Pre-exp No CPA	Pre-exp CPA	Post-exp No CPA	Post-exp CPA	Plunge No CPA	Plunge CPA
Viability (%)	98.7 ± 0.3	99.2 ± 0.18	98.9 ± 0.2	96.8 ± 1.4	3.1 ± 2.9	19.4 ± 4.6

B) Relative and absolute viability (Direct–thaw samples)

Temperature °C / Viability (%)	–10	–15	–25	–35	–45
Relative viability	73.1 ± 13.2	80.6 ± 7.0	81.08 ± 3.3	67.5 ± 6.6	57.42 ± 6.2
Absolute viability	65.4 ± 9.8	72.79 ± 2.8	77.2 ± 5.4	64.8 ± 6.8	52.4 ± 4.1

C) Relative and absolute viability (Plunge–thaw samples)

Temperature °C / Viability (%)	–10	–15	–25	–35	–45
Relative viability	10.9 ± 2.9	20.6 ± 8.5	22.24 ± 2.3	35.6 ± 7.5	49.7 ± 11.2
Absolute viability	10.5 ± 2.71	20.4 ± 7.13	22.89 ± 1.91	35.1 ± 7.06	45.04 ± 6.12

Graded freezing in the presence of 5% DMSO and 6% HES on Rinzl coverslips

A) Controls

Controls	Pre-exp No CPA	Pre-exp CPA	Post-exp No CPA	Post-exp CPA	Plunge CPA
Viability (%)	95.3±3.5	98.74 ± 0.16	99.16 ± 0.2	99.16 ± 0.2	14.4 ± 2.2

B) Relative and absolute viability (Direct–thaw samples)

Temperature °C / Viability (%)	–5	–10	–15	–25	–35	–45
Relative viability	95.4 ± 0.9	93.8 ± 1.4	93.5 ± 2.1	88.9 ± 0.17	88.6 ± 1.3	87.7 ± 5.09
Absolute viability	89.3 ± 5.8	84 ± 6.9	98 ± 2.0	92.3 ± 3.8	91.0±2.0	90.8 ± 10.2

C) Relative and absolute viability (plunge–thaw samples)

Temperature °C / Viability (%)	–5	–10	–15	–25	–35	–45
Relative viability	60 ± 19.3	44.33 ±13.9	67 ± 5.5	79 ± 11.3	81.33 ± 10.6	88.6 ± 9.9
Absolute viability	48.79 ± 13.3	37.05 ± 11.25	56.8 ± 8.3	66.05 ± 6.2	79.7 ± 4.4	81.4 ± 0.3

Chapter 5

Viability of HUVECs after CPA serial dilution

A) Controls

Controls	Pre-experiment /No CPA	Pre-experiment /CPA	Post experiment /NO CPA	Post experiment /CPA	Post-experiment /After removal
Viability (%)	99.4 ± 0.1	99.7 ± 0.1	98.6 ± 0.3	99.3 ± 0.2	98.7 ± 0.4

B) Viability before and after CPA removal

Sample	Immediately after thaw (before CPA removal)	After CPA removal
Viability (%)	79.6 ± 1.5	18.9 ± 9.4

Viability after graded freezing in the presence of CryoStor

A) Controls

Controls	Pre-experiment /No CPA	Pre-experiment /CPA	Post experiment /NO CPA	Post experiment /CPA
Viability (%)	99.7 ± 0.2	99.3 ± 0.2	99.8 ± 0.3	99.4 ± 0.08

B) Viability before CPA removal

Sample	Immediately after thaw (before CPA removal)
Viability (%)	4.2 ± 2.2

Viability after graded freezing in the presence of 2% CS, 5% DMSO and 6% HES

A) Controls

Controls	Pre-experiment /NO CPA	Pre-experiment /CPA	Post experiment /NO CPA	Post experiment /CPA	Post experiment/after removal
Viability (%)	99.7±0.03	99.2±0.1	98.1±0.1	97.2±1.2	99.6±0.09

B) Viability before and after CPA removal

Sample	Immediately after thaw (before CPA removal)	After CPA removal	24 hours post-thaw
Viability (%)	99.6±0.08	96.6±2	0.6±0.1

A) Graded freezing in the presence of 2% chondroitin sulfate, 5% DMSO and 6% HES (cooling rate 0.2 °C/min)

Temperature °C / Viability (%)	–5	–10	–15	–25	–35	–45
Relative viability (Direct thaw)	96.0 ± 1.5	98.8 ± 0.2	96.3 ± 1.4	98.5 ± 0.9	93.1 ± 4.4	98.5 ± 0.06
Relative viability (Plunge thaw)	96.7 ± 0.8	99.5 ± 0.3	97.6 ± 1.1	98.9 ± 0.5	96.7 ± 1.4	97.7 ± 1.0

B) Viability after CPA removal

Samples	Pre/no CPA	Pre/CPA	Post /no CPA	Post/ CPA	Immediately after thaw	After CPA removal	24 hours after CPA removal	CPA removal after 24 hours
Viability (%)	98.4 ± 0.5	97.1 ±2.5	98.1 ± 1.0	99.2 ± 0.05	98.5 ± 1.0	93.3 ± 3.1	0.92 ± 0.2	81.7 ± 9.0