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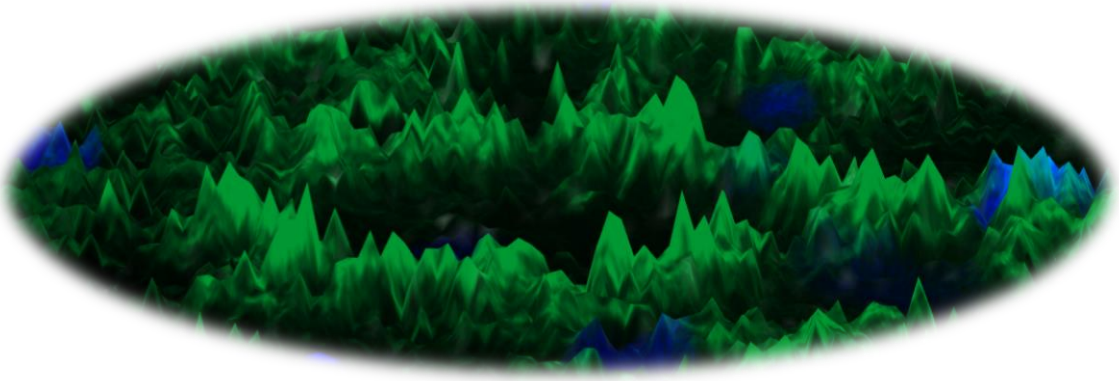

ESTABLISHMENT OF A PROTOCOL FOR THE CRYOPRESERVATION OF CELL SHEETS OF ADIPOSE TISSUE STEM CELLS

UNIVERSIDADE DO ALGARVE

DEPARTAMENTO DE CIÊNCIAS BIOMÉDICAS E MEDICINA

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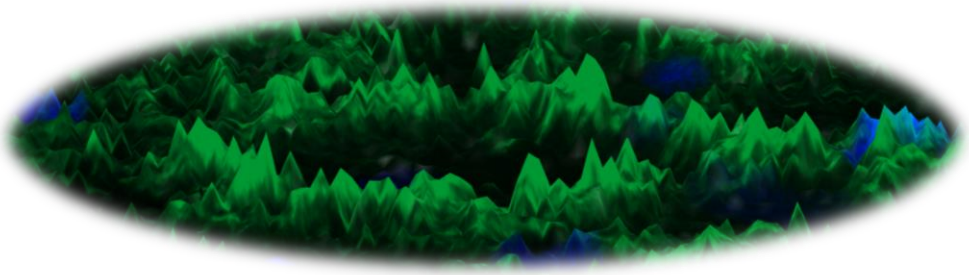

ESTABLISHMENT OF A PROTOCOL FOR THE CRYOPRESERVATION OF CELL SHEETS OF ADIPOSE TISSUE STEM CELLS.

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2016

Declaração de autoria de trabalho

Declaro ser o autor deste trabalho, que é original e inédito. Autores e trabalhos consultados estão devidamente citados no texto e constam das listagens de referências incluídas.

Universidade do Algarve, ___ / ___ / _____

Assinatura: _____

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Resumo

A Engenharia de Tecidos é uma área interdisciplinar da Medicina Regenerativa que visa criar e desenvolver substitutos biológicos para reparar, manter ou melhorar a função de tecidos lesados, com base em princípios da engenharia conjugados às ciências da vida. A Engenharia de Tecidos tira partido das propriedades de estruturas tri-dimensionais (3D) que combinadas com células estaminais pretendem recriar um ambiente semelhante ao nativo de um tecido. Estas estruturas 3D (scaffolds) são produzidas com materiais de origem natural ou sintéticos, que idealmente terão as propriedades físicas, mecânicas e químicas para promoverem o melhor desempenho dessas células e portanto a regeneração dos tecidos.

Na última decada as células estaminais mesenquimais foram amplamente utilizadas na Engenharia de Tecidos, pois têm o potencial de proliferarem e se manterem indiferenciadas com a capacidade de se auto-renovarem e/ou diferenciarem em diferentes tipos de células. Existem várias fontes de células estaminais com caracteristicas diferentes utilizadas em TE, em que as que apresentam maior impacto são as derivadas da medula espinal, do sangue e do tecido adiposo, entre outras localizadas em diferentes zonas do corpo humano.

A combinação entre *scaffolds* e células estaminais mesenquimais apresentam algumas limitações tais como a indução de uma resposta inflamatória após transplante e o facto da grande maioria dos biomateriais utilizados não serem biofuncionais.

A Engenharia de Cell sheets é a alternativa, pois utiliza a matriz extracelular depositada pelas células como *scaffold* natural para a regeneração de diferentes tecidos.

O conceito de *cell sheet* foi introduzido por Teruo Okano e os seu colaboradores nos anos 90 no Japão. Estas *cell sheets* são produzidas em superficies revestidas com um polímero não iónico sensível à temperatura. Quando a temperatura é inferior a 32ºC a superficie fica hidrofílica, promovendo o destacamento das células em folha (*cell sheets*) sem recorrer ao uso do tradicional tratamento enzimático. Assim, esta tecnologia permite obter *cell sheets* com uma organização celular própria e coesiva, dado que as interações célula-celula e célula-matriz extracelular são mantidas.

Em estudos anteriores no nosso laboratório foram produzidas *cell sheets* a partir das células estaminais humanas derivadas do tecido adiposo (hASCs). As *cell sheets* de hASCs quando transplantadas para feridas excisionais em pele de ratinho, induziram a formação de cristas epiteliais, normalmente apenas encontradas em pele humana, e a formação de um número significativo de folículos pilosos.

Tendo em consideração estes resultados e antevendo as possibilidades de ter estas *cell sheets* disponiveis para uso imediato na clínica (*off-the-shelf*) a sua criopreservação seria vantajosa. Assim, o objectivo deste estudo foi definir um método de criopreservação que não só permita a preservação da viabilidade das hASCs mas também a integridade da matriz extracelular das cell sheets, que se sabe ser critico para garantir a sua funcionalidade, após transplantação.

De modo a minimizar um potencial efeito adverso do processo de criopreservação, o método testado teve como base o método *standard* slow cooling rate, utilizado na criopreservação de células em suspensão. Foram então definidas duas condições de criopreservação, a condição standard, com 10% do crioprotector Dimethylsulfoxide (DMSO), e a condição exprimental, com 5% DMSO. Com o objectivo reduzir a toxicidade para as células criopreservadas.

O efeito das condições de criopreservação na viabilidade celular foi analisado depois das *cell sheets* serem dissociadas, tendo sido demonstrado que ambas as condições de criopreservação não afectam de forma significativa a viabilidade celular. No entanto, verificou-se que a organização do citoesqueleto das células na *cell sheet* sofreu alterações depois da criopreservação em ambas as condições, verificando-se uma desorganização mais acentuada na condição *standard*. Verificou-se ainda que ambas as condições de criopreservação afetam a integridade da matriz extracelular das *cell sheets*, embora pareça que a condição *standard* afecte de um modo mais significativo. Mais ainda ambas as condições de criopreservação afectaram a quantidade total de proteínas. Potencialmente, este resultado está associado com as proteínas da matriz laminina, fibronectina e colagéneo I. De facto, a expressão destas proteínas excepto o colagéneo foi afectado tanto a nível molecular e proteico.

Mais ainda verificamos a expressão dos seus genes por reacção em cadeia da polimerase (PCR). Onde a nível molecular o gene da laminina está sobre expressa em ambas as condições de criopreservação, o gene da fibronectina apenas na condição exprimental

e o gene do colagéneo não sofre alterações significativas em ambas as condições de criopreservação.

Considerando que as propriedades mesenquimais das células que compõe as *cell sheets*, são determinantes nos resultados previamente observados, a expressão dos marcadores típicos após a criopreservação foi analisado a nível genético e proteico, usando PCR e citometria de fluxo respectivamente.

Com base nos resultados obtidos, que demonstram que a matriz extracelular é significativamente afectada pelo processo de criopreservação, será necessário testar diferentes protocolos e diferentes métodos de criopreservação, no sentido de se obter uma melhor preservação da integridade estrutural da *cell sheet*, e portanto garantir a sua funcionalidade após transplantação.

Palavras-Chave: Cultura de Tecidos, Células Estaminais do tecido Adiposo, *Cell Sheets*, Criopreservação, DMSO, *Off-the-Shelf*, Engenharia de Tecidos, Medicina Regenerativa.

Abstract

Regenerative Medicine (RM) englobes the multidisciplinary and interdisciplinary field of Tissue Engineering (TE) that aims to repair or enhance tissue or organ function. The field of TE takes advantage of the properties of three-dimensional (3D) structures that combined with different cells allow to recreate the native environment of a tissue. These 3D structures are produced with synthetic or natural materials, and aimed to have the ideal physical, mechanical and chemical proprieties for a better performance of cells, thus promoting the tissue regeneration. In context of TE, stem cells (SCs) are combined with the 3D structures or *scaffolds*, allowing the creation of viable and complex substitutes for tissue regeneration. The SCs have been largely used in TE due to its high proliferative rate, self-renewal capacity, ability to differentiate into different cell lineages. In the last decade the mesenchymal stem cells have been widely used in tissue engineering, they have the potential to proliferate and maintain undifferentiated with the ability to self-renew and / or differentiate into different cell types. There are various sources of stem cells with different characteristics used in TE, where they have the greatest impact are derived from spinal cord, blood and adipose tissue, among others located in different areas of the human body.

The use of scaffolds, might promote an inflammatory response after transplantation, and the major part of used biomaterials are not biofunctional. One of the alternatives to solve this problem is the production of constructs without the use of traditional biomaterials. The Cell Sheet Engineering is the alternative because it uses the extracellular matrix deposited by the cells as a natural scaffold for the regeneration of different tissues.

Cell sheet technology was originally proposed by Okano and co-workers, in early 90's. This technology takes advantage of thermo-responsive culture dishes that enable reversible cell adhesion to and detachment from the dish surface by a controllable hydrophobicity of the surface. By temperature change, a cell sheet with organized cellular entities and cohesive cell-to-cell and cell-ECM interactions is obtained.

In previous studies in our laboratory we generated cell sheets from human stem cells derived from adipose tissue (hASCs) that after transplantation in mice full-thickness excisional skin wounds, induced the formation of rete ridged-like structures and a significant number of hair follicles. Considering these results and envisioning the possibility of having cell sheets available off-the-shelf for immediate use in the clinic, to have these structures cryopreserved would be advantageous.

The goal of this work was to define a cryopreservation methodology that allows the preservation of both cells viability and the properties of CS extracellular matrix (ECM).

hASCs obtained from three different donors, were cultured in UP cell thermoresponsive dishes, to form hASCs-CS. Different cryopreservation conditions were considered, by varying the concentration of DMSO: i) standard condition with 10% of DMSO used to cryopreserve cell suspension; and ii) experimental condition with 5% of DMSO to reduce the cytotoxicity.

The effect of cryopreservation conditions over cell viability was analysed after dissociation of the CS. The results showed that both cryopreservation conditions do not significantly affect cell viability. However the cytoskeleton of cells suffered alterations after cryopreservation in both conditions, which were more evident in the standard condition. It was also found that both cryopreservation conditions affect the integrity of the extracellular matrix of cell sheets, although it appears that a standard condition affecting more significantly. Furthermore, after cryopreservation the amount of total protein, decreased to half, which indicates that both conditions of cryopreservation affects the extracellular matrix content.

Potentially, this result is associated with matrix proteins laminin, fibronectin and collagen type I. In fact, these proteins other than collagen was affected both molecular and protein level.

Moreover we found the expression of their genes by polymerase chain reaction (PCR). Where the molecular level, the laminin gene is over expressed in both the cryopreservation conditions, the fibronectin gene only in experimental condition and collagen gene does not change significantly in both the cryopreservation conditions.

Whereas the properties of mesenchymal cells that comprise the cell sheets are determining the results previously reported, the expression of typical markers following

cryopreservation was examined at the genetic and protein level using PCR and flow cytometry, respectively.

Based on the results obtained, showing that extracellular matrix is significantly affected by cryopreservation, is to experiment with different protocols and different methods of cryopreservation. In order to obtain a better preservation of the structural integrity of the cell sheet, and thus ensuring its functionality after transplantation.

With this thesis, it was possible to open routes to target a suitable cryopreservation methodology applied to hASCs-CS, which enables an off-the-shelf TE and RM strategy.

Keywords: Tissue Culture, Adipose Stem Cells, Cell Sheets, Cryopreservation, DMSO, Off-the-Shelf, Tissue Engineering, Regenerative Medicine.

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EQUATION

Abbreviations

3H – Three hours
7AAD - 7-amino actinomycin D
90/10 – 90% of FBS plus 10% of DMSO
95/5 – 95% of FBS plus 5% of DMSO

α-MEM – Minimum Essential Alpha
β-actin – Beta - actin
ºC – Celsius degrees
%SD – Percentage of Standard Deviation

AF – Alexa Flour

BSA - Bovine Serum Albumin

Cal - Calcein
CD - Cluster of differentiation
CPA- Cryopretective agent
CS- Cell sheet

DAPI- 4',6-diamidino-2-phenylindole
DMSO- Dimethylsulfoxide
DNA – Deoxyribonucleic acid

ECM- Extracellular matrix

FBS – Fetal Bovine Serum

hASCs- human Adipose Stem Cells
hASCs-CS – human Adipose Stem Cells- Cell Sheets
HRP- Horse Radish Peroxidase

ICC - Immunocytochemistry
ISCT- International Society for Cellular Therapy

LCST- Lower Critical solution temperature

mRNA - messenger RNA
MSCs- Mesenchymal Stem cells

ON- overnight

PBS – Phosphate Buffered Saline
PCR – Polymerase Chain Reaction
PFA- Paraformaldehyde
Pi – Protease innibitor
PI – Propidium iodide
PIPAAm – Poly(N-isopropylacrilamide)
PVDF – Poly(vinylidene diflouride)

qRT-PCR – Quantitative reverse transcription PCR

RM- Regenerative Medicine
RT- Room temperature
RT – Room Temperature

SDS- Sodium docedyl sulfate
SDS-PAGE- Sodium docedyl sulfate polyacrilamide gel electrophoresis
SVF- Stromal Vascular Fraction

TBS- Tris Buffered Saline
TE- Tissue Engineering
TCPS – Tissue Culture Polystyrene
TR - Thermoresponsive

CHAPTER I

INTRODUCTION

I. Introduction

1.1. Regenerative Medicine

Regenerative Medicine (RM) is area of research which attempts to develop methods to regenerate damaged tissues, creating substitutes in the laboratory and safely transplanting them into the patient when the body cannot heal itself [1,2,3,4,5].

RM is a biomedical technology, aimed at the regeneration and repair of the body. Based on biology and inspired in technology RM integrates regenerative, stem cell (SCs) and tissue biology. RM involves tissue regeneration, cell proliferation, apoptosis, differentiation, dedifferentiation, transdiferentiation and extracellular matrix and not only isolation, propagation, directed differentiation and transplantation of embryonic or adult SCs.

A main strategy used in RM is cell-based therapy based on SC, which can self-replicate for undefined periods and under the proper environment can differentiate into mature cells that have characteristic and specialized functions of a tissue[6].

RM includes the multi- and inter-disciplinary field of Tissue Engineering (TE) which was first defined by Langer and Vacanti in 1993 as "a field that applies the principles of engineering and life sciences towards the development of biological substitutes to restore, maintain, or improve tissue function"[7]. In the context of this work, MSC are of particular interest for TE applications[8–11].

1.2. Mesenchymal Stem Cells and applications

Mesenchymal Stem Cells (MSCs) were first isolated from bone marrow by Friendenstein in 1976. MSCs are tight adherent spindle-like cells which are able to form single-cell colonies (Zhao et al). These cells have the ability to differentiate into a number of cell types.

MSC are ubiquitous and have been found in adipose tissue[12], muscle[13], liver[14], lung[15], umbilical cord blood [16,17], amniotic fluid[18], among others [19,20].

Defining their cellular phenotype has proved problematic. In 1999 Pittenger et al proposed that hematopoietic MSCs are positive for CD105, CD166, CD54, CD55, CD13

and CD44. Conversely, they must be negative for CD34, CD45, CD14, CD31 and CD133[21]. In 2006 a standardised definition multipotent MSC was by the International Society for Cellular Therapy. A MSC the must express the cell surface markers CD105, CD73 and CD90, and lack the expression of the cell surface markers CD45, CD34, CD14 or CD11b, CD79a or CD19 and HLA-DR. Additionally, MSCs must differentiate into osteoblasts, adipocytes and chondroblasts *in vitro* [22,23].

However, MSC are highly heterogeneous and have variable expression of surface markers. Therefore, they can be considered as a mix of progenitor cells with varying degrees of replicative/differentiation potential[24,25]. Zhao et al. [26] recently reported the existence of a very small subset of MSCs from bone marrow, that are capable of extensive self-renewal and possess pluripotency. Collectively, MSC have shown the ability to differentiate (in *vitro* or *in vivo)* into adipocytes, chondrocytes, osteoblasts, myocytes, neurons, hepatocytes, and pancreatic islet cells[13,27].

MSCs are being actively investigated for regenerative medicine purposes due their plasticity, multipotentiality, high expansion rates and their immunosuppressive properties[28,29,30] They can be easily obtained from multiple sources and isolated in laboratory[31], and hold great promise for treating many diseases and disorders. The therapeutic application involves the transplantation of autologous or allogeneic MSCs into patients, by local delivery or systemic infusion. Efficiency of delivery remains a problem: in the case of systemic infusion less than 1 % of the total injected cells reach and home to the desired location due to the lack of MSCs homing receptors [32].

MSCs therapy approaches rely on their multilineage differentiation capacity and their immunomodulatory characteristics. MScs can secrete cytokines and growth factors that are anti-apoptotic, proangiogenic and have the potential to reduce scarring and inflammation. The main examples of therapeutic applications with MSCs are related with the myocardial infraction[33], the osteogenesis imperfecta[34], large bone defect[35], metachromatic leuko dystrophy (MLD) and Hurler syndrome[36], and severe idiopathic aplastic anemia[37].

MSCs can be combined with biodegradable scaffolds, with desired mechanical properties, and cell-biomaterial constructs further cultured *in vitro* culturing prior to *in vivo* implantation[7,38,39,40]. However, biomaterials pose a number of technical challenges.

A different and innovative tissue engineering strategy has arisen with the advent of cell sheet engineering technology, an approach that avoids the use of scaffolds.

1.2.1. Adipose Stem Cells

One of the main sources of MSCs is adipose tissue, which contains a supportive cell rich stroma that can be easily isolated through liposuctions procedures from the inferior abdomen[41,22]. Adipose tissue provide a significant number of ASCs, exhibiting a yield 40-fold greater than bone marrow, and with a stable growth and proliferation kinetics in culture[42]. These MSCs isolated from fat tissue are commonly termed Adipose derived-Mesenchymal Stem Cells (ASCs). They present the same potential to differentiate in vitro under proper conditions, into adipogenic, osteogenic and chondrogenic lineage[43]. ASCs isolated from adipose tissue maintains the similarity with bone marrow MSCs, in terms of surface markers. ASCs express CD29, CD44, CD90, and CD105 but are negative for CD13, CD31, CD34, CD45, and HLA-II[44]. They also show an hypoimmunogenic status that enables them to evade immune recognition and thus modulating the immune responses after in vivo transplantation[45]. ASCs are also able to stimulate the recovery of an injured or diseased tissue, in paracrine way, secreting cytokines and growth factors[46] Thus ASCs are promising type of adult SCs for regenerative medicine in cell therapy and TE.

1.3. Cell Sheet engineering technology

Cell Sheet (CS) engineering , originally proposed by Okano and co-workers, in early 90's[47],avoids the shortcomings associated with traditional TE approaches which use materials to form scaffolds, as it is a scaffold-free approach.

Scaffold free technology aims to explore the inherent capacity of cells to produce extracellular matrix (ECM) to create functional 3D tissues[48] and avoids the use of exogenous biomaterial scaffolds. The process can be promoted by exogenous stimuli, such as growth factors, enzymes, and mechanical stimulation, and often follows standard steps of cell condensation, cell proliferation, cell differentiation, ECM production, and tissue maturation.

This technology takes advantage of thermoresponsive (TR) culture dishes that enable cell detachment from the dish surface (Figure 1). This is achieved by coating ordinary

polystyrene surfaces such as Petri dishes with a TR polymer such as poly(N-isopropylacrylamide) (PIPAAm)[49,50]. PIPAAm is a temperature-dependent anionic reversible polymer with separation phase in aqueous solutions. At 37°C, the isopropyl side groups are hydrophobic, improving cell adhesion, proliferation and differentiation. At 20°C (below the lower critical solution temperature (LCST) of the polymer) the surface becomes hydrophilic. By lowering the temperature to 20°C, cell adhesion is inhibited and the cells detach as a sheet, avoiding the traditional proteolytic enzyme treatment (Figure I-1)[51,52,53,54]. The ECM secreted by the cells in culture is the natural scaffold of CS and therefore the use of external materials to create a structure is avoided. This procedure allows maintenance of cell-to-cell and cell-ECM junctions to keep the structure of the cell sheet intact and artificial tissue-like structures with organized cellular entities and cohesive cell-to-cell and cell-ECM interactions can be obtained[81,81,83].

Other surfaces engineered with smart polymers which react to different stimulus as voltage as gold surfaces[55], pH[56,57] and Ionic strength[58] are being developed. However, these surfaces have not reached clinical application.

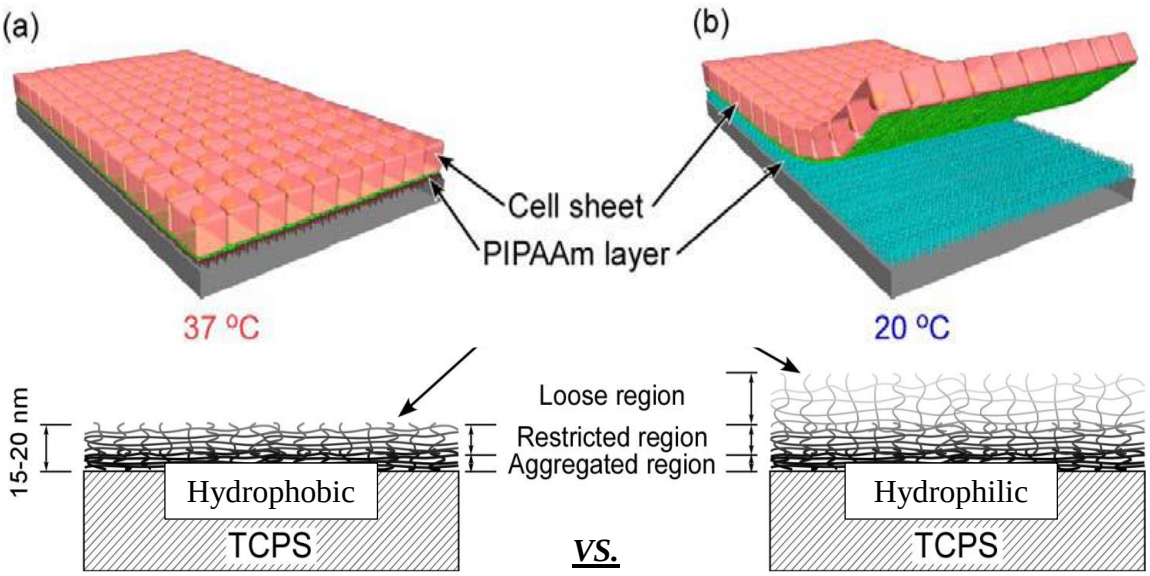

Figure 1 - Features and functioning of surfaces. Adapted from[59].

1.3.1.1. CS Applications in Regenerative Medicine

CS have great potential for regenerative medicine applications where cell surfaces are required, for example in tissues such as cardiac[60], and skin[61,62] and corneal epithelium[63] grafts, as well as to regenerate urothelium[64] and periodontal ligament[65].

The first example of creation of functional tissue for direct transplantation to host tissues[53,66–68,69] for clinical application of CS engineering was cornea replacement. Corneal ephetilial SCs were isolated from corneal epithelia, expanded in TR dishes and recovered by temperature decrease to transplant as a sheet in patients with ocular trauma[70]. Besides improved visual acuity, this alternative strategy avoids the opacity of traditional scaffolds or substrate carriers[66,63]. An autologous strategy for direct transplantation has been also proposed for skin[71], periodontal ligaments and bladder regeneration (Figure 2A)[72].

Three-dimensional constructs are required when thick and cell dense tissues are needed in regenerative medicine applications. Therefore, CSs have been manipulated into three-dimensional (3D) constructs by layering them (Figure 2). For example, one approach used cardiac myocytes CSs to recreate cardiac tissue[60], preserving their functionality and synchronized pulsations (Figure 2B). A 3D four-layered CS construct of neonatal rat cardiac myocyte was subcutaneously transplanted into rats. Microvascular networks developed and the implant was capable of spontaneous beating[66].

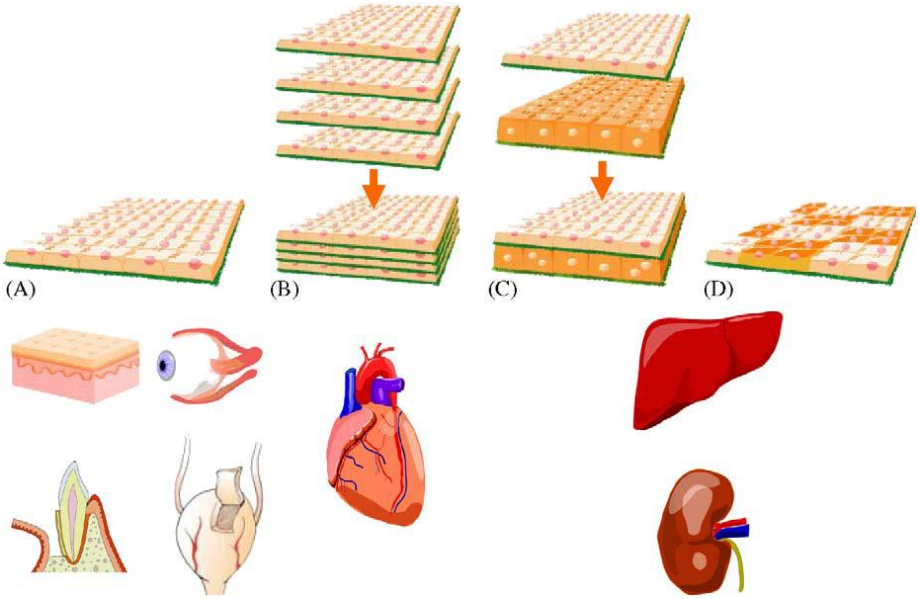

Figure 2 – Tissue-like substitutes obtained by CS engineering. (A) Application of Single CS transplantation for cornea replacement and regeneration of skin, periodontal ligament and bladder. (B) Stacking of several CS for the improved performance of cardiac tissue[73]. (C and D) The Liver and the kidney, tissues with laminar and higher-order structures, can also be recreated with CS engineering. Adapted from[66].

A good example of success was the work of transplantation of CSs into recipients was developed by Masayuki Yamato, who developed CS with functional Conexin 43 positive gap junctions which mediate the electrical communication through layered cell sheets similarly to what happens in the heart[54]. In another work T. Shimizu developed a construct composed by 30 cardiac myocytes sheets staked (approx. 1mm) was successfully implanted in recipients by polysurgery, successfully avoiding hypoxia, nutrient insufficiency and/or waste accumulation[74]. Bone tissue regeneration is another area where the CS technology has shown promising results. Pirraco et al. [75] have used osteogenic CS to promote the new bone formation when implanted subcutaneously into nude mice. Cerqueira *et al.* showed the use of CS technology to engineer heterotypic CSs based human keratinocytes (hKC), dermal fibroblasts (hDFb), and the dermal microvascular endothelial cells (hDMEC); the resulting CS can participate in wound re-epithelialization, remodulation of the ECM and modulation of angiogenesis within the dermal vascular network[62]. This author further developed cell sheets from human stem cells derived from adipose tissue (hASCs) that after transplantation into mice with full-

thickness excisional skin wounds, induced the formation of rete ridged-like structures and a significant number of hair follicles.

CS constructs are trending towards greater complexity, vascularization and thickness. As the majority of the tissues present within the human body comprise several cell layers of parenchyma and epithelia surrounded by ECM, CSs have the potential to create functional structures with proper thickness for cell dense tissues as the heart, the liver, the muscle and the kidney[68]. The layering of CSs will also allow the creation of heterotypic CSs. Further progress may come from combining CS technology with nanofabrication techniques, including micropatterning[76–79] and biomolecule-immobilization[80,81] to develop new cell culture surfaces. Micropatterning methods allow two polymers with different LCSTs to be coated on the same surface, permitting selective cell adhesion under temperature controlled environments[82]. In addition, the immobilization of bioactive molecules on TR surfaces of culture dishes may remove contaminant components from mammalian sources, allowing the fabrication of CSs under serum-free conditions[83,84].

In sum, CS technology holds great promise. However, for this approach to be practical in a clinical setting, cryopreservation techniques and procedures must be developed that preserve both the cellular and extracellular components of the structure.

1.4. Cryopreservation

Cryopreservation of living cells and tissues basic technology used in biology, biotechnology, plant and animal breeding programmes, cell culture, microbiology and also in modern medicine[85].

In regenerative medicine approaches the time between tissue construct production and clinical application can be long. For the technology to be practical, effective preservation methods and off-the-shelf strategies[86,87,88,89,90] are crucial. Cryopreservation methods to preserve cells in suspension are well established, but the preserve of complex multicellular living structures, such as biological tissues, remains a challenge.

Ideally, cryopreservation methods should avoid ice crystal formation and cell shrinkage inside bulky constructs[91].

Currently, two major cryopreservation processes are in use. The, first is freezing method based on gradual cooling at variable rates; the second is vitrification [92].

1.4.1. Cryoprotetants

Cryoprotetants (CPA) are substances that minimize the formation of ice crystals intracellular and/or extracellularly during the freezing process, resulting in increased cell viability and metabolic activity, as well as conservation of morphologic structure and tissue architecture upon thawing. Cryoprotetants can be used individually or in combination[122, 93].

A crucial parameter is the quantity of CPA used in cryosolutions. High concentrations of CPA favour glass formation and minimize damage induced by ice crystal formation,

The presence of solutes in cryosolutions promotes the decrease of the freezing point, which increases exposure at low temperatures and consequently decreases cell damage during the cryopreservation process.

Another effect is the direct alteration of the semi-permeability of cell membranes and consequently the osmotic equilibrium during the freezing process.

CPA are divided into two main groups based on permeability: **permeable** and **non-permeable.** Furthermore, the non-permeable are classified by their molecular weight

(MW): (i) low MW compounds; (ii) intermediate MW compounds (sugars); (iii) and high MW compounds (Table1)[92].

Table 1. Cryoprotetants. Adapted from [92].

Permeable	***Non-permeable***	
Low molecular weight MW < 100 Da	**Sugars** 180 < MW < 594 Da	**High molecular weight** MW >1000 Da
Ethylene glycol	Glucose	ε-Poly-L-Lysine (ε-PLL)
Dimethyl sulfoxide (DMSO)	Fructose	Polyvinyl pyrrolidone (PVP)
Propylene glycol	Lactose	Dextran
Glycerol	Sucrose	Polyethylene glycol (PEG)
1,2-/2,3-butandiol	Trehalose	Polymer polyvinyl alcohol (PVA)
Formamid, acetamid	Raffinose	Hydroxyethyl starch (HES)

1.4.1.1. Permeable Cryoprotetants

The majority of permeable CPA are hydro-soluble, avoiding the water-to-water hydrogen bonding and consequently the ice formation at low temperature[122, 23]. The high concentration of salts is reduced in the presence of permeable CPA, as glycerol, which possess high viscosity levels at low temperatures and kinetically retards ice crystal growth. Despite all these protective properties, permeable CPA have shown deleterious effects to cells, mainly at high concentrations, and it is possible to distinguish between the osmotic and the chemical toxicity[94]. Osmotic toxicity occurs when permeable CPA is added in proper concentrations and cross the biological membranes entering at a rate largely different to the exit of water. If the CPA enters slower than water exits the cell, the osmotic force becomes higher in the extracellular space causing a damage to cell by over shrinking. If the CPA enters faster than water, cell cytoplasm will swell until disruption of the membrane occurs. Chemical toxicity is more complex due to the large range of existing permeable CPA (Table 1) and to the fact that each one of them interacts in a different way with different cells. Moreover, permeable CPA are relatively

innocuous in comparison to solutes with similar concentrations, causing a time and temperature-dependent effects in the majority of cases[89,94].

Examples of permeable CPAs are methanol, ethylene glycol, dimethyl sulfoxide (DMSO), propylene glycol and glycerol (Table 1). Among these, the most effective permeable CPAs for cryopreservation are glycerol, a small poly-hydroxylated solute, and DMSO, an organosulfur compound. Both are highly soluble in water and show low toxicity during short-term exposure to living cells[95]. Dimethyl sulfoxide (DMSO) is the most efficient and more frequently used CPA to cryopreserve different types of cells, tissues, organs, as well as tissue-engineered constructs. DMSO is a polar solvent that can stabilize cellular proteins by increasing the freezing energy of unfolding, via preferential exclusion mechanisms, and can also stabilize the plasma membrane through interacting electrostatically with the phospholipids bilayer at higher concentrations [96,97]. Additionally, DMSO was recently shown to scavenge oxygen free radicals, which enhances CPA action[98]. On the other hand, cryopreservation with DMSO may cause gene mutation in cells[99] and the interaction between the DMSO and the hydrophobic residues of cell proteins may promote their denaturation and destruction[100]. To avoid these negative effects the levels of DMSO are reduced in the freezing solutions, thus eliminating the need for CPA removal, making the thawed cells available for direct implantantion[101].

1.4.1.2. Non-permeable Cryoprotectants

Non-permeable CPA are mainly used in vitrification protocols due their capability to promote a fast dehydration before the direct exposure to liquid nitrogen (LN)[92]. The amount of non-permeable CPA required for dehydration prior to vitrification should be carefully dosed in order to avoid an over-dehydration of biological material to a point beyond recovery. Non-permeable CPAs act on the extracellular surface of the cell, creating a hydrated layer that causes osmotic shrinkage of the cells and join an ice front during freezing[92]. The low presence of intracellular water prevents completion of intracellular ice formation, acting as a mechanism of protection during cryopreservation. Both non-permeable CPA sugars and polymers have a low toxicity risk since they cannot penetrate the cell membrane[93,100]. The use of high concentrations of non-permeable

CPA demands a permanent evaluation of small variations of MW or purity level, because these parameters can dramatically alter cell survival and viability during the vitrification procedure. Regarding sugars, the principal influence is the alteration of osmotic equilibrium of buffers[93,92]. These small carbohydrate sugars, such as trehalose, sucrose and maltose have the capacity to stabilise the bilayer membranes during the hypertonic exposure and ice crystals formation since they interact with the polar head of phospholipids. In addition, they can stabilize and preserve proteins, as well as to form a stable glassy state[92,93].

Among the sugars, the most effective as non-permeable CPA is trehalose, a non-reducing disaccharide of glucose that is found at high concentrations in a wide variety of organisms that are capable of completely survive to dehydration. Furthermore, when trehalose is combined with other permeable CPAs, mainly DMSO, it improves the protective capabilities of cryosolutions[102].

Trehalose is a small carbohydrate sugar (a non-reducing dissacharide of glucose) used as a non-permeable cryoprotectant in solutions of cryopreservation. It is one of the most effective non-permeable CPA. By interacting with polar head of phospholipids[88] it stabilises cellular membranes during hypertonic exposure.

Moreover, trehalose has the ability to stabilize and preserve proteins, viruses, and bacteria as well to form a stable glassy state. Trehalose is often used in combination with DMSO, resulting in an alteration of osmotic equilibrium of cryopreservation buffers[92,93].

In the case of polymers, the hydrogen bonding sites of molecules of water interact with the polymer side chains, and their high MW increases the viscosity at low temperatures impeding intracellular ice formation[92]. A polymer that shows a high efficiency on cryopreservation is the carboxylated poly-L-lysine (ε-PLL). Ε-PLL is a cationic polymer that is adsorbed electrostatically to cell surfaces due to its positively charged hydrophilic amino groups [85] .

ε-PLL is a non-permeable CPA used in cryosolutions, due its antifreeze proprieties of low toxicity. It is a cationic polymer positively charged by hydrophilic amino groups. In presence of water it is adsorbed electrostatically to the surface of cells. The high molecular weight increases the viscosity at low temperatures which allow the inhibition

of ice recrystallization and a successful protection of cellular membranes during cryopreservation[92].

1.4.2. Methods of Cryopreservation

1.4.2.1. Freezing

1.4.2.1.1. Slow Cooling

The slow cooling rate is the conventional method of cryopreservation that preserves cells using liquid nitrogen without notorious damages to the cryopreserved cells[98]. The optimal cooling rate of 1ºC per minute allows a partial frozen state that maintains the osmotic equilibrium between the extracellular and intracellular compartments in a process called dehydration. The process requires a controlled rate freezer[103]. However, slow cooling has the disadvantage of subjecting the cells to temperature stress and unfavourable culture conditions leading to intracellular ice formation, and consequently mechanical stresses. All these events increase the possibility of fracture causing irreparable damages on the cryopreserved cells/tissues[104].

1.4.2.1.2. Rapid Cooling

The rapid cooling method appears as a profitable procedure to optimize the process of cryopreservation. However, the rapid rate of cooling enables the permeation of water inside the cells, leading to intracellular ice formation and eventually cell damage and death[105]. The thawing process can also result in extracellular ice formation, due the insufficient solute concentration [92]. The cryopreservation with rapid cooling method of living cells is more efficient supported by adding cryopreservation solution to prevent ice formation during the entire procedure but it still leads to significant cell damage[106].

1.4.2.2. Vitrification

Vitrification is defined as glass-like solidification that is achieved by partial replacement of water by penetrating agents which are easy glass formers and subsequent fast dehydration of biological material by non-penetrating cryoprotectant[92]. It is an alternative method to traditional cryoprotetant based freezing [107]. This method does not require the use of specialized material to control freezing rate and the solutions do not crystalize, thus preserving in a more effective way the cells and tissues during the cooling process. Contrarily to what happens in the slow cooling rate, the high concentration of intracellular solutes during dehydration is avoided[107]. Despite this, improvements to this cryopreservation are needed in order to maintain a constant physiological temperature along the entire procedure, including in the equilibration and dilution processes.

1.4.3. Cryopreservation in Tissue Engineering

Cryopreservation is a tenable solution for the maintenance of “living” biomaterials being produced and applied in TE. The major obstacle found in TE is the preservation and the storage for long-term without the loss of the desirable properties that constructs have prior cryopreservation[85].

1.5. Objective

The goal of this work was to develop a cryopreservation methodology that would allow the preservation of both cell viability and extracellular matrix (ECM) properties in CS.

1.6. Experimental Strategy

hASCs obtained from three different donors, were cultured in UP cell thermoresponsive dishes, to form hASCs-CS. Two cryopreservation conditions were tested, by varying the concentration of the cryoprotectant DMSO: i) a standard condition with 10% of DMSO, commonly used to cryopreserve cell suspensions; and ii) an experimental condition with 5% of DMSO to reduce the cytotoxicity.

CHAPTER II

MATERIALS AND METHODS

II. Materials and Methods

Materials

2.1. Tissue Culture Polystyrene Surfaces

Tissue Culture Polystyrene Surfaces (TCPS) T150 cm2 flasks, were used for isolation and expansion of hASCs.

2.2. Thermoresponsive Dishes

Thermoresponsive dishes, grafted with poly (N-isopropylacrylamide), were used to generate cell sheets of ASCs. This technology avoids proteolytic enzyme treatment allowing the harvesting of confluent cells as intact CS along with their deposited Extracellular Matrix (ECM). A poly(vinylidene difluoride) (PVDF) membrane (Milipore, U.S.A.), with 2 cm of diameter to carrier the cell sheets.

2.3. Cryopreservation

Cryopreservation of hASCs-CS by slow cooling rate was possible due the Controlled-rate freezing container alcohol-free CoolCell (BioCision LLC, USA), freezing at – 1 ºC per minute, and successful cryopreservation. The paten-pending Cool Cell technology utilizes a thermo-conductive alloy and highly-isolative outer materials to control the rate of heat removal and make cell cryopreservation reproducible.

To retain optimal viability of cell sheets, cryopreserved during 7 days at -196ºC, was used the Liquid Nitrogen (LN) tank.

During all process of cryopreservation cell sheets were kept in 2mL cryovials (Nunc,U.S.A.).

2.4. Characterization

2.4.1. Flow Cytometry

All assays of flow cytometry were performed on FACSCalibur flow cytometer (BD Biosciences, Belgium) and data was analyzed using Cell Quest Pro version 4.0.2 (BD Biosciences, Belgium) software.

2.4.2. Quantification of Total Protein

For quantification of total protein were used the Commercial Kit BCA DCTM Protein Assay (Bio-Rad/USA).

2.4.3. Complementary Deoxyribonucleic acid synthesis

The purity of Ribonucleic acid (RNA) of the different samples was assessed using Nanodrop" ND-1000 spectrophotometer (NanoDrop Technologies, U.S.A.).

This was carried out with the qScript cDNA synthesis kit (Quanta Biosciences, U.S.A.), according to the manufacturer's instructions in a MJ MiniOpticon Real-Time PCR Detection System (Bio Rad, U.S.A.).

2.4.4. Microscopy Analysis

All samples were observed under AxioImager Z1M microscope (Zeiss, Germany) and images were acquired and processed with Zen Blue 2012 software (Zeiss, Germany).

2.4.5. Transmission Electron Microscopy

TEM was performed on Institute for Molecular and Cell Biology (IBMC) facilities, following the standard protocol.

Ultrathin sections (40-60 nm thickness) were prepared on a LKB 2188 NOVA Ultramicrotome (LKB NOVA, Bromma, Sweden) using diamond knives (DDK, Wilmington, DE, USA). The sections were mounted on 300 mesh nickel grids, stained with uranyl acetate and lead citrate, and examined under a JEOL JEM 1400 **TEM** (Tokyo, Japan). Images were digitally recorded using a Gatan SC 1000 ORIUS CCD camera (Warrendale, PA, USA).

Methodologies

2.5. Stem Cells isolation and culture from adipose tissue

Human subcutaneous adipose tissue samples were obtained from liposuction procedures at Hospital da Prelada (Porto, Portugal). Samples were obtained after informed consent and under a collaboration protocol with 3B's Research Group. That was approved by the ethical committees of both institutions, ensuring the patient's agreement for the collection of samples, as well as patient's anonymity.

Human subcutaneous adipose tissue samples were transported under controlled temperature conditions in Phosphate Buffer Saline (PBS) with 10% (v/v) Antibiotic (AB) (Alfagene, Lifetecnhologies, U.S.A.), and processed within 24 hours after collection. Samples were washed with PBS to make sure that the majority of the blood was removed, and digested with 0.05% (v/v) collagenase type II (Sigma, U.S.A.) under agitation, for 45 minutes at 37ºC [108]. After that, samples were filtered and centrifuged at 800g during 10 minutes to obtain a pellet corresponding to the stromal vascular fraction (SVF). Red blood cells in the SVF were lysed by incubation with blood cell lysis. The buffer were composed by 155mM of ammonium chloride, 12mM of potassium bicarbonate and 0.1M of ethylenediaminetetraacetic acid (all the reagents were supplied by Sigma-Aldrich, Germany) in deionized water, for 10 minutes at room temperature (RT). After centrifugation at 300g for 5 minutes, the red blood cells-free SVF was resuspended in Minimum Essential Medium Eagle (α-MEM) (Lifetecnhologies, U.S.A.) supplemented with 10% (v/v) Fetal Bovine Serum (FBS) and 1% (v/v) AB (Alfagene, Lifetecnhologies, U.S.A.), and plated in tissue culture polystyrene (TCPS) flasks. The cell medium was replaced every 48 hours of culture. Adherent human adipose stem cells (hASCs) were selected after 5 days of culture and harvested at 90% (v/v) confluence along the passages. All the experiments were independently performed with 3 donor samples (Table 2).

Table 2. Donor information.

Sex	***Age***
Female	55
Female	59
Female	68

2.6. Cell sheets Fabrication

hASCs, at passage 2 , were plated in UP cell 35mm TR dishes (Nunc, Thermo Scientific, Denmark) or six well plates (Falcon, Corning, U.S.A.) at a cell density of $3x10^5$ cells/cm^20, and cultured for 5 days in α-MEM medium, supplemented with 10% (v/v) FBS, 1% (v/v) AB and 50µg/ml of ascorbic acid (WAKO, Japan) at 37ºC, in a humidified atmosphere with 5% CO_2, according to what was previously described[71]. Culture medium was replaced after 3 days of culture.

After 5 days, CSs were recovered from TR dishes by temperature decrease. hASCs were washed twice with fresh culture medium and then left in 100µL of PBS.

A poly (vinylidene difluoride) membrane (Milipore, U.S.A.), with 2cm of diameter was placed on top of CS-hASCs and maintained for 20 minutes at RT. After this time, CS border was carefully folded onto the membrane and CS was recovered. Protocol was replicated for cells cultured in six-well plates and recovered mechanically.

2.7. Cell Sheets Cryopreservation

Cryopreservation of cells in suspension commonly performed using solutions with Dimethyl Sulfoxide (DMSO) as principal cryoprotetant at a concentration of 10% (v/v), using the slow freezing method.

For cryopreservation was used two different cryosolutions, the condition commonly adopted for cryopreservation of cellular suspensions, namely 90% (v/v) FBS and 10% (v/v) DMSO (90/10). Alongside, envisioning a decrease of DMSO amount, other solution composed by 95% (v/v) FBS and 5% (v/v) DMSO (95/5) was also investigated.

The CSs were retrieved, and then carefully placed into 1.5mL of cryopreservation solution in 2 mL cryotube™ vials (Thermoscientific, Denmark). Cryotubes were then placed in a controlled-rate freezing container CoolCell (BioCision LLC, U.S.A.), which was then quickly placed in a –80ºC freezer allowing a slow cool rate freezing at –1ºC per minute overnight. Cryotubes were transferred to a Liquid Nitrogen (LN) tank at -196ºC and left for 7 days before thawing and analysis of CS.

2.8. Cell Sheets thawing

After 7 days of cryopreservation in LN, cryotubes were placed on ice until be thawed at 37ºC following the rapid-thawing methodology. This principle of a quick heat shock avoids major damages to hASCs-CS.

The recovery process was performed by washing and rehydrating, mainly consisting on CS removal with the carrier from the cryotube and rinsed 3 times in α-MEM. The CSs were incubated within α-MEM in three different temperatures, each taking 30 minutes, including RT, followed by an increasing of temperature up to 37ºC, and a stabilization period at the same temperature. All the samples were collected after 3 hours (3H) of equilibrium.

2.9. Flow Cytometry Analysis

Flow cytometry allows to characterize the phenotype of any cell population through the quantification of expression of surface and intracellular characteristic markers. Single cells flow in a fluid stream through a beam of laser light to analyse and simultaneously measure the relative size, internal complexity and fluorescence intensity[109].

In this study, this technique allowed the characterization of several surface markers related with the mesenchymal phenotype after isolation of the different hASCs populations, selection and expansion at different passages. To perform flow cytometry analysis, hASCs (section II-2.5) cultured in complete α-MEM were trypsinized and dispensed $1.5x10^5$ of cells per each flow cytometry tube (Falcon, U.S.A.).

2.9.1. Preparation of cellular suspension from Cell Sheets

Before and after cryopreservation CS were transferred to 15mL tubes containing 400µL of Collagenase Type II (Sigma, U.S.A.) 7µg/mL (Pzactivity0.9LU/mL) diluted in α-MEM and incubated for 20 minutes at 37ºC under agitation. Afterwards, 0.25% (v/v) Trypsin-EDTA were added and the CS was further incubated for 10 minutes at 37ºC with agitation.

The activity of collagenase and trypsin was inactivated using PBS with 10% (v/v) FBS, and the obtained cell suspension was centrifuged for 5 minutes at 1300 rpm. The supernatant was discarded, and the pellet was re-suspended in 500µL of PBS with 10% (v/v) FBS to obtain a single cell suspension.

2.9.2. Cells Labelling

2.9.2.1. Surface marker labelling

In order to confirm a mesenchymal phenotype, the expression of mesenchymal markers CD105, CD73, CD90 and of hematopoietic markers CD45, CD34 and CD31 was assessed. Moreover, it was evaluated the expression of human leukocyte antigen-DR (HLA-DR). For direct staining, different primary fluorophore-conjugated antibodies were used (Table 3). Antibodies were added to a 100µL cell suspension of 150000 cells, dissociated hASCs-CS, and incubated for 20 minutes at RT in the dark. After the incubation time, 2mL of PBS were added to each flow cytometry tube which were then centrifuged at 1800 rpm for 5 minutes at RT. Supernatant was carefully removed and the cell pellet was re-suspended in 500 µL of Acquisition Buffer (1% (v/v) paraformaldehyde in PBS).

2.9.2.2. 7AAD/Draq5 Stainning

7-AAD is a non-permeable cell membrane dye, thus generally excluded from viable cells, that binds to double stranded DNA by intercalating between base pairs in G-C-rich regions. Aiming at assessing the viability of the cells forming a CS before and after cryopreservation, cell suspension obtained after dissociation of the CS (section II-2.9.1) were incubated with a 7-amino actinomycin D (7-AAD) viability staining solution. In this case, 3µL of 7AAD viability staining solution was added to a $3x10^5$ cells in PBS 10% (v/v) FBS and incubated during 10 minutes at RT. In order to gate adequately the nucleated cells, fluorescent probe DRAQ5™ Far-Red Fluorescent Live Cell Permeant DNA Dye (eBioscience, Germany) was used. This reagent stains specifically DNA, to distinguish the nucleated cells from cellular debris. All assays were performed on FACSCalibur flow cytometer (BD Biosciences, Belgium) and data was analysed using Cell Quest Pro version 4.0.2 (BD Biosciences, Belgium) software.

Table 3. List of antibodies and dilutions used to perform Flow cytometry.

Cells	*Antibody/Host/Brand*	*Antibody Dilution*
hASCs	CD105-FITC, Rabbit (BD Biosciences, Germany) CD73-PE, Rabbit (BD Biosciences, Germany) CD90-APC, Rabbit (BD Biosciences, Germany)	1:25
	HLA-DR-FITC, Rabbit (BD Biosciences, Germany) CD31-APC, Rabbit (BD Biosciences, Germany) CD45-FITC, Rabbit (BD Biosciences, Germany) CD34-PE, Rabbit (BD Biosciences, Germany)	

2.10. Quantification of Total Protein

Before and after cryopreservation (section II-2.8) CS was stored in 150 µl of RIPA Buffer [187 mL dH2O; 50 mM Tris – 1.1725 g; 150 mM NaCl – 2.1915 g (dissolve); pH= 7.4 (with HCl 6M); 1% Triton X-100 – 2.5 mL; 0.5% Sodium deoxycholate – 1.25 g; 0.1% SDS – 0.25 g; Add dH2O until 250 mL; filter with 0,22 µm], supplemented with Protease Inhibitor Cocktail (Sigma, USA) in a 1:100 proportion.

Total protein was extracted by ultrasonication in an ice bath. After 3 minutes of centrifugation at 13000 rpm, 75 µl of the supernatant containing the total protein extract was diluted with 75 µl of Lamelli buffer in a 1:1 proportion and stored at -80ºC.

The quantification of total protein was performed with a colorimetric method based on the use of Bicinchoninic Acid (BCA), using a commercial Kit BCA DCTMProtein Assay (Bio-Rad, USA). The method is based on the ability of proteins, in an alkaline medium, to reduce $Cu2^{+}$ to $Cu1^{+}$, forming a purple complex. BCA assay was performed in a 96 well plate with remaining 75 µl supernatant of samples, mixed with 200 µl of the working reagent from commercial Kit BCA DCTM Protein Assay (Bio-Rad/USA). Working reagent was composed by BCA Reagent A [sodium carbonate, sodium bicarbonate, bicinchoninic acid and sodium tartrate in 0.1M sodium hydroxide] and BCA Reagent B [4% cupric

sulfate] in a 50:1 proportion. The optical density was measured at 562 nm. A standard curve of OD vs protein concentration was obtained using Bovine Serum Albumin (BSA) solutions in the range of 0 to 2000 mg/ml.

2.11. Real time reverse-transcriptase- Polymerase Chain Reaction (RT-PCR)

Real time RT-PCR provides a sensitive, reproducible, and accurate method for determining small quantities of RNA targets [110]. The principle is based on two principal finding, first the discovery of Taq polymerase that possess 5'-3' exonuclease activity. Second, the discovery of dual-labelled fluorogenic oligonucleotide probes, which emit a fluorescent signal only upon cleavage, where the amount of fluorescence released is directly proportional to the amount of product generated in each PCR[110].

The standard protocol is based on five steps: initial denaturation; denaturation; annealing; extension; and data acquisition. To analyse the data, the software of instruments of RT-PCR calculates the variation of fluorescence emission ($^{\Delta}$Rn), which is plotted against cycle number, determined as a threshold value (Ct). To normalize this quantification the choice of a reliable housekeeping gene is very important[110]. In our case, to calculate the relative gene expression the method proposed by Pfaffl (Equation 1)[111] was used, which is valid only when the amplification efficiencies of target and reference genes are similar.

Equation 1. Method of Pfaffl.

$$\text{Ratio} = \frac{(E_{target})^{\Delta CT,\ target\ (calibrator - test)}}{(E_{ref})^{\Delta CT,\ ref\ (calibrator - test)}}$$

2.11.1. Total RNA isolation

RNA of hASCs-CS was extracted using TRIzol® reagent to maintain the integrity of RNA during cellular components disruption. Therefore, 1.5mL of TRI® reagent (Sigma, Germany) was added to each hASCS-CS, prior and after cryopreservation. Samples was homogenized, kept overnight at -80ºC and then, chloroform (200µl/mL TRIzol®) was added. Samples were centrifuged at 13000 rpm for 15 minutes at 4°C, and the aqueous phase of each tube containing the RNA was transferred to a new Eppendorf. The precipitation of the nucleic acids was achieved by adding 750µl of isopropanol after homogenization and incubation at RT. The suspension was centrifuged for 10 minutes at 13000 rpm and 4°C in order to remove the isopropanol. The pellets were further washed by vortexing with RNAse-free 70% ethanol, at a 1:1 ratio to the volume of trizol. Once dried, the pellets were resuspended in a volume ranging between 10-50 ul of deionized water – RNAse free, depending on the size of the pellet.

2.11.2. Complementary Deoxyribonucleic acid synthesis

The purity of Ribonucleic acid (RNA) of the different samples was assessed using "Nanodrop" ND-1000 spectrophotometer (NanoDrop Technologies, U.S.A.). Only samples with a 260/280 ratio between 1.6 and 2.0 were used for complementary Deoxyribonucleic acid (cDNA) synthesis.

cDNA synthesis was performed from 2µg of RNA in a final volume of 40µL of samples before and after cryopreservation . For samples cryopreserved with 95/5 condition, 125ng of RNA in a final volume of 7.5µL were used, due the short amount of extracted RNA. This was carried out with the qScript cDNA synthesis kit (Quanta Biosciences, U.S.A.), according to the manufacturer's instructions in a MJ MiniOpticon Real-Time PCR Detection System (Bio Rad, U.S.A.).

2.11.3. Quantitative PCR (qPCR)

The expression of the genes of interest was measured by qPCR on system Real-Time PCR Mastercycler (Eppendorf, Germany) in 96-well plates, using the Perfecta SYBR® Green Fast Mix (Quanta Biosciences, U.S.A.). This method allows the amplification quantification of a target gene, through the emission of fluorescent dye that intercalates into DNA strands. Previously, all samples and primers were diluted in a proportion of 1:20[1] in water RNA-free. For the amplification reaction a mixture composed by 10µL of SYBR® Green (Quanta Biosciences, U.S.A.), 1.2µL forward (FW) and reverse (RV) primers, 6.6µL of H2O sterile and 1µL of cDNA was prepared per well for each gene of interest (Table 4). The specificity of the amplified fragments was confirmed by analysing the melting curves that were obtained by an increase of temperature from 55 to 95°C (0.033 °C/s). The relative expression of ECM genes and mesenchymal gene markers, between samples prior and after cryopreservation was calculated using the comparative method proposed by Pfaffl. Normalization of the results was performed with the internal control Beta-2-Microglobulin mRNA expression.

Table 4. Sequence of specific Primers and annealing temperatures used in Real-Time PCR for Amplification.

Gene and accession number	*Sequence (5'-3')*		*Annealing temperature (°C)*
CD90; NM_006288.3	FW	CAGCATCGCTCTCCTGCTAA	59
	RV	ACTGGATGGGTGAACTGCTG	
CD105; NM_001114753.2	FW	CTCCCTCTGGCTGTTGCC	60
	RV	ATATGTCACCTCGCCCCTCT	
CD45; XM_006711474.1	FW	TTGTGGCTTAAACTCTTGGCA	57
	RV	GGCTTTGCCCTGTCACAAAT	
CD34; NM_001025109.1	FW	ACCCTGATTGCACTGGTCAC	59
	RV	ATAAGGGTCTTCGCCCAGC	
CD73; NM_001204813.1	FW	TTTTGCACACCAACGACGTG	58
	RV	GAACCTTGGTGAAGAGCCGA	
HLA-DRA; NM_019111.4	FW	CCTGTCACCACAGGAGTGTC	60
	RV	TCCACCCTGCAGTCGTAAAC	
Beta-2-Micra;	FW	TGGAGGCTATCCAGCGT	60
XM_005254549.1	RV	CGGATGGATGAAACCCA	
Collagen; XM_006719242.1	FW	GTTCACGTACACTGCCCTGA	59
	RV	TCCACACCGAATTCCTGCTC	
Fibronectin;	FW	CTGGCCAGTCCTACAACCAG	60
XM_005246417.1	RV	CATGAAGCACTCAATTGGGCA	
Laminin; NM_005559.3	FW	TCACGGTGAACAGGTGCTAC	59
	RV	AGAGCTGCATATCCGCTTCC	

2.12. Cacein and Propidium Iodide Staining

Calcein-AM and Propidium Iodide (PI) assay are fluorescent dyes that enable to distinguish live from dead cells[113]. Calcein-AM is highly lipophilic and it is easily transported into living cells through the cell membrane. After entering a living cell is activated through an enzymatic reaction by the enzyme esterase, giving rise to another molecular product, Calcein-AM. In this form, Calcein-AM emits green fluorescence with excitation of 490nm and emission wavelength of 515nm, respectively visualization in live cells[113]. PI as a membrane impermeant dye binds to DNA by intercalating between the bases with little or no sequence preference and with a stoichiometry of one dye per 4–5 base pairs of DNA, when cellular membrane are disrupted. Because PI emits red

flourescense with an excitation between ~30-40 nm is commonly used as a counterstain to identify dead cells[114].

To assess cell viability, Calcein AM (Invitrogen, U.S.A.) (1µg/ml) and PI (Invitrogen, U.S.A.) (1µg/ml) were dissolved in α-MEM without FBS. The solution was then incubated with cells sheets of hASCs for 30 minutes at 37°C in the dark. hASCs-CS were observed under an AxioImager Z1M microscope (Zeiss, Germany) and the images were acquired and processed with Zen Blue 2012 software (Zeiss, Germany).

2.13. Phalloidin Staining

Phalloidin-Tetramethylrhodamine B isothiocyanate (Sigma, U.S.A.) was used to stain cytoskeleton F-actin fibers of hASCs-CS. Phalloidin conjugates have similar affinity for both large and small filaments and bind in a stoichiometric ratio of about one phallotoxin per actin subunit and do not bind to monomeric G-actin. After fixation with formalin, CS prior and after cryopreservation were incubated with Phalloidin-TRITC (1:100 in PBS) for 1 hour at RT and washed 3 times with PBS. Nuclei were counterstained with DAPI. Samples were then observed under AxioImager Z1M microscope (Zeiss, Germany) and images were acquired and processed with Zen Blue 2012 software (Zeiss, Germany).

2.14. Immunocytochemistry

This method follows the principle of antigenic antibody binding. The binding of specific antibodies allows the identification and localization of a specific protein in a cell structure or tissue. In this work, specific antibodies were used to target proteins present in the ECM and mesenchymal markers of hASCs-CS in order to confirm the maintenance of matrix organization before and after cryopreservation and demonstrate mesenchymal phenotype.

CS were washed and fixed with 10% (v/v) buffered formalin for 30 minutes at RT, washed with PBS and if necessary stored in PBS at 4°C. To avoid non-specific binding cells were incubated with a freshly prepared 3% (v/v) bovine serum albumin (BSA, Sigma, Canada) solution in PBS (BSA/PBS) for 40 minutes at RT. After this, CS were washed with PBS and then incubated with primary antibodies diluted in 1% (v/v) BSA/PBS for 1 hour at RT or

overnight at 4ºC, at a concentration defined as optimal for each antibody (Table 5). Cells were washed 3 times with PBS and incubated for 1 hour at RT with AlexaFlour 488 (AF488) conjugated secondary antibody (Molecular Probes, U.S.A.) diluted 1:500 in 1% (v/v) BSA in PBS. After the final PBS washing, cell nuclei were counterstained with 3µg/mL DAPI solution for 30 minutes at RT. Samples were then observed in an AxioImager Z1M microscope (Zeiss, Germany) and images were acquired and processed whith Zen Blue 2012 software (Zeiss, Germany).

Table 5. List of antibodies used to perform immuno staining on CSs.

Primary Antibody (Dilution)	***Secondary Antibody (Dilution)***
Anti-Fibronectin, Rabbit (abcam, U.K.) (1:1000)	AlexaFlour 488 anti-Rabbit (Molecular Probes, U.S.A.) (1:500)
Anti-Laminin, Rabbit (abcam, U.K.) (1:100)	AlexaFlour 488 anti-Rabbit (Molecular Probes, U.S.A.) (1:500)
Anti-Collagen I, Rabbit (abcam, U.K.) (1:100)	AlexaFlour 488 anti-Rabbit (Molecular Probes, U.S.A.) (1:500)
CD105, Mouse (eBiosciences, Germany) (1:100)	AlexaFlour 488 anti-Mouse (Molecular Probes, U.S.A.) (1:500)
CD90, Mouse (Biolegend, U.S.A.) (1:100)	AlexaFlour 488 anti-Mouse (Molecular Probes, U.S.A.) (1:500)
CD73-PE, Rabbit (BD Biosciences, Germany) (1:10)	–
CD45, Mouse (BD Biosciences, Germany) (1:100)	AlexaFlour 488 anti-Mouse (Molecular Probes, U.S.A.) (1:500)
CD34, Mouse (Biolegend, U.S.A.) (1:100)	AlexaFlour 488 anti-Mouse (Molecular Probes, U.S.A.) (1:500)
CD31, Mouse (eBiosciences, Germany) (1:100)	AlexaFlour 488 anti-Mouse (Molecular Probes, U.S.A.) (1:500)
HLA-DR-FITC, Rabbit (BD Biosciences, Germany) (1:10)	–

2.15. Transmission Electron Microscopy

Transmission Electron Microscopy (TEM) was used to observe fine details of the internal structure of the hASCS-CSs prior and after cryopreservation. This technique is based on light phenomena, creating a field of electrons, when the beam passes through the sample leading to an image. This results in one image, where the dense structures are black (scattered electrons) and non-dense structures are bright (unscattered electrons). To analyse hASCs-CS by TEM, we fixed in 2% glutaraldehyde and 1% paraformaldehyde overnight at RT[115]. The remaining protocol was performed at HEMS (Histology and Electron Microscopy Service) - Institute for Molecular and Cell Biology (IBMC), following this steps: hASCs-CSs dehydration with acetone solutions; embedding the material within an epoxy resin; Ultrathin sections (40-60 nm thickness) were prepared on a LKB 2188 NOVA Ultramicrotome (LKB NOVA, Bromma, Sweden) using diamond knives (DDK, Wilmington, DE, USA). The sections were mounted on 300 mesh nickel grids, stained with uranyl acetate and lead citrate, and examined under a JEOL JEM 1400 **TEM** (Tokyo, Japan). Images were digitally recorded using a Gatan SC 1000 ORIUS CCD camera (Warrendale, PA, USA).

2.16. Statistical analysis

Statistical analysis of all results was performed with GraphPad® Prism 4.02 software. All measurements were obtained from three different donors (n=3) with at least triplicates *per* donor. Variation is reported as standard error. Results that showed a normal distribution were analysed by one and two-way ANOVA, or unpaired two-tailed t-Student tests, as indicated. For results did not follow a Gaussian distribution, unpaired two-tailed Mann Whitney t-test was used. Normal distribution was tested using the D'Agostino&Pearson test. The post hock test Bonferroni was used to determine if the difference between the cryopreserved samples (90/10 and 95/5 conditions) and the not-cryopreserved samples was significant through an analysis of the variance setting. The significance levels were set at: *p<0.05, **p<0.01, and ***p<0.001.

CHAPTER III
RESULTS - CRYOPRESERVATION OF CELL SHEETS OF ADIPOSE TISSUE STEM CELLS: LIMITATIONS AND SUCCESSES

III. Results

3.1. Effect of cryopreservation on viability and cytoskeleton organization for permeable condition of cryopreservation.

As a first step, it was important to determine the effects of cryopreservation under different conditions on cell viability and cytoskeletal integrity. The viability of the cells composing the CS prior and after cryopreservation was assessed by live/dead staining followed by flow cytometry. CS were subjected to two cryopreservation conditions: 5% DMSO in FBS or 10% DMSO in FBS and stored for 7 days at -80^{o}C. Samples were retrieved form storage, and immediately thawed in a 37^{o}C water bath until the last frozen crystal disappeared. Samples were then transferred to a culture dish and equilibrated in culture media for either 3 hours (3H) or overnight (ON). Results are presented in Table 6.

Table 6: Flow cytometry analysis with 7AAD staining to evaluate the cell viability of hASCs-CS prior cryopreservation and after cryopreservation with conditions 95/5 and 90/10 followed 3 Hours and ON of equilibration time. A decrease on viability for samples cryopreserved with condition 90/10 and equilibrated during ON. *p<0.05 for differences between samples Prior Cryopreservation and samples cryopreserved with condition 90/10.

Condition	*No. of Samples (3Donors)*	*Equilibration Time (after thawing)*	*Cell Viability (%±SD)*
Prior Cryopreservation	n=4	0 Hours	90,16±6,31**
95/5	n=4	3 Hours	91,32±2,79
	n=4	ON	75,24±4,44**
90/10	n=4	3 Hours	82,41±5,46
	n=4	ON	77,19±1,96**

Our results indicate no difference in cell viability between treatments; however, equilibration times affected viability. These results were confirmed by live dead assays on samples equilibrated for 3H (Figure 3A).

We then evaluated preservation of organization the cytoskeleton by phalloidin staining (Figure 3B). A higher disorganization of actin filaments was observed in samples cryopreserved in 10% DMSO in FBS than in 5% DMSO in FBS (Figure 6B). All samples equilibrated for 3H.

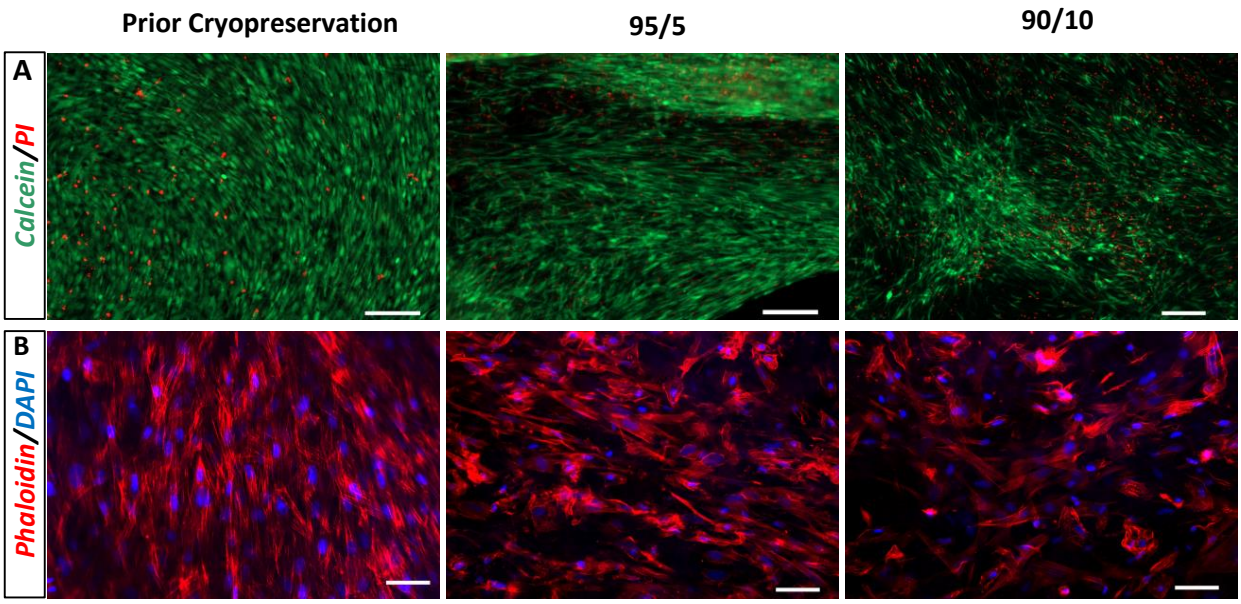

Figure 3: hASCs-CS prior cryopreservation and after 7 days of cryopreservation (90/10; 95/5). (A) Live/Dead assay with Calcein (green) Propidium Idodide(PI-red). Scale bar=200µm. (B) Analysis of cytoskeleton organization of hASC-CS prior and after cryopreservation. Staining of actin filaments by Phalloidin–TRITC (red) and nucleis by Dapi (blue). Samples prior cryopreservation scale bar=200µm. 95/5 and 90/10 samples scale bar=50µm.

3.2. Effect of cryopreservation over ECM.

In order to determine the effect of cryopreservation on expression of ECM proteins, we analysed the expression of collagen type-I, fibronectin and laminin before and after cryopreservation.

3.2.1. Gene expression analysis.

Our analysis revealed that expression of ECM related genes fibronectin and laminin was upregulated significantly (**$p<0.01$) when cryopreserved in 5% DMSO. The expression of laminin was significantly increased (*$p<0.05$) when cryopreserved in 10% DMSO. Collagen type-I and fibronectin seemed upregulated in both conditions, but the difference was not statistically significant (Figure 4).

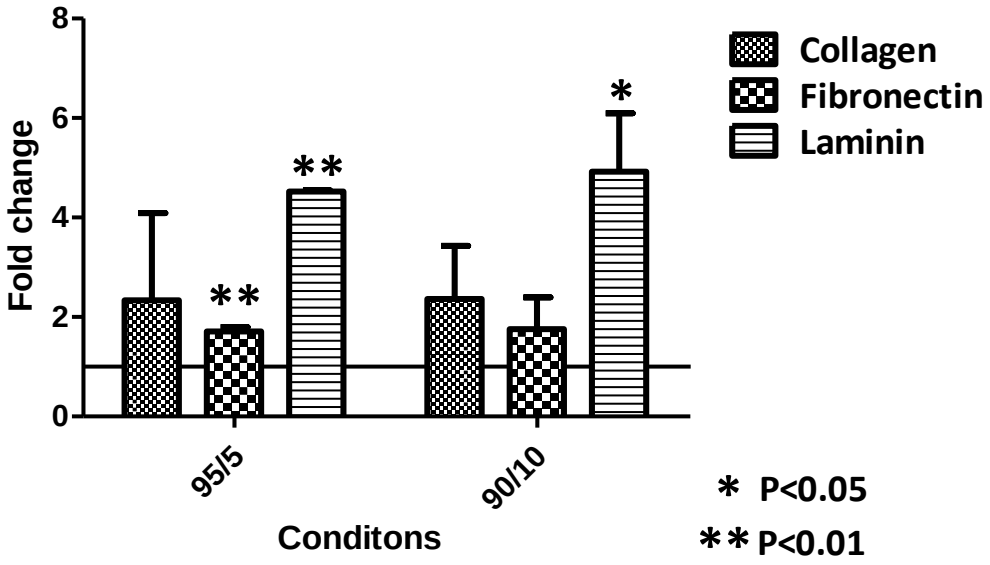

Figure 4: Real time qRT-PCR to determine relative expression of principal proteins in Extracellular Matrix Collagen type-I (COL1), Fibronectin (FN1) and Laminin (LAMA1) in hASC-CS. The Y-axis shows fold-change difference in relative expression of principal proteins in ECM between 95/5 and 90/10 conditions of cryopreservation compared with hASC-CS prior cryopreservation. * p<0.05 for differences between samples Prior Cryopreservation and samples cryopreserved with condition 90/10; **p<0.01 for differences between samples Prior Cryopreservation and samples cryopreserved with condition 95/5.The measurements are the result of 3 biological replicates (with 3 technical replicate).

3.2.2. ECM protein secretion.

In order to study how the cryopreservation protocol affected protein stability, we performed a BCA protein assay to determine total levels of protein before and after the procedure.

As shown in Figure 5, we observed a significant decrease of total protein (***p<0.001) after 7 days of cryopreservation in both conditions.

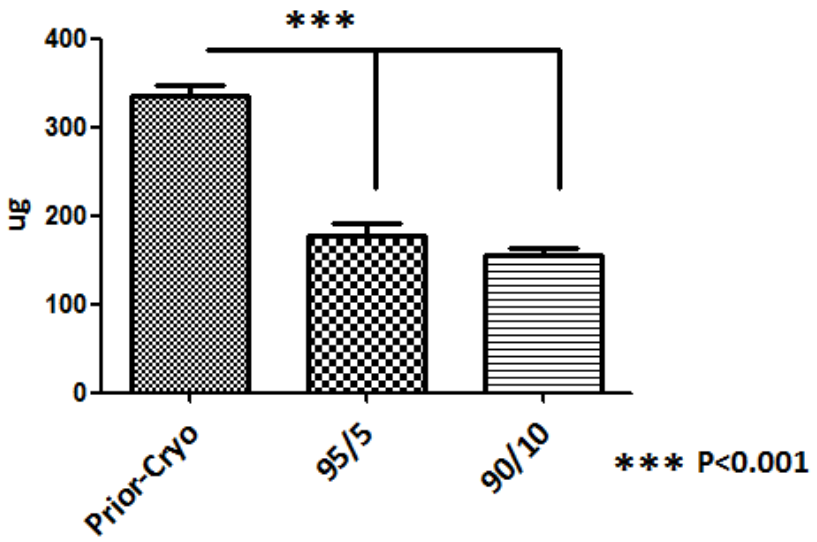

Figure 5: Quantification and comparison of total protein extract in RIPA Buffer, between hASC-CS prior cryopreservation and cryopreserved during 7 days in conditions 90/10 and 95/5. ***p<0.001 for differences between samples Prior Cryopreservation and samples cryopreserved with conditions 90/10 and 95/5.

To determine the condition of ECM proteins in the hASCs-CS, we performed immunocytochemistry against collagen type-1, fibronectin and laminin. Collagen type-I was well maintained in the cryopreserved samples, while laminin was severely affected by cryopreservation in both conditions. Fibronectin was better maintained in 90/10 than in 95/5 (Figure 6).

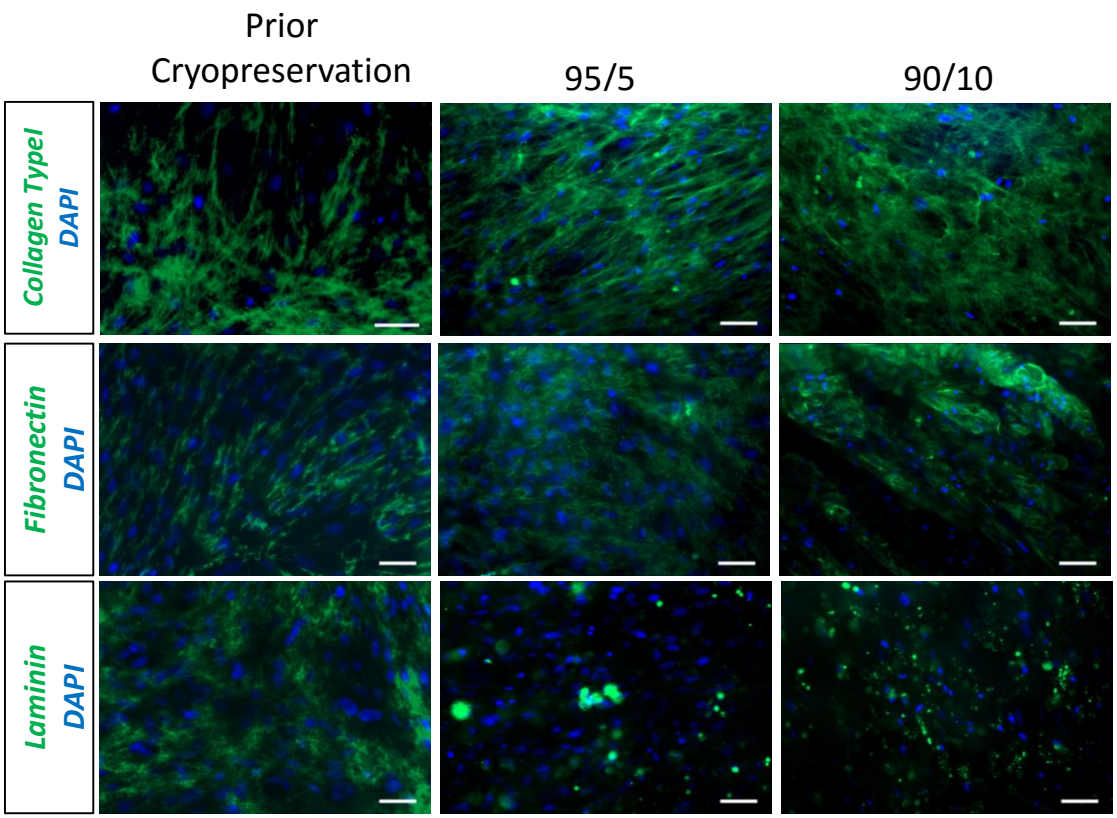

Figure 6: Analysis of ECM in hASCs-CS. Immunocytochemistry analysis of principal proteins of Extracellular Matrix of hASC-CS. Staining with anti-Collagen Type I; anti-Fibronectin; anti-Laminin. All samples are marked with Dapi for nucleis (blue) and anti-rabbit Alexa 488 as secondary antibody (green). Scale bar = 50µm.

We then evaluated the effect of our cryopreservation protocols on the CS morphology at the ultrastructural level. TEM revealed major disorganization of nuclei and matrix in hASCs-CS cryopreserved within 10% DMSO (Figure 7). In contrast, CS cryopreserved in 5% DMSO maintained similar and regular morphology in comparison with samples prior cryopreservation (Figure 7).

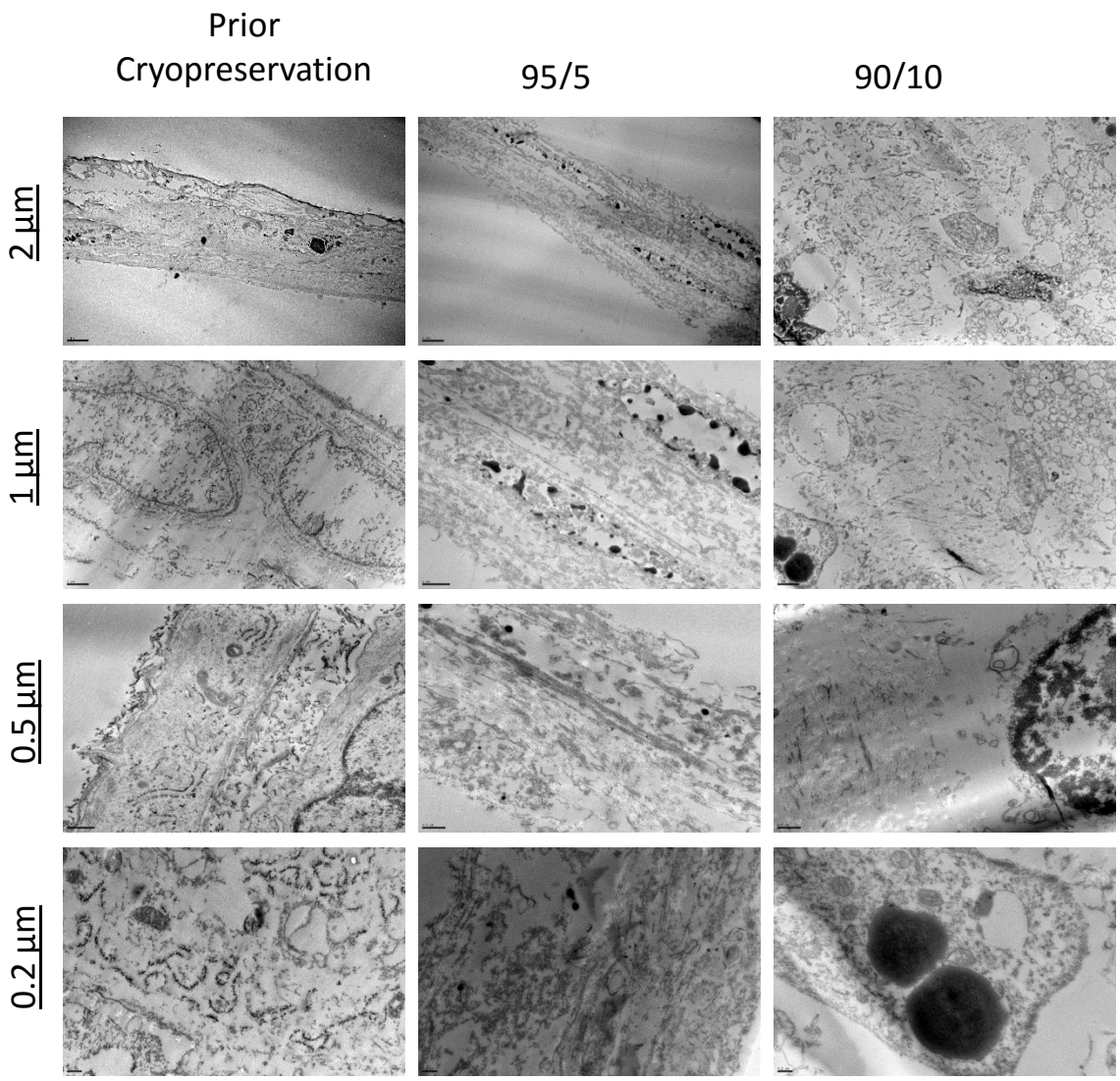

Figure 7: Structural morphology of haverested hASCs-CS by TEM for analysis of maintenance of Cell sheets morphology. hASC-CS prior cryopreservation; hASC-CS in condition 90/10; hASC-CS in condition 95/5. Scale bar from 2 µm to 0.2 µm.

We the sought to understand how our cryopreservation protocols affected the mesenchymal phenotype of the cells in the CS.

3.3. Effect of cryopreservation over mesenchymal phenotype.

It was important to determine whether cryopreservation preserved the minimal phenotypic criteria defined by ISCT for mesenchymal stem cells[71,22].

To do so we characterized the expression of surface mesenchymal markers by flow-cytometry using CS obtained and cryopreserved from 3 different donors. 95% of the cells were positive for markers Thy-1 (CD90), ecto-5'-nucleotidase (CD73) and Endoglin (CD105).

The hematopoietic markers CD45 (Protein tyrosine phosphatase, receptor type C) and CD31 (Platelet endothelial cell adhesion molecule or cluster of differentiation 31) were under 2%, as expected. CD34 (Cluster of differentiation 34) shows a percentage above the 2% defined as minimal criteria, but this value was the result of an abnormally high value in one of our donors (data not shown). Our results showed our samples to be immune-privileged, as they were negative for marker HLA-DRA (Major histocompatibility complex, class II, DR alpha) across all three donors (Table 7).

Table 7: Flow-cytometry analysis of the Mesenchymal markers among 3 different population of hASC, CD90; CD73; CD105; CD34; CD45; CD31; HLA-DRA.

All Populations (n=3)	*Markers*	*(%±SD)*
hASCs	CD90	98,26± 2,90
	CD73	95,89±6,62
	CD105	96,95±4,98
	CD34	8,67±12,80
	CD45	1,07±1,03
	CD31	0,6±0,30
	HLA-DR	0,66±0,38

Overall, our results showed the expected mesenchymal immunophenotype. To confirm our results, we performed qPCR analysis for mesenchymal surface markers of hASCs-CS before and after cryopreservation for 7 days in both conditions across 3 donor samples. We analysed the expression of positive genetic markers CD90, CD73 and CD105 and verified no significant difference in expression before and after cryopreservation, comparing with samples prior cryopreservation. As housekeeping we use the same Beta-2-Microglobulin (β2M), as previously described. Our results are shown in Figure 8.

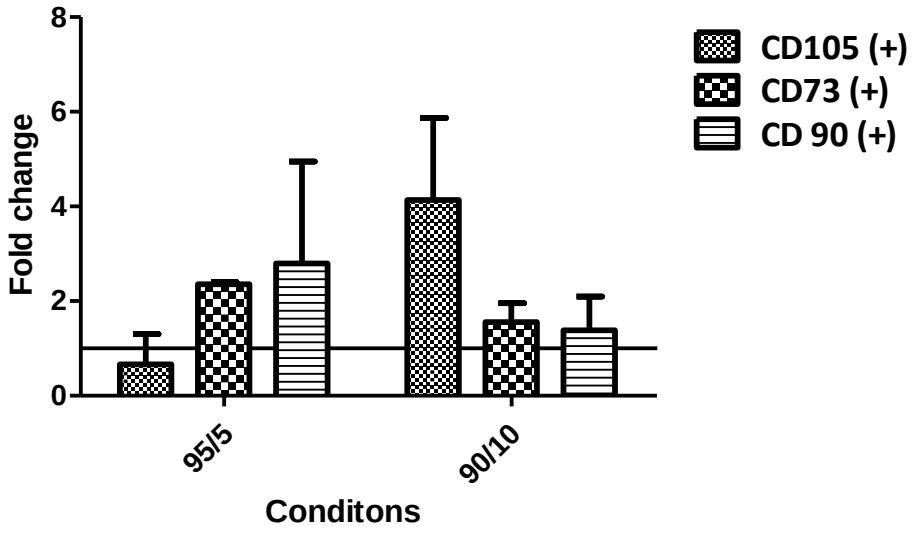

Figure 8: Analysis of principal surfaces Mesenchymal Markers of hASCs-CS. (A) Real time qRT-PCR to determine relative expression of positive mesenchymal markers Thy-1 (CD90); ecto-5'-nucleotidase (CD73); Endoglin (CD105), in hASC-CS. The Y-axis shows fold-change difference in relative expression of mesenchymal markers between 95/5 and 90/10 conditions of cryopreservation compared with hASC-CS prior cryopreservation. All results were statistically non-significant.

We further confirmed these results by immunocytochemistry (ICC) on samples cryopreserved for 7 days under both selected conditions (5 % and 10% DMSO). Our results show that cells remained positive for CD90, CD73 and CD105, (Figure 9) while being negative for hematopoietic markers CD34, CD31, CD45 and HLA-DR (Figures 10).

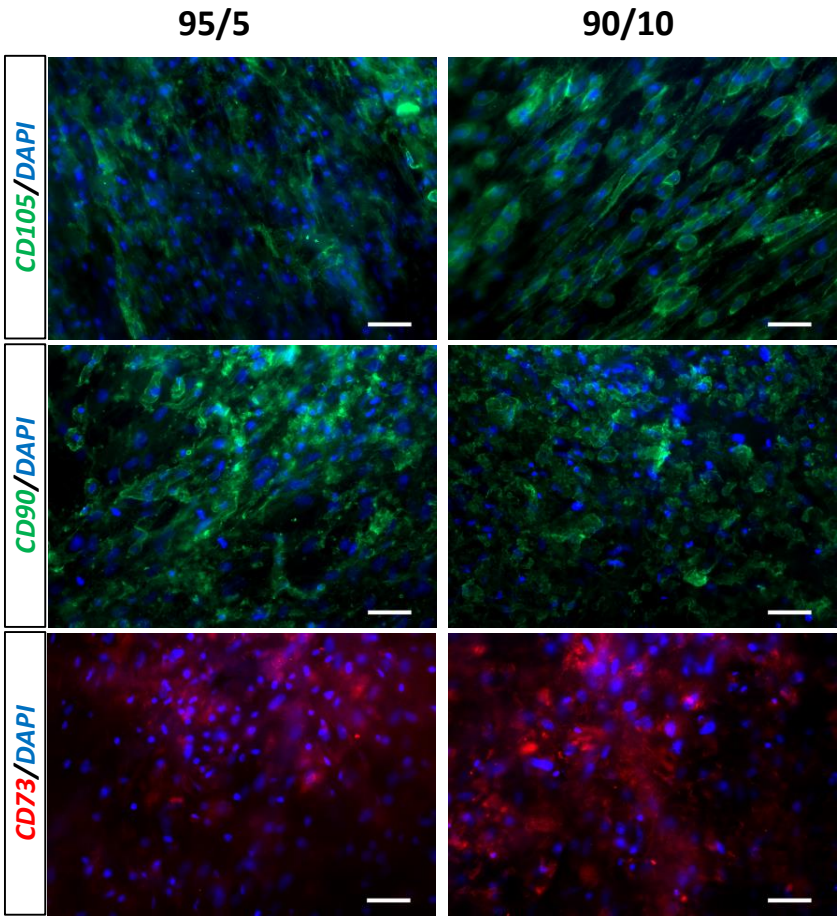

Figure 9: Analysis of principal surfaces Mesenchymal Markers of hASCs-CS. (B) Immunocytochemistry analysis of principal mesenchymal markers of hASC-CS. Indirect staining with anti-CD90, anti-CD105 and direct staining with PE CD73.

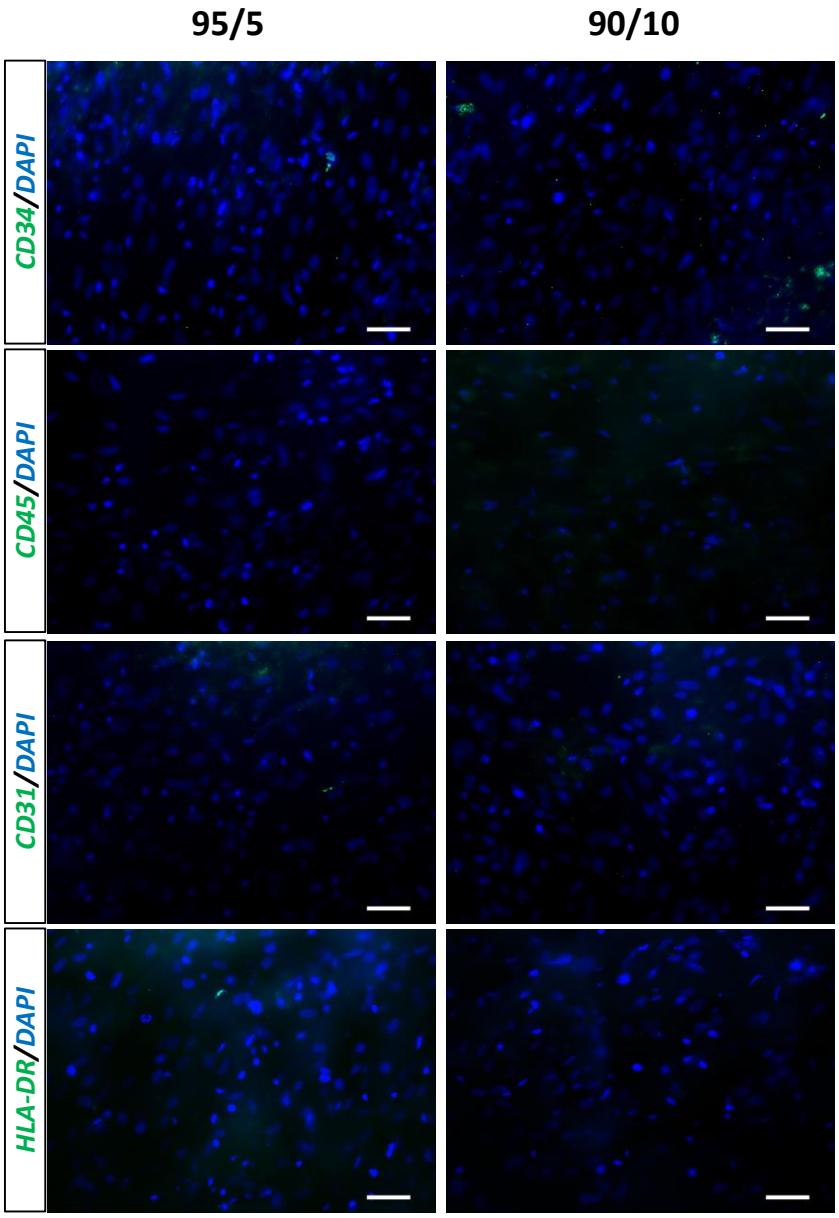

Figure 10: Analysis of principal surfaces Mesenchymal Markers of hASCs-CS. (C) Immunocytochemistry analysis of hematopoietic markers of hASC-CS. Indirect staining with anti-CD34, anti-CD45, anti-CD31 and direct staining with FITC HLA-DR. All samples are marked with DAPI for nuclei and anti-mouse Alexa 488 as secondary antibody. Scale bar=50µm.

CHAPTER IV

DISCUSSION & CONCLUSIONS

IV. Discussion & Conclusions

hASCs are a very attractive cell type for RM applications, in part because they present immune-privileged features, as they lack the expression of human leukocyte antigen-DR and stimulate the production of anti-inflammatory cytokines. hASCs have the capacity to form a cell sheets, constituting a promising research area for development of wound healing and other potential applications in TE[71].

The present work has focused on the development of a protocol for cryopreservation of hASCs-CS with the goal of decreasing the production time of hASCs-CS and offering an off-the-shelf solution for future TE applications.

Integrity of the ECM is critical for a good functional outcome of CS transplants. Using standard cryopreservation procedures as a starting point, our main objective was on the maintenance of viability, morphology and mesenchymal phenotype after 7 days of cryopreservation. We wanted to evaluate if the standard condition (10%DMSO) usually used for cryopreservation of hASCs cell suspensions would also work in the same way in our hASCs-CS. Moreover, we intended to decrease the percentage of DMSO from 10% to 5%, in order to reduce possible cytotoxic effects on our hASCs-CS.

Our goal was to maintain cell viability while decreasing cell cytotoxicity during cryopreservation. These conditions with DMSO (90/10 and 95/5) enabled the maintenance of over 70% cell viability of hASCs-CS when cryopreserved for 7 days (Table 6 and Figure 3A).

In view of these results, these two conditions were used for further study: the 90/10 as standard condition and 95/5 as experimental condition.

We first produced and characterized hASCs-CS in terms of mesenchymal phenotype, cellular viability and morphological stability. We isolated hASCs from the stromal vascular fraction (SVF) of human subcutaneous adipose tissue, following the protocol previously reported[71]. Prior to the creation of cell sheets, we confirmed the maintenance of the mesenchymal nature of hASCs among three isolated populations by determining presence or absence of the mesenchymal markers CD90, CD73, CD105, and the hematopoietic markers CD45, CD34, CD31, HLA-DR, as established by the International Society for Cellular Therapy[22].

Previous work in our group[71] confirmed that the human adipose derived stem cells (hASCs) are immunologically compatible and multipotent. Moreover they show a high growth rate in culture, and produce their own extracellular matrix (ECM). They formed a functional cell sheet scaffold-free in only 5 days of culture.

A critical aspect of creating hASCs cell sheets is that secretion of ECM has to be sufficient to confer the ideal robustness and organization to allow cell sheet detachment. The hASCs cultured for 5 days on TR dishes using a standard medium gave rise to a fragile monolayer of cells, with an ECM composed by collagen type-I, fibronectin and laminin that was not sufficient to allow the cell sheet manipulation[71]. It was therefore necessary to stimulate the proliferation rate of ECM, adding ascorbic acid to the culture media[71,116]. The use of high concentrations of ascorbic acid resulted in an increase of contractibility of the cell sheets after detachment; however, this is not expected to have any consequences in clinical application, which involves the use of PVDF membrane or gelatin coated manipulators to avoid the shrinkage, and that are removed after cell sheet adheres to host tissues.

Cryopreservation of cellular suspensions of hASCs in 10% of DMSO showing high viability when thawed has been previously reported[101]. The optimal condition for cellular suspension's cryopreservation is the combination with 90% of Fetal Bovine Serum (FBS) and 10% of DMSO, used as the standard condition in our laboratory. The FBS component works as a buffer of osmotic pressure and as a cell membrane protector, reducing the risks of damage by ice crystal formation during cryopreservation and thawing process[85]. DMSO is the most efficient cryoprotective agent used in cryopreservation of many types of cells and tissues. However it is cytotoxic in a time, temperature, and concentration dependent manner. It permeates cells and can cause osmotic stress or chemical toxicity when used in high concentrations[90,95].

The first condition tested was the standard condition composed with 90% of FBS and 10% of DMSO (condition 90/10). The second condition tested was composed of only 5% DMSO, combined with 95% of FBS (condition 95/5), with the intention of reducing DMSO cytotoxicity. The hASCs-CS cryopreservation was performed using the slow cooling method in the rate of -1ºC/min until reach -80ºC ON, followed of 7 days at -196ºC in liquid nitrogen for both conditions.

In storage of cells at low temperatures the lethal interval of temperature is between 15ºC and -60ºC. Between these temperatures the damage and the loss of the cryopreserved cells viability is maximal. To avoid this situation we used the previously referred to slow cooling rate which allows a progressive dehydration of cells passing the critical interval slowly enough to avoid intracellular ice formation[95]. Once -80ºC was reached the cryovials were transferred to the liquid nitrogen (LN) tank at -196ºC. After the 7 days of cryopreservation in LN with both conditions, samples were rapidly thawed in water bath at 37ºC, and transferred into culture dishes for equilibration of samples, in order to avoid the cell damage from osmotic stress and removing the DMSO. The equilibration of samples was performed during 3 hours and ON for both conditions.

We observed that cell viability of hASCs-CS after ON equilibration of the samples decreased (although in in a non-significant manner) in the case of cryopreservation condition 95/5. In the case of cryopreservation condition 90/10 we observed a significant decrease of cell viability when compared to hASCs-CS viability prior to cryopreservation (Table 6). The cell viability of hASCs-CS was not significantly affected with 3 hours of sample equilibration for both conditions.

These first observations were sustained by the Cal/PI assay which sustained (Figures 3A). Thus, these results reveal a clear influence of the equilibration time on cell viability and based on that we perform all the subsequent assays with samples equilibrated for 3 hours. In general, our results follows the normal criteria of cell viability that is applied in clinical approaches of cryopreservation[118], as more over 60% of cell viability after cryopreservation is obtained.

Despite both conditions of cryopreservation not affecting cell viability after 3h of equilibration, evaluation of cytoskeleton organization revealed some disorganization in samples cryopreserved with both conditions comparing with hASCs-CS prior to cryopreservation. Our results reveal a major incidence of disorganized cytoskeleton in hASCs-CS cryopreserved with condition 90/10. These results indicate that the process of cryopreservation of the cytoskeleton is sensitive to the % of DMSO used (Figures 3B)[119]. The observation that 10% DMSO affected cytoskeletal organization may explain the lower cell viability obtained after ON of equilibration time.

We verified a better organization of cytoskeleton of cryopreserved hASCs-CS (Figures 3B) using only 5% of DMSO. This agreed with a study performed by Xu *et al.*2012, on

effects of cryopreservation in cell suspension of adherent mesenchymal stem cells which suggested that cooling rates are a critical factor in preservation of cytoskeletal organization during cryopreservation. We conclude that the -1ºC/min used in our study is the optimal cooling rate[120].

However, the rate -1°C / minute is not ideal for cells in cell sheets. The equilibrium time is also a stress factor for hASCs-CS, since hASCs are adherent cells and equilibration is done in suspension. The distribution of cells is not affected, as shown by DAPI staining[53].

In the scaffold-free CS engineering-based approach, the organization of ECM is of crucial importance, as it works as a construct in CS technology. This is due ECM proteins such as collagen, laminin and fibronectin that form networks on the surface of the cells. The organization of the matrix can also influence the organization of the cell cytoskeleton, as well that can mechanically influence cell behaviour by the activation of intracellular signalling pathways, interacting with cell-surface receptors[121].

In hASCs-CS the maintenance of ECM is crucial and we intended to understand how cryopreservation alters the expression of ECM proteins. The expression of collagen, fibronectin and laminin was analyzed the levels prior cryopreservation comparing with cryopreserved samples. Our results revealed a clear decrease of the total quantity of protein after 7 days of cryopreservation that is statistically significant in both conditions when compared to samples prior cryopreservation (Figure 5). However at the molecular level cryopreservation appears to promote an up-regulation in the principal genes that code for ECM proteins as collagen type-I, fibronectin and laminin. Nevertheless not all these genes presented a statistically significant up-regulation (Figure 4).

In a study performed by Rodriguez-Ares et al. 2009 with frozen tissue-suspension obtained from processed human amniotic membrane, a reduction of total amount of protein after cryopreservation was verified[122], due to the dehydration promoted by DMSO during process of cryopreservation[122,123]. In the condition 95/5 the up-regulation was significant for fibronectin and laminin genes (Figure 4). In the condition 90/10 only laminin gene suffer a significant up-regulation with a $p < 0.05$ (Figure 4). However, at the molecular level, the mRNA of collagen type-I does not undergo significant upregulation in either conditions of cryopreservation.

We analyzed levels of collagen, laminin and fibronectin by ICC. The protein most affected by cryopreservation was laminin, which revealed a weak and punctuated signal (Figure

6) in ICC images for both cryopreservation methods. ICC also revealed some levels of disorganization of fibronectin in both cryopreservation conditions (Figure 6). However, hASCs-CS preserved in condition 95/5 seemed more affected then those cryopreserved in condition 90/10 (Figure 6). The only protein apparently not affected by cryopreservation in either condition was collagen type-I (Figure 6).

We then analyzed mRNA levels of these 3 proteins (Figure 4). mRNA of fibronectin was significantly upregulated in condition 95/5. Laminin mRNA, on the other hand, was significantly upregulated in both condition of cryopreservation. In contrast, collagen type 1 mRNA was not significantly upregulated in either cryopreservation condition. These results could suggest interruption of a negative feedback loop for fibronectin and laminin genes after cryopreservation and thawing. Overall our results are similar to those of the Rodriguez-Ares et al. 2009[122].

The organization of the ECM before and after cryopreservation was evaluated by TEM in order to determine potential effects of the method over matrix composition and integrity. After thawing the structure of cell sheet was lost for hASCs-CS cryopreserved with both conditions in comparison with samples prior to cryopreservation. However comparing the results obtained in both conditions of cryopreservation, the condition 95/5 appears to promote a better preservation than condition 90/10 (Figure 7). Moreover, aggregates of proteins were observed along all the structure of hASCs-CS cryopreserved with both conditions (Figure 7). The loss of cell sheet structure after thawing and cryopreserved with 10% of DMSO was already observed by Kito K. et al. 2005[95] in cryopreservation of cultured corneal epithelial cell sheet in Rabbit.

As we intended to understand if our process of cryopreservation alters the mesenchymal phenotype of cryopreserved hASCs-CS, we proceed to analyse molecular markers and did not verify any significant alteration of mesenchymal markers for cryopreserved hASCs-CS in comparison with samples prior to cryopreservation. We also confirmed the maintenance of mesenchymal phenotype by ICC (Figures 9 and 10). Overall, the process of cryopreservation did not affect the expression of mesenchymal phenotype of our hASCs-CS at P1 with both conditions of cryopreservation during 7 days in LN. After the rapid thawing process and 3H of equilibration, the hematopoietic markers remained negative throughout all the procedure.

In this sense, an off-the-shelf hASCs-CS was achieved after cryopreserving with DMSO at 5% DMSO. However the cryopreserved hASCs-CS still fall short of ideal conditions for a real clinical application. Therefore, further optimizations need to be performed to better preserve the hASCs-CS ECM. To achieve this, less aggressive compounds of cryopreservation, such as Poly-L-Lysine, should be investigated. Furthermore, the use of adequate cooling rates to cryopreserve hASCs-CS is crucial. Finally, their functionality needs to be characterized *in vivo* after transplantation to better characterize the CS final structure and functionality.

CHAPTER VI

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VI. References

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