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Determination of optimal cryoprotectant agents for 3D cell-based products cryopreservation by slow freezing

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Estando al final de mi doctorado, me pregunto por qué empecé, y creo que como algunos empezamos la carrera, sin una idea clara de lo que elegimos estudiar, yo comencé la tesis. El buen ambiente que vi en el laboratorio, más el que siempre me ha gustado cacharrear y hacer experimentos decantaron la balanza hacia el doctorado.

¿Qué ha sido y es la tesis para mí?

Es un periodo donde vuelcas mucha energía, y en el que tienes muchas subidas y bajadas. Además, la frustración es un frecuente compañero de viaje, y el constante cambio y aprendizaje están al orden del día. Por ello, creo que la tesis es una locura, donde la gente que le gusta lo difícil y que tiene un ligero toque lo realiza. Siento que soy uno de estos casos, y me definiría como lo hizo Dalí:

"la única diferencia entre un loco y yo,
es que el loco cree que no lo está,
mientras que yo sé que lo estoy".

Rizando el rizo de este tema, hace poco se publicó un artículo donde se aseguraba que los doctorandos somos seis veces más propensos a desarrollar ansiedad o depresión en comparación con la población en general. ¿Entonces, es la tesis perjudicial para la salud, o es que los doctorandos ya venimos con nuestra locura y la tesis saca a relucir nuestros problemas? ¿Qué es primero el huevo o la gallina?

Aun creyendo que para hacer la tesis hay que estar un poco loco, estoy muy contento del camino que he realizado. Cuando hecho la mirada atrás y pienso en cómo era cuando empecé el doctorado y como salgo de aquí, pienso que ha pasado una vida entera. En este tiempo, ha cambiado mucho mi manera de pensar, de actuar y de sentir. Para ello, he tenido que gestionar mi tiempo y mi locura (cosa que aún tengo que mejorar), viajar a congresos para presentar mi trabajo, frustrarme, ser creativo, errar y empezar de nuevo una y otra vez. Además, en todo este camino me ha acompañado mucha gente; desde mi familia y mis amigos, hasta mis compañeros de trabajo y directores de tesis. Algunos me habéis ayudado con vuestros consejos o enseñándome a ser un investigador, otros aguantado mi locura y otros simplemente acompañándome.

Gracias, ya que vosotros lo habéis hecho posible.

Y ahora que acaba esta etapa y comienzo otra, siento vértigo otra vez. Lo que sé es que salgo diferente de la tesis, algo más optimizado, y que seguiré disfrutando de mi locura en este nuevo camino.

Caminante, son tus huellas
el camino y nada más;
Caminante, no hay camino,
se hace camino al andar.
Al andar se hace el camino,
y al volver la vista atrás
se ve la senda que nunca
se ha de volver a pisar.
Caminante no hay camino
sino estelas en la mar.
Antonio Machado

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GLOSSARY

Aβ: beta amyloid plaques
AD: Alzheimer disease
APA: alginate-poly-L-lysine-alginate
APP/Ps1: Alzheimer disease transgenic mice
Arpe-19: human retinal pigment cell line
BHK: baby hamster kidney cell line
BMSC: bone marrow stem cells
β-TC: beta cell insulinoma cell line
CCK-8: cell counting kit-8
CFU-F: Fibroblast colony forming units
CD: choroid plexus
CHO: Chinese hamster ovary
CIOCD: cryopreservation induced onset cell death
CNS: central nervous system
CPA: cryoprotectant agent
CPC: calcium phosphate cements
CRF: controlled rate freezers
CYP: cytochrome P450
C2C12: immortalized mouse myoblast cell line
Da: Daltons
DM: Diabetes mellitus
DMEM: Dulbecco´s modified Eagle´s medium
DMSO: dimethyl sulfoxide
DPBS: Dulbecco-s phosphate buffered saline
DXM: dexamethasone
D1MSC: D1 mesenchymal stem cells
EG: ethylene glycol
FLF: fulminant liver failure
FBS: fetal bovine serum
G: α-L-guluronic acid
GLP-1: glucagon like peptide 1
GMP: good manufacturing procedures
HA: hyaluronan
hESCs: human embryonic stem cells

hPSCs: human pluripotent stem cells
HS: human serum
HSV-TK: herpex virus thymidine kinase
ICH: intra cerebral haemorrhage
IIF: intracellular ice formation
IL-6: interleukin 6
LDH: lactic dehydrogenase
Low MW-HA: low molecular-weight hyaluronan
M: Manuroric acid
MSC: mesenchymal stem cells
MSC-Epo: D1MSC engineered to release EPO
OA: osteoarthritis
PBS: phosphate buffered saline
PCL: PEG-coated poly(ε-caprolactona)
PD: Parkinson´s disease
PDL: poly-D-lysine
PEG: poly-ethylene-glycol
PEX: hemopexin-like protein
PLGA: poly(lactic-co-glycolic) acid
POL: poly-L-ornithine
PRP: platelet rich plasma
PRP-SF: platelet rich plasma-synovial fluid
PVP: poly-vinyl-pyrrolidone
SC: stem cells
SF: synovial fluid
SFGNESTGL: green fluorescent protein, herpex virus thymidine kinase and firefly Luciferase triple reporter system
TECs: tissue engineered constructs
TGF-β: transforming growth factor beta
TNF-α: tumour necrosis factor alpha
VEGF: vascular endothelial growth factor
ZVAD: benzyloxycarbonyl-Val-Ala-DL-Asp-fluoromethylketone
1.1B4: human insulin secreting cell line 1.1B4

Index

1

Introduction

Advances in cell encapsulation technology and its application in drug delivery

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ABSTRACT

Introduction: Cell encapsulation technology has improved enormously since it was proposed 50 years ago. The offered advantages compared to other alternative systems, such as the prevention of repetitive drug administration, have triggered the use of this technology in multiple therapeutic applications.

Areas covered: In this article, the improvements of the cell encapsulation technology and the strategies to overcome the drawbacks that move away this technology from the clinic have been summarized and discussed. Besides, the different studies and clinical trials that have been performed in several therapeutic applications have also been described.

Expert opinion: Authors believe that the future translation from bench to bedside of this technology requires the optimization of diverse aspects: (1) biosafety, controlling and monitoring cell viability, (2) biocompatibility, reducing pericapsular fibrotic growth and hypoxia suffered by the graft, (3) control over drug delivery, (4) and the final scale up. On the other hand, an area that deserves more attention is the cryopreservation of encapsulated cells since it will facilitate the arrival of these biosystems to the clinic.

Keywords: Microencapsulation, cryopreservation, therapeutic applications, cell source, drug delivery, biocompatibility, biosafety, alginate

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1. INTRODUCTION

Cell encapsulation is a strategy based on the immobilization of cells in a biocompatible matrix. The matrix is covered by a semipermeable membrane, which protects the inner cells from the host´s immune system and the mechanical stress (Figure 1) [1]. The polymeric scaffold, in addition of enabling the bidirectional transport of nutrients, oxygen and residues, also controls the release of the therapeutic factors produced “de novo” by the cells and provides a suitable microenvironment that promotes and controls their viability and proliferation [2,3]. Furthermore, the immunoisolation of cells within the polymeric matrix allows the transplantation of non-human cells for clinical use [4]. Undoubtedly, this is an important benefit due to the limited availability of human donor tissues. Moreover, it gives the possibility of entrapping genetically modified cells in order to express any desired therapeutic molecule [5]. This technology also suppresses, or at least minimizes, the chronic administration of immunosuppressant agents, preventing the side effects that often occur when tissues or organs are transplantanted [6]. Consequently, this advantages have promoted the development and employment of cell encapsulation in organ replacement, tissue engineering and regenerative medicine as drug and cell delivery therapies [1,7].

The final objective of cell encapsulation conditions the matrix characteristics. If a cell delivery system is designed, then the matrix should be somehow degradable, but if the goal of the therapy is the release of a therapeutic agent from the entrapped cells, the matrix should be more robust. Thus, if the encapsulation goal is drug delivery, the matrix is coated with a semipermeable membrane in order to increase the mechanical stability and achieve long lasting matrices. Moreover, cell encapsulation can be applied in geometries of macrocapsules (size up to cm) or microcapsules (diameter up to micrometer) [8]. Macrocapsules are able to enclose cells in relatively large diffusion chambers with semipermeable properties. They can have conformations of flat sheets, hollow fibers or disks and can be classified in intra- or extravascular devices [9,10]. Intravascular devices are associated with thrombosis risk, doing this therapy unacceptable [11]. Therefore, the most used devices are the extravascular ones, which are implanted subcutaneously or in the peritoneal cavity. They can be administered with minor surgery and explanted without difficulty in the case of side effects or when replacement is needed [12,13]. Obviously, as encapsulated cells are implanted in an invasive way, all used materials and reagents in this process need to be sterilized and the encapsulation process is performed under sterile conditions.

The disadvantage of macrocapsules is their relatively small surface-to-volume ratio, requiring high amounts of nutrients and oxygen supply. Moreover, an adequate diffusion gradient is needed and also the encapsulated cell density cannot exceed 5–10% of the capsule

Article highlights.

- Cell encapsulation, as a drug delivery system, provides a broad range of therapeutic applications due to the fact that the entrapped cells, which release the '*de novo*' therapeutic factors, are immunoisolated from the environment allowing the implantation of allogeneic, xenogeneic and genetically modified cells.
- The emerging search of new biomaterials for the matrix and the coatings of microcapsules, in order to enhance the mechanical properties, biocompatibility or cell behavior, will broaden the future therapeutic applications of this technology. Several clinical trials with encapsulated cells have been performed whereas others are ongoing, showing promising results mainly in type I diabetes but also in other pathologies such as Parkinson disease or intra cerebral hemorrhage.
- Cell microencapsulation presents several still unresolved drawbacks that include issues of biosafety, such as the pericapsular fibrotic overgrowth, or biocompatibility.
- The preservation of encapsulated cells will represent a notorious advantage as it will facilitate the 'on demand' access in the clinic and will reduce the costs, which is a pivotal need in the final translation of this technology to the clinic.
- The final step to the clinic will go through the scaling up of cell encapsulation and will have to comply with the GMP and the requirements of healthcare governments.

This box summarizes key points contained in the article.

volume fraction. Therefore, large numbers of cells are required with macrocapsules for clinical therapy, being necessary more than one device per clinical treatment [14]. However, microcapsules have a larger surface volume ratio, having comparatively a greater mass diffusion than macrocapsules [11]. Thus, most research groups have focused their efforts in the development of microcapsules for diverse applications [15-18].

We will focus in this review on cell microencapsulation technology as a drug delivery system. Moreover, we will describe the different strategies, therapeutic applications and future challenges to approach the clinical use of this technology.

2. IMPROVEMENTS IN CELL ENCAPSULATION TECHNOLOGY

The biomaterials employed in cell encapsulation must provide proper biocompatibility and physical properties in the organism to yield an efficient therapy [19]. One key for the success of cell encapsulation depends on the chosen polymers which can be distinguished in polymers from natural sources (polysaccharides, polypeptides, and polynucleotides) and synthetic polymers [20]. A large range of biomaterials has been proved, such as agarose, hyaluronic acid, fibrin, collagen or polyethyleneglycol (PEG) [21-25] for immobilizing cells into a matrix. However, the biomaterial more widely studied that qualifies for clinical application is alginate, due to its favorable properties, including its biocompatibility, easy manipulation and great safety characteristics demonstrated in the field of transplantations [26-29].

Alginates are natural anionic polysaccharides that create three dimensional structures when they react with divalent ions like calcium or barium. They are formed by mannuroic (M) and α-L-guluronic acid (G) residues, of widely varying compositions and sequential structures depending the G and M ratio of the alginate source [30,31]. A primordial parameter from alginate to be determined and standardized is the G/M ratio because it has a significant

impact on some of the alginate gel properties including biocompatibility, stability, mechanical resistance and permeability among others [32-34]. Another parameter not to be ignored is alginate purity. Low purity alginates contain endotoxins, proteins and polyphenols that reduce the biocompatibility of implants [35,36]. Thereby, different purification methods have been developed obtaining highly purified alginates which decrease alginate immunogenicity in vivo [37-39].

Ca-alginate beads under physiological conditions tend to suffer osmotic swelling, increasing their permeability, destabilization and finally, the rupture of the matrix. This is mainly because the affinity of calcium ions is higher towards some chelating agents, such as phosphate, than towards alginate. In addition, the exchange of calcium ions with other non gelling ions, such as sodium, could destabilize the alginate gel [40]. Thus, alginate microcapsules are coated with a polycation layer after gelification in order to increase mechanical stability of the capsule and allow the control of the molecular weight cut-off of the membrane. Different coatings such as chitosan, oligochitosan or poly-methylene-coguanidine have been proved, but the most used polycations for alginate beads coating, both in research and in clinical trials, are poly-L-lisine (PLL) and poly-L-ornithine (PLO) [41-44]. However, PLL and PLO are known to be immunogenic and therefore the microcapsule system requires a final coating with alginate to form the alginate-poly-L-lysine-alginate (APA) microcapsules [45]. Some groups have shown that PLO shows higher mechanical stability, better biocompatibility and regulates strictly the permeability or that gives less cell adhesion than PLL in vivo [46,47]. However, another study was performed comparing with similar material and methods, the biocompatibility of different polycations. It was showed that PLL is more stable and more biocompatible than PLO, which showed more protein adhesion in vivo [41]. Since then, many efforts have been made in order to develop more biocompatible, strong and stable systems but an optimization of the APA biosystem is still required [48,49].

Currently, several variations are under development in order to improve the technological properties of the APA microcapsules. For example, the mix of ionically crooslinked alginate with covalently crooslinked polyacrylamide (photocrosslinked alginate) improves the mechanical strength and stretchability of the hydrogels [50]. Other groups have increased the degradation rate of alginate hydrogels by reacting alginate with sodium periodate and synthesizing oxidized alginates [51]. Thus, as the photocrosslinked alginate improves mechanical properties of alginate but reduce its degradation rate, some groups have oxidized the photocrosslinked alginate obtaining hydrogels with better properties and correct degradation rates [52]. Nevertheless, the oxidation of alginates in cell encapsulation is mostly used for controlling the degradation rate for cell delivery strategies, which is

not the article scope. Another variation of the alginate matrix that allows enhancing its mechanical properties is the enzymatic conjugation of alginate with tyramine, which permits the control of the swelling behavior of hydrogels without increasing the cytotoxicity to the cells [53,54]. In this way, the covalently cross linked alginate matrices (e.g. photocrosslinked or tyramine conjugate) could improve alginate stability when they are implanted. New trends on cell encapsulation are based on the biofunctionalization of the matrices with the purpose of improving and controlling cell behavior within them. Short synthetic peptides such asarginine-glycine-aspartic acid (RGD) or proteins, such as collagen or fibronectin are added to the matrix, mimicking the physical and biomechanical characteristics of native environment. Our group has studied biofunctionalized APA capsules with RGD containing C2C12 myoblasts, showing an improvement in 30% of cell viability compared to classic APA capsules [55,56].

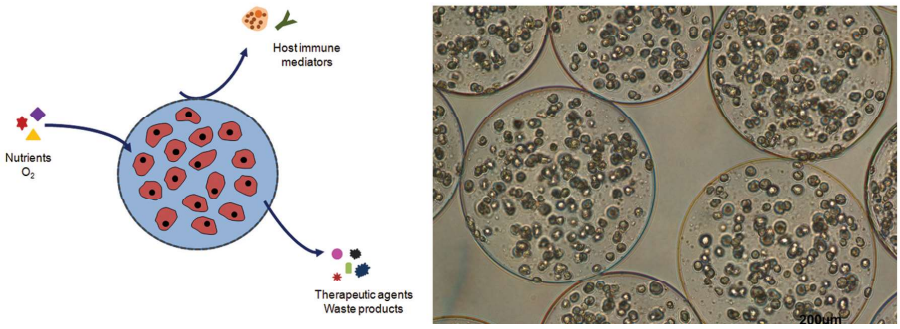

Figure 1: (A) Schematic illustration of the encapsulated cells within the semipermeable membrane. (B) Microscopy images of C2C12 myoblasts in APA capsules. Scale bar: 200 µm

Microcapsule coatings have also been subject of optimization in order to improve mechanical properties and biocompatibility of the capsules. Although the polycation coating is neutralized with a second alginate coat, it does not seem to stealth completely the positive charges of PLL or PLO. In a recent work, a lepirudine-based human whole blood model was used as a tool for measuring the biocompatibility of different microcapsules. It has been described that alginate-polycation (AP) or APA capsules trigger the complement activation whereas Ca/ Ba alginate do not. As it is demonstrated in Figure 2, the deposition of complement component 3 (C3) on bead surface is higher in AP or APA beads than Ca/Ba beads [57]. Thus, new approaches with external coatings focused on avoiding the immune response and improving mechanical properties are being developed. For example, protamine is being studied to replace the classical polycations in APA capsules. In a recent study, alginate/protamine sulphate/alginate capsules containing pancreatic islets has shown an

increase of 30% on cell viability than classical APAs, restoring normoglycemia in chemically induced diabetic mice [58]. Moreover, genipin, a natural and biodegradable cross-linker with low cytotoxicity is being researched. Genipin improves the biocompatibility and mechanical strength of APA microcapsules when is cross-linked with a PLO layer compared to the classic APA capsules [59]. In addition, after extraction of implanted empty capsules, genipin crosslinked capsules show a reduced fibrotic response. Recently Spasojevic et al, have proved to reduce the inflammatory response of alginate-PLL capsules by masking PLL with PEG-b-PLL diblock copolymers [60]. They demonstrated that adding the PEG-b-PLL100 at the capsule surface does not affect cell viability neither during the time that the study was performed nor the molecular weight cut-off of the membrane. In addition, 1 month later when alginate-PLL-PLL100-b-PEG capsules were retrieved from the Balb/c mice, the cellular adhesion at the surface was reduced three times compared to the alginate-PLL capsules. Thus, this could be an alternative method to mask pro-inflammatory components on the surface of microcapsules and reduce the host immune response.

3. THERAPEUTIC APPLICATIONS OF ENCAPSULATED CELLS

Cell type plays a paramount role in the treatment of different diseases with encapsulated cells. Although the cell lines are more resistant, easier to culture and manipulate, they may show erratic and uncontrollable behavior, whereas primary cells are safer but have limited lifespan. In addition to the cell type, the cell source is another limiting parameter due to the large amount of cells that are required. For example, the acquirement of human cells is difficult, expensive and most importantly, limited due to ethical and health-government restrictions. Thus, the utilization of xenogenic cells is extended in cell encapsulation research as they are immunoisolated by the capsule. However, the chemokines and antigens that are released by cells may produce the activation of the immune response and the initiation of an inflammatory process, concluding in graft rejection. Consequently, stem cells (SC), and most especially the mesenchymal stem cells (MSC), are nowadays being employed as an alternative because of their hypoimmunogenic, immunoprotective and plasticity properties [61]. Some of the therapeutic applications with microencapsulated cells will be next summarized.

3.1. Diabetes

Diabetes mellitus (DM) is characterized by the lack of response to high glucose blood levels due to defects on insulin secretion, insulin action or both. Current research efforts on cell encapsulation are mainly aimed to treat type I diabetes, a disease that develops as a consequence of the destruction of pancreatic islets including the insulin secreting β-cells.

Cell encapsulation research has focused on restoring and regulating the insulin supply. First, different in vivo studies were performed with allogeneic islets. However, obtaining large amounts of functional human islets is a hard task, and therefore, several research groups have investigated the use of xenogeneic islets or engineered modified cells to supply insulin [62-64]. In the 1980s, it was shown that implanted microencapsulated xenogenic islets in rats restored hyperglycemia for 2 weeks [65]. Since then, many studies have been carried out with different encapsulation techniques, biomaterials, cell sources, administration routes and animal models but only a few clinical trials have moved forward [64,66-68]. On those trials, mainly pancreatic islets have been entrapped in alginate capsules in order to verify efficiency, biosafety, immunoisolation and the survival of the implants.

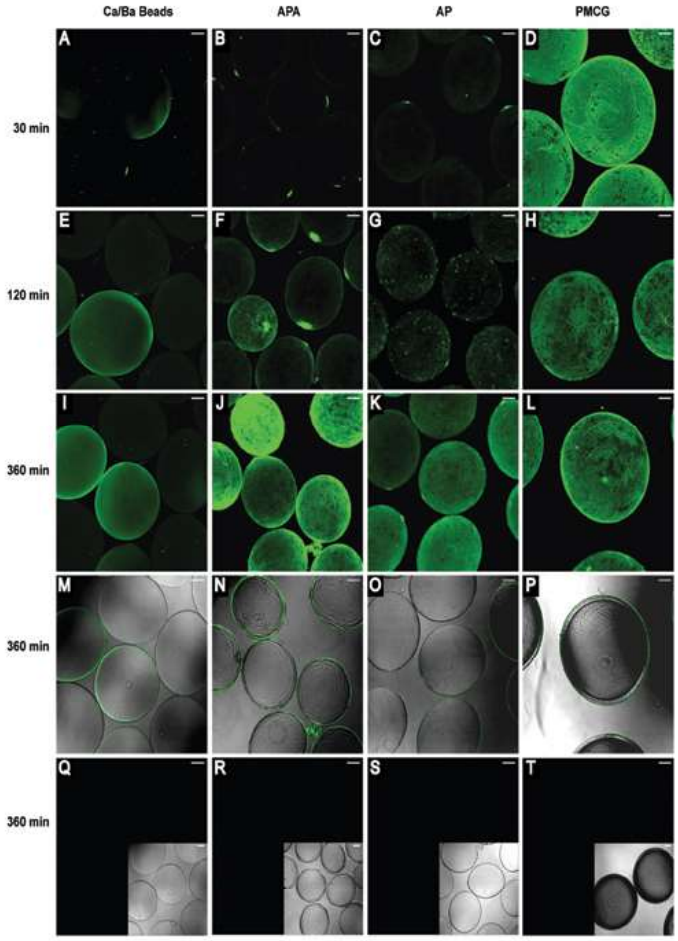

Figure 2: Deposition of C3 on the microsphere surface after incubation in human lepirudin anti-coagulated whole blood. (A–L) 3D projections made by sectioning entire microspheres after incubation for 30, 120 and 360 min. (M–P) Projections through the equator overlaid with transmitted light images after 360 min. (Q–T) Controls are given in the lower panels as projections (black pictures). The inserts show transmitted light equatorial sections for visualization. Bars are 100 μm. Reprinted and adapted with permission from [58] Notes; APA: alginate-poly-L-lysine-alginate; AP:alginate-poly-L-lysine; PMCG: poly(methylene-co-guanidine); Ca/Ba Beads: Alginate beads polymerized with Calcium or Barium without coatings.

In 1996, neonatal porcine islets enclosed in APA capsules were transplanted into the peritoneal cavity of a type 1 diabetic man without immunosupression, achieving a modest decrease of exogenously administered insulin for 12 weeks. One decade later, the implant was explanted and no immune response or fibrosis was perceived, with partial survival of the graft [4]. But, the first clinical trial in non immunosuppresed patients with allogenic immunoisolated islets was launched in 2003 [28]. They used the alginate-PLO-alginate system and standardized procedures for the encapsulation and transplantation of human islets [69]. In this clinical trial, the independence of exogenous insulin therapy was not achieved but patients´ glycemic control was improved for three years. Importantly, the frequency of hypoglycemic episodes decreased and adverse events were not reported [27]. Another clinical trial was performed in non immunosuppressed patients, in order to verify the safety and viability of microencapsulated islets. Human islets enclosed in alginate capsules were implanted in four cases, and no changes in insulin requirements or glycemic control were reported. When implants were explanted, the capsules were intact but enclosed in a fibrotic tissue and contained necrotic islets [70]. In contrast, other studies with pancreatic islets are showing promising results such as the phase IIa clinical trial run by DIABECELL® (Living Cell Technology) company, whose results show an insulin dose reduction of 20% and up to 70% unaware of hypoglycemic events [71]. Although pancreatic islets are the most widely used cell type, others are also being employed (Table 1).

In recent years, different in vitro/ in vivo studies have tried to overcome the drawbacks of the clinical microencapsulated islets transplantation. For example, they have intended to resolve the lost of islets viability due to hypoxia. In this sense, it has been proposed the reduction of the diameter of the microcapsules through the use of an air-driven encapsulator which enhance the nutrients diffusion throughout de capsule [72]. Islets have been co-encapsulated with MSCs in order to improve the graft outcome, as MSCs have shown effects on revascularization and preservation of islet morphology in mice [73]. The co-encapsulation with MSCs resulted in the improvement of islet function in vitro and graft survival in vivo [74]. Other studies are searching for new insulin producing cells/tissue alternatives sources. For example, immortalized fetal human liver (FH-B-TPN) cells have been engineered for the treatment of type I diabetes by inserting the pancreatic and duodenal homeobox 1 gene. In the study in order to differentiate FH-B-TPN cells in insulin secreting ones, the cell culture was made in serum free medium, cell were aggregated and finally microencapsulated. The insulin secretion was exhibited in vitro and in vivo and the mean glucose of diabetic non-obese diabetic mice was lowered in a few days [75]. Alternatively, the use of human embryonic SCs (hESCs) or induced pluripotent stem cells (iPSCs) has been proposed. A two step procedure to differentiate hESCs into mature insulin beta cells in vivo has been described [76]. Also,

an in vitro procedure to differentiate the human pluripotent stem cells (hPSC) to glucose-responsive beta cells has been proposed recently. This procedure could generate large quantities of these cells, doing the beta cells availability a past drawback [77]. On the other hand, iPSCs could also be generated from patient fibroblasts and differentiated into beta cells without ethical implications [78]. Although it has been proposed, the microencapsulation of these cells has not been accomplished yet [79].

Performed by	Biomaterial	Cell type	Immunosupresssion	Results	Year	Ref.
St Vincent Medical Center	Alginate poly-L-lisyne	Human islets	Yes	Insulin independence 9 months (1 patient)	1994	[158]
Living Cell technologies	Alginate	Neonatal porcine islets	No	30% insulin reduction for 14 months. Partial graft survival 9.5 years later (1 patient)	2007	[4]
Novocell	Conformal PEG coating	Human islets	No	Stopped in 2007 (absorbed by Viacyte)	2007	[159]
Australian Foundation for Diabetes Research	Alginate	Human islets	No	No changes in insulin requirements or glycemic control	2009	[70]
University of Perugia	Alginate- poly-L-ornithine -alginate	Human islets	No	Insulin consumption decreased and improved glycemic control (6 cases)	2006/11	[27,28,69]
Cliniques universitaires Saint-Luc- Université Catholique de Louvain	Alginate monolayer (1 – 3 cm^2)	Human islets	Phase Ia: Yes Phase Ib: No	Recruiting patients started in 2008 (NCT00790257)	2011	[160]
β O_2 technologies	Multiple-layer Alginate structure	Human islets	No	Improvements in hemoglobin A1c and reduction in insulin requirement (1 patient)	2013	[161]
Viacyte (VC-01™)	Encaptra® (24 cm^2)	Pancreatic progenitors cells derived from human embryonic stem cells	No	Recruiting patients started in 2014 (NCT02239354)	2014	[162]
Living Cell technologies (Diabecell®)	Alginate	Neonatal porcine islets	No	20% insulin reduction and 70% unaware hypoglycemic events	2014	[71]

Table 1. Clinical trials performed as ongoing for the treatment of type I idabetes cell encapsulation.

3.2. Hepatic diseases

Nowadays, allotransplantation is the most effective therapy for the treatment of various liver diseases and metabolic liver disorders. Unfortunately, the lack of human organ donors restricts its application. Alternative sources to liver tissue have been searched and developed for human liver transplantation, such as bioartificial livers and whole liver xenotransplantation [80,81]. As another alternative, the transplantation of isolated xenogenic hepatocytes has been proposed to treat severe diseases such as acute, chronic or fulminant hepatic failure, due to their unlimited availability and its cost-effectiveness [82]. Porcine

hepatocytes seem to be a proper cell source due to their similarities with human physiology and the great number of them that can be obtained. In fact, they have been used in studies with small animals and primates [83,84]. Even so, host immune response and graft rejection represents an important concern. Different groups have taken advantage of cell encapsulation technology and have developed strategies for the therapy of liver failures with porcine hepatocytes [85].

In the 1990s, it was the first time that an allo-transplantation of hepatocytes in alginate capsules was performed in rats with galactosamine-induced fulminant liver failure (FLF). An increase on the survival rate up to 80% and a decrease on serum bilirubin levels for 4 weeks was observed [86,87]. Since then, different in vitro and in vivo studies have been performed to characterize the encapsulation of porcine hepatocytes [88,89]. In addition, some groups have employed different cryopreservation systems to store encapsulated porcine hepatocytes in order to approach this therapy to the clinic, and prove if banks of encapsulated hepatocytes could be developed in a future [43,90-95]. In mice induced to FLF with acetaminophen administration followed by a 30% hepatectomy, encapsulated and cryopreserved porcine hepatocytes were implanted. Encapsulated hepatocytes provided liver specific metabolic functions and improved regeneration of liver tissue without immunosupression with survival rates up to 70% [91,93]. These results show that biobanks of encapsulated hepatocytes are not beyond the bounds of possibility for clinical use in FLF. However, there are no clinical trials performed with microencapsulated porcine hepatocytes yet.

Other groups have co-encapsulated hepatocytes with bone marrow SCs (BMSC) as BMSCs seem to maintain the specific function and phenotype of hepatocytes. Different xeno/singenic in vivo studies have showed that the co-encapsulation of hepatocytes and BMSCs prolongs the viability of hepatocytes and enhances their ability to correct congenital hyperbilirubinemia in Gunn rats [96,97]. Other studies have compared the survival rate of singenic encapsulated MSCs to encapsulated hepatocytes after transplantation in partially (90%) hepatectomized rats. Encapsulated MSCs showed a survival rate up to 90% and released hepatotrophic grown factor and IL-6, maintaining TNF-α at low levels, unlike the implanted free MSCs [98-100]. Moreover, on these studies, it was also compared two transplantation sites, showing that intrasplenic transplantation of bioencapsulated MSCs increases the recovery rates of hepatectomized rats compared to intraperitoneal transplantation. These findings together with the simplicity to isolate and expand porcine MSCs suggest that MSCs could be an alternative to hepatocytes in the treatment of hepatic failure.

3.3. Central Nervous System (CNS)

Cell encapsulation also has high potential on the treatment of diverse

neurodegenerative diseases. Some of these diseases have been associated not only with neuronal damage, but also with an initial vascular damage that could trigger a neuronal damage too. In this sense, neurotrophic factors, angiogenic proteins or the combination of both have been used for their neuro-angiogenic protection and neuronal proliferation. These factors have a short half-live in the organism and show notable side effects when they are systemically administered. Therefore, the encapsulation of cells that release these agents seems to be a suitable therapy for local implantation in the injured site.

One of the most prevalent neurodegenerative diseases is Alzheimer's disease (AD), the most common form of dementia. The progressive deterioration of cognitive and mnemonic ability is its most remarkable characteristic, and they occur as a result of the degeneration of basal forebrain cholinergic neurons. Another feature in AD is the formation of beta amyloid plaques (Aβ) and tau protein aggregates in brain, which some researchers claim as responsible of the neuronal loss and others as the results of it. Nowadays, the treatment of AD consists on the pharmaceutical treatment of the symptoms, in plus of the psychosocial attention and care giving of the patient. The neuronal protection and clearance of Aβ plaques could be a possible treatment of the AD and it is currently under research [101]. Thus, we encapsulated vascular endothelial grown factor (VEGF) secreting fibroblasts and implanted into AD transgenic mice (APP/Ps1) achieving an improvement on angiogenesis that helped on the clearance of Aβ plaques in the damaged brain. Moreover, neuronal apoptosis was decreased and cognitive defects ameliorated in the treated mice [102]. Subsequently, we demonstrated that the implantation of encapsulated fibroblasts secreting VEGF increased the number of neuronal precursors during 6 months in the treated mice, as well as the cellular proliferation in the hippocampal dentate gyrus [103].

Another prevalent neurodegenerative disease is Parkinson disease (PD). PD patients show progressively motor impairments as result of the dysfunction and death of dopaminergic neurons in the substantia nigra. Some research groups have encapsulated porcine choroidal plexus cells, that secrete most of neurotrophins present in cerebrospinal fluid [104]. They have shown significant neurological improvements in animal models of brain diseases after implantation with choroidal plexus (CP) in alginate-PLO microcapsules [105-108]. When they tested the recovery of neurological functions in a non-human primate model of PDs with the administration of encapsulated CP for 6 months, the striatal neural fiber network was restored and neurological behaviors enhanced due to the secretion of neuroprotective and neurorestorative factors into the affected site [109]. Currently, Living Cell Technology is recruiting patients to perform a phase I clinical trial with the product named NTCELL®, based in encapsulated eCPc [110].

But microencapsulation technology has also been studied in other CNS injuries,

apart of the neurodegenerative diseases, such as the intra cerebral hemorrhage (ICH). Glucagon like peptide 1 (GLP-1) has exhibited neuroprotective and neurotrophic activity as well as an anti-apoptotic effect on neurons [111]. Thus, GLP-1 secreting MSCs have been entrapped in alginate capsules and implanted in the right ventricle of an animal model of traumatic brain injury [112]. Results showed that after transplantation there was a reduced hippocampal neuronal cell loss and less cortical glial and cyto-skeletal abnormalities. Taking into account this research, in 2011 it was accomplished a phase I/II clinical trial with 11 patients suffering from ICH. The principal purpose was to assess the safety of GLP-1 cell beads® which consist on GLP-1 secreting allogenic MSCs. Beads were implanted in the brain after surgical evacuation of the hematoma. Afterwards, capsules were removed in a second surgery after 14 days of treatment. The study has been terminated and no side effects have been detected neither from surgical interventions or from the implants themselves [113]

3.4. Another diseases

Besides the therapeutic applications mentioned above, microencapsulated cells have also other uses as drug delivery systems in the treatment of diverse pathologies. Parathyroid allotransplantation constitutes the last therapeutic alternative for patients suffering from postsurgical hypoparathyroidism [114]. Therefore, in order to avoid transplant rejection, diverse attempts have been performed to microencapsulate parathyroid tissue [115]. In the first study, two cases have been reported declining by half daily in calcium and vitamin D replacement therapy [116]. In the study performed in 2009, two different implantation of alginate microspheres were performed in a patient with continuous intravenous requirement of calcium to survive [117]. Twenty months later, functionality of the graft was demonstrated and no requirement of intravenous calcium was achieved. Taking these results into account, authors suggested that this procedure could be used as a therapeutic alternative in severe hypoparathyroidism

On the other hand, cell microencapsulation could be applied in heart regeneration. As commented previously, angiogenic factors can improve new blood vessel growth and restore perfusion in damaged or ischemic myocardium [118]. Therefore, the employment of angiogenic factor in the infarcted area could be suitable as an alternative therapy in patients in whom conventional revascularization is not recommended [119]. In a study by Zang et al, xenogeneic CHO cells modified to express VEGF were immobilized in APAs and were implanted into the injured myocardium [120]. The in vivo experiment was performed for 21 days showing that microencapsulated CHO cells could augment angiogenesis, increase global heart function in the post-infarcted myocardium and maintain microencapsulated

CHO structure. Thereby, these experiments suggested that microencapsulated xenogeneic cell-based gene therapy might be a novel alternative strategy for therapeutic angiogenesis in ischemic heart disease.

Last but not least, cell microencapsulation has been employed for cancer treatment. The vast majority of the research has focused in the treatment of malignant brain tumors [121]. The procedure in the treatment would be straightforward: during surgery, just after removing the tumor mass, the encapsulated cells releasing therapeutic agents would be implanted in the immediate vicinity of the excised tumor. In the subsequent days, the therapeutic agents released from the microencapsulated cells would inhibit the growth of the malignant cells that were not retrieved by the surgery. In brain tumor models, few pre-clinical studies have shown the efficacy of cell encapsulation technology. Several studies have been made with cells that release different therapeutic agents such as endostatin and TNF apoptosis inducing ligand among others, obtaining promising results [122-125]. However, there are no clinical studies performed for malignant brain tumors.

On the other hand, in pancreatic carcinoma, a clinical trial has been performed [126]. Genetically modified allogeneic cells were encapsulated in cellulose sulphate (NovaCaps®) to over-express a CYP enzyme. Fourteen patients suffering from pancreatic cancer were treated and the results were promising: the tumors of four patients regressed after treatment, and those of the other ten individuals who completed the study remained stable. Median survival was doubled in the treatment group by comparison with historic controls, and 1-year survival rate was three times better. These data demonstrate the usefulness of cell microencapsulation in the treatment of diverse tumors.

4. STEPS TO CLINICAL TRANSLATION

Cell microencapsulation represents a promising technology for the clinical treatment of several diseases. However, some aspects such as biosafety or the scale up from lab to factory need to be more deeply studied to get the approval from government healthcare agencies and reach clinic use.

In cell encapsulation, the “de novo” release of therapeutic agents should be controlled, with the possibility of interrupting it if adverse effects are detected. Thus, different systems that regulate cell behavior have been developed. For example, the tet on/off system stops the transcription of the codified gene after stimuli with doxicicline, allowing the production of therapeutic agent during the wished period of time [127]. Moreover, the cell source is an important biosafety issue to be taken in account. Different SCs types have shown notorious tumorigenic potential, such as immortalized MSCs, ESCs or iPSs, being

necessary the control of cell behavior within capsules after implantation [128]. A system that can prevent the abnormal cell growth is the inclusion of the thymidine kinase suicide gene in the enclosed cells, which produce highly toxic triphosphates in the presence of ganciclovir leading to cell death. This system allows the inactivation of the encapsulated cells genetically modified with the Herpex Virus thymidine kinase (HSV-TK). Our group has demonstrated the effectiveness of this system by decreasing drastically the viability and secretary function of encapsulated Epo secreting C2C12 myoblasts after ganciclovir administration [129].The study demonstrated that HSV-TK system represents an effective alternative to control the abnormal growth of encapsulated cells. Another suicide system is based on an inducible caspase-9 protein that is activated using a specific chemical inducer of dimerization which triggers cell apoptosis. It has been characterized in vitro and in vivo in MSCs and subsequently tested in a phase I study with modified T cells [130,131]. With a single dose administration of the dimerizing drug, MSCs and their progeny were selectively eliminated in 24 hours while 90% of the modified T cells were eliminated in 30 minutes in the Phase I study. These results confirm that the introduction of a transgene regulating cell apoptosis, could overcome the fear of stem cells employment for their tumorigenic potential, enhancing their use in the clinic. On the other hand, Bhujbal et al, have recently designed a novel immunoisolating encapsulation system to overcome cell protussion [132]. Baby hamster kidney (BHK) cells expressing anti-tumor protein sLrig1 were firstly mixed with 3.4% intermediate-G alginate, transferred into droplets with an electrostatic bead generator and coated with 0,05% PLL. Later, these Alginate-PLL capsules were suspended in a 2% high-G alginate solution and passed through an air droplet-generator to form multilayer capsules. In this study, they demonstrated that the multilayer capsules are able to maintain cell viability for a long time and strongly reduce cell protrusion. This new method can be an alternative process to control the biosafety of encapsulated cells, but further studies in pre-clinical models in vivo are needed to determine its efficacy.

A biocompatibility issue takes place at the early stages after implantation, when a deeply hypoxic and proinflamatory microenvironment that can affect the enclosed cells, which may limit the survival of the graft, is generated. Thus, the search of an optimal administration site is of great relevance for the implant success. Intraperitoneal cavity (IC) and the subcutaneal cavity (SC) are the most suitable delivery sites if the released therapeutic agent has systemic effect because these sites present low hypoxic and proinflamatory enviroments [133,134]. In contrast, for a local effect of the released therapeutic agent, implantation must be performed next to the affected/ objective tissue, where hypoxia and inflammation post-implantation can have drastic outcome on graft survival. Dexamethasone (DXM)-loaded poly(lactic-co-glycolic) acid (PLGA) microspheres and encapsulated C2C12

myoblasts embedded in hydrogels that mediate the prolonged release of DXM, reduce the post-transplantation inflammation and foreign body reaction and prolong the implant lifetime [135], represent an interesting alternative to circumvent these early drawbacks. Moreover, another mayor concern occurs at the removal stage of the graft, since microencapsulated cells can spread in the administration site, being difficult their retrieval. In order to overcome this drawback, encapsulated cells can be monitored. Luciferase is a good non-invasive biosystem for monitorization of encapsulated cells. Our group has fused the properties of three genes, HSV1-TK, the green fluorescent protein and the Firefly Luciferase, and implemented the triple reporter system (SFGNESTGL) into enclosed C2C12 cells. The triple reporter vector allows the in vivo monitoring of encapsulated cells after the administration of D-Luciferin, whereas cell viability can be controlled with the administration of ganciclovir [136]. Another alternative is the delimitation of microencapsulated cells within injectable scaffolds, such as implants based on calcium phosphate cements (CPC) or hydrogels. We have studied the incorporation of APA capsules into UPLVG alginate hydrogels with an in situ prepared scaffold, improving the microcapsules retention, reduction of post-transplantation inflammation, in plus of facilitating transplantation procedure and implant retrieval [135]. Alternatively, biodegradable microcapsules can be employed allowing the host immune to clear cells without the need of retrieval. In this sense, a system consisting on PC12 cells entrapped in alginate-glutamine degradable poly-L-lysine microcapsules, and subsequently placed in PEG-coated poly (ε-caprolactona) (PCL) nanoporous chambers, showed to be biodegraded and cleared once the therapeutic effect of the released dopamine by PC12 cells was achieved [137].

On the other hand, the scale up of the microencapsulation technology is also another pitfall to bear in mind as it will be the final step in order to move from bench to bedside. Currently, just one three dimensional microfluidic system to obtain large amounts of encapsulated cells has been described. The system produces hydrogel microspheres ranging from 500–700 μm in diameter. This new microencapsulation approach can potentially reduce the classical human islet microencapsulation time in several orders of magnitude, from 8 to 64 times [138].

While the production of large amounts of encapsulated cells is resolved, the long-term storage of biosystems is also a crucial task. One long-term storage technique is the cryopreservation, which is defined as the use of very low temperatures to preserve structurally intact living cells, tissues or maybe even whole organs in the future [139-141]. Cryopreservation has also shown good results in preserving tissue-engineered constructs (TECs) such as encapsulated cells [142]. In this process, cells, TECs or tissues are suspended in a salt solution with one or more cryoprotectants (CPAs), cooled to very low subzero

temperatures in liquid nitrogen and stored for a long period of time. When the sample is required, it is warmed rapidly to recover its normal function [143]. The aim of adding CPAs in this process is the suppression of ice formation replacing the water in cells/tissues and increasing the total concentration of all solutes. However, CPAs use is limited by their toxicity[144].

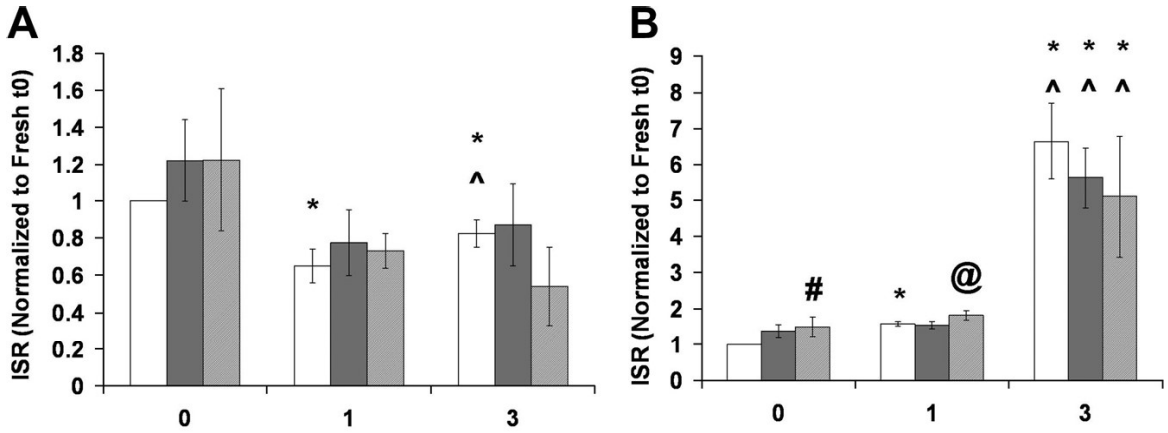

Figure 3: Metabolic activity over time in Fresh and Cryopreserved stable C2C12 cells encapsulated in hydrogels and cultured A) 1 (RGD C1) or B) 4 (RGD C4) days post-encapsulation. Cryopreserved groups and Fresh controls were assessed up to 3 days post-warming. All groups were normalized to the Fresh group at t0. The Fresh group is represented by the white bars, the Frozen group by the dark gray bars, and the DPS-vitrified group by the light gray bars. *p<0.05 compared to same group at t0, ^p<0.05 compared to same group at one day post-warming, #p<0.05 compared to Fresh group at same time point, @p<0.05 compared to Frozen group at same time point. n=3. Notes; RGD C1: RGD-alginate beads cultured for 1 day prior to cryopreservation; RGD C4: RGD-alginate beads cultured for 4 day prior to cryopreservation. Reprinted and adapted with permission from [156]

The dangers associated with freezing cells, TECs or tissues include mechanical damage due to intracellular ice crystal nucleation, osmotic damage by water influx and efflux, and solute effect, described as chemical damage owing to an increase in the intracellular ions concentration. The damage can be produced both during the cooling and the thawing process. Therefore, the rate of temperature change as well as the CPA concentration and election, have to be extensively controlled during CPAs addition and removal [145-149]. Mainly, two different freezing methods are currently in use: the slow freezing protocol, that uses slow cooling rates and low concentration of CPAs, minimizing the creation of intracellular ice and the toxic and osmotic damage of cells to exposure to CPAs, and the vitrification, where the sample is rapidly cooled with high concentrations of CPAs achieving an ice-free/ vitreous state, not only on cooling but also on thawing. Slow cooling protocol has mostly studied with encapsulated hepatocytes, pancreatic islets and MSCs [43,67,91-93]. We tested

several dimethyl sulfoxide (DMSO) concentrations in the long term storage of encapsulated C2C12 recombinant myoblasts concluding that concentrations of 10% DMSO preserves the cell capacity to modulate the hematocrit [150]. Vitrification has also been employed for the preservation of encapsulated hepatocytes, pancreatic islets and MSCs [94,95,151,152]. For example, MSCs have been vitrified in alginate-fibrin capsules, demonstrating that proliferation and differentiation capacity was maintained, in plus of no detecting microstructure damage in capsules [153].

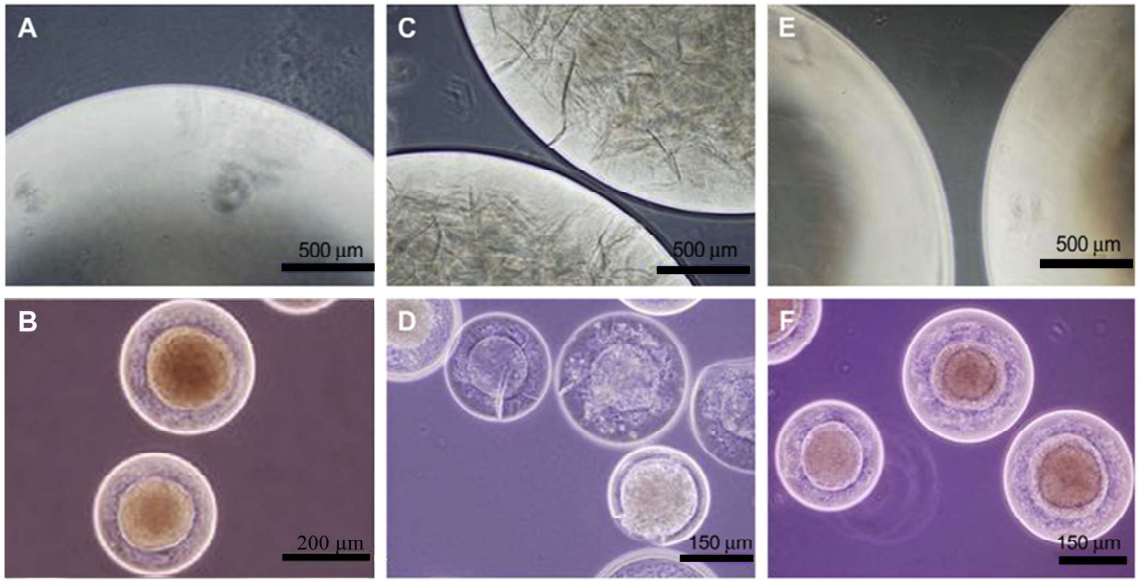

Figure 4: Morphology of agarose beads after cryopreservation and thawing process. Agarose beads without cryopreservation as a control (A), control containing islets (B), freezed with DMSO (C), freezed with DMSO containing islets (D), vitrified with KYO-1 solution (E) and vitrified with KYO-1 solution containing islets (F). Scale bars: 500 µm for (A,C,E), 150 µm for (D,F), and 200 µm for (B). Notes; KYO-1: vitrification solution consisted in Ethylene Glycol (5,38M), DMSO (2M), Poly-ethylene glycol (PEG) 1000 (0,1M) and Polyvinylpyrrolidone K10 (0,00175M) diluted in EuroCollins solution. Reprinted and adapted with permission from [67]

Unfortunately, not many studies have directly compared vitrification and conventional freezing protocols. The use of different cell lines, freezing protocols, biomaterials and CPAs solutions among studies does not make easy the comparison between the results. Recombinant insulin-secreting C2C12 myoblasts and mouse beta cell insulinoma cell lines (β-TC) have been encapsulated and vitrified or freezed in two different studies [154,155]. From both studies, it can be concluded that slow cooling is more appropriated due to the simplicity of the procedure and the maintenance of insulin secretion compared to the

vitrified group that reduced insulin secretion (Figure 3). In contrast, Agudelo et al [66,156] showed that vitrification was better than slow cooling due to the maintenance of the integrity of the agarose capsules, a higher insulin secretion of islets and better cell functionality maintaining blood normoglycemic levels in diabetic mice for 32 and 46 days (1000 and 2000 islets implant respectively) (Figure 4). Altogether, it seems clear that for each type of cell and biomaterial, a specific study regarding the choice of the cryopreservation protocol and the optimal CPAs solutions should be done. In this sense, the mathematical model proposed by Sambanis group would be a useful tool [157].

5. CONLUSIONS

Cell encapsulation has been widely studied and developed during the last years with several clinical trials conducted, resulting a promising technology for the treatment of diverse diseases. However, technological properties and biosafety concerns need to be improved. The synergies between disciplines such as chemistry, cell engineering, pharmaceutical technology, biology and materials science hopefully will lead to finally bring cell microencapsulation a little closer to the clinic. Although, we have described in this review several therapeutic applications, this technology has more therapeutic potential to be discovered.

6. EXPERT OPINION

Since in the 1960s it was proposed the microencapsulation of cells within polymeric matrix, this technology has evolved enormously. The main advantage that encapsulation technology provides is the immunoisolation of the enclosed cells which yield multiple therapeutic applications. Immunoisolation allows the “de novo” secretion from the entrapped cells of the therapeutic agent, instead of its repetitive administration, such as in the case of the need of insulin administration in type I diabetes. Moreover, the potential hold by this technology is augmented since the entrapped cells can be genetically modified to express and release the desired therapeutic agent. Another advantage provided by this technology is that allogeneic or xenogeneic cells can be entrapped into microcapsules with a minor rejection by the body. This latter advantage allows the transplantation of new cell sources that can replace the limited disponibility of human donor cells. For example, encapsulation of SCs, such as hESCs and iPSCs, is under study. However, they show the limitations of having a tumorogeneic potential in both cell types or the low obtaining efficiency of IPSCs. More extended is the use of MSCs due to their capacity to immune modulate the microenvironment. Thus, MSCs have widely studied, raising progressively their research in encapsulation.

In spite of the enormous possibilities that microencapsulation offers, there are still

several bearings that need to be improved or resolved. First, the survival of the enclosed cells after implantation should be improved. Microcapsules matrices should mimic the cell source microenvironment, and in this sense alginate has been modified or mixed with natural as well as artificial extracellular matrix. Second, the hypoxia suffered by the graft and the fibrotic pericapsular growth after implantation have not been resolved yet, being responsible of implant failures in several clinical trials. Thus, hypoxia has been approached by the induction of pre-vascularization with pro-angiogenic molecules, oxygen suppliers, oxygen carriers within the microcapsules or arterial connections. On the other hand, to prevent the biocompatibility issue of the pericapsular fibrotic overgrowth, the employment of more biocompatible coatings as well as microcapsules embedded with anti-inflammatory drugs have been studied [59,135]. Third, envisioning the use of hESCs or iPSCs, systems that allow controlling the viability of the cells would result of great interest. Moreover, if the release of the therapeutic agent is not desired anymore, these systems would allow the inactivation of the graft. The use of suicide genes provides a suitable system to resolve this biosafety pitfall. Another alternative is the removal of the graft, but the spreading of microcapsules could result in a new biosafety hurdle. To resolve this, graft monitoring systems would allow detecting if any remaining microcapsule has not been removed from the patient. Finally, we believe that the control of drug delivery by encapsulated cells in a sustained or sequential way, in response to external stimuli or environmental changes, is another relevant point to explore and develop in cell encapsulation. In this regard, conditional promoters allow the management of sustained/pulsatile drug release and represent a new step in drug delivery control. However, there is still much to do and more accurate systems are needed to achieve a fit control of drug release from encapsulated cells. Among the drawbacks mentioned above, we believe that biosafety and biocompatibility are still the pivotal aspects that need to be notably optimized in order to move this therapy into the clinic.

Thinking on clinical translation, the preservation of encapsulated cells will represent a notorious advantage. The establishment of biosystem banks may facilitate the “on demand” access of patients to the treatment in a clinic far from the manufacturer. Thus, encapsulated cell banking would allow the transport of encapsulated cells between labs and clinical centers making a notorious step in reaching the clinic. In addition, this process would reduce the costs of production which is a pivotal need in the final translation of this technology from bench to bedside. The main methodologies for preservation of encapsulated cells are vitrification, that prevents ice formation within the sample, and the slow freezing protocol in which the intracellular ice formation is minimized but the extracellular formation is not. Importantly for cell microencapsulation, the formation of ice could also compromise the biomaterial or capsule integrity, being vitrification a priori the best method of cryopreservation for

multicellular systems. Independently of the best cryopreservation method, their optimization will entail simplified processes, the maintenance of capsule integrity and cell viability, the avoidance of animal products and organic compounds, the reproducibility of the system and the possibility to preserve large quantities of microcapsules.

The final step to clinic will be the scaling up of cell encapsulation from lab to factory. Previously, the standardization and definition of protocols must be done in order to minimize lab to lab variability. In addition, the process has to guarantee the reproducibility and safety but also needs to meet the high-output requirements of healthcare governments, while accomplishing the good manufacturing practices (GMP). In conclusion, we consider that the tirelessly pursuit of developments on cell encapsulation would end up in more ongoing clinical trials reaching the clinic and the ultimate goal, the amelioration of patients diseases.

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Advances in the slow freezing cryopreservation of microencapsulated cells

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ABSTRACT

Over the past few decades, the use of cell microencapsulation technology has been promoted for a wide range of applications as sustained drug delivery systems or as cells containing biosystems for regenerative medicine. However, difficulty in their preservation and storage has limited their availability to healthcare centers. Because the preservation in cryogenic temperatures poses many biological and biophysical challenges and that the technology has not been well understood, the slow cooling cryopreservation, which is the most used technique worldwide, has not given full measure of its full potential application yet. This review will discuss the different steps that should be understood and taken into account to preserve microencapsulated cells by slow freezing in a successful and simple manner. Moreover, it will review the slow freezing preservation of alginate-based microencapsulated cells and discuss some recommendations that the research community may pursue to optimize the preservation of microencapsulated cells, enabling the therapy translate from bench to the clinic.

Keywords: cryoprotectant (CPA), dimethylsulfoxide (DMSO), slow freezing, drug delivery, cell microencapsulation, alginate

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1. INTRODUCTION

The increasing use of cell therapies for the treatment of diverse diseases has promoted the research of the areas that will enable the advance and spread of these therapies. In this way, a process that will allow correct storage, shipment and delivery of cell-based products has also gained attention. One of the possible methods for the delivery of cell-based products is refrigerated shipment or short-term preservation. However, this method will only permit storage for a few days and rules out the possibility of being a desirable method for long-term storage or delivey. Thus, the preservation at deep cryogenic temperatures (ranging from -196°C to approximately -150°C) where cells, tissues, cell-based products or organs are suspended in a tissue medium with one or more cryoprotectants (CPAs), a process named cryopreservation, is the only technology that enables "holding the biological clock" of cell-based products and facilitates the "on demand" access of patients to the treatments in a clinic far from the manufacturer [1,2]

Theoretically, if cryopreservation works successfully, it should provide a quality product that ensures the previous characteristics are kept. However, during the cryopreservation process several mechanisms lead to cell damage and compromise product quality and integrity. The size of the samples, as an example, represents an obstacle for the penetration of CPAs and can interfere with cooling and warming profiles [3]. The exposure of the embedded cells to the CPA depends on the cell location in the structure, which could lead to different cell viabilities throughout the cell-based construct. In this sense, there are different protocols for storing cell therapies, offering both advantages and drawbacks that should be taken into consideration to ensure the correct storage of the cell-products (Table 1). Currently, the most used procedures are either slow freezing or vitrification. Vitrification is a rapid freezing method at which a solution, using high amounts of CPAs (excess of 40% w/v), is cooled below its glass transition temperature without ice crystal formation leading to an amorphous matrix that could also be defined as an extremely cold viscous liquid [4]. In this method, ice formation is avoided throughout the entire sample, but these high CPA concentrations are normally toxic to mammalian cells, oblying to minimize the exposure to CPAs complicating the handling process [5-7]. The controlled rate or slow freezing has been developed over the past 40 years, establishing protocols to preserve samples such as embryos, cells, blood products or stem cells among others [8-10].

In contrast to vitrification, samples are cooled in a controlled way (for mammalian cells -1˚C/minute) using lower concentrations of CPAs, and thus, producing ice crystals. The intracellular ice formation (IIF) is avoided with the use of slow cooling rates and the dehydration of cells, and even if ice crystals are nucleated in the samples, cell viability and

function are preserved in different cells, tissues, and cell-based products [11,12]. This method is also used for larger samples and technically is a simpler procedure than vitrification [4]. Moreover, slow freezing does not need advanced equipment, and in the case of alginate cell-based products, almost all samples are preserved using this protocol [12-15]. This review will discuss the slow freezing method for the preservation of cell-based products. The steps for a successful slow freezing will be summarized and the preservation of the alginate-based cell products will be discussed.

	Advantages	Drawbacks
Slow freezing	Low concentrations of CPA	Ice creation through sample
	Simple handling and equipment	Possible structure/tissue damage
	Possibility to storage large volumes	
Vitrification	Ice creation is prevented	High amounts of CPAs
	Preservation of tissues structures	CPAs related toxicity
		Complex addition and removal of CPAs

Table 1: Advantages and drawbacks of the different cryopreservation methods

2. CELL INJURY IN SLOW FREEZING

Undestanding of the mechanisms that could damage cell viability and function is essential for the cryopreservation of cell-based systems. During the slow freezing process, the ice crystal formation in the whole sample cannot be avoided. Therefore, a successful protocol with an optimal cooling rate that prevents major injury mechanisms (e.g. IIF, solute toxicity and osmotic damage) should be employed.

Cryoprotectant (CPA) is a term coined to describe “any additive which can be provided to cells before freezing and yields a higher post-thaw survival than can be obtained in its absence” [16,17]. CPAs reduce the water content in cells/tissues and increase the total concentration of all solutes. They are added before cryopreservation to the freezing solution to prevent the IIF in the process, which is associated with lethal cell injury in the majority of cases [18]. However, the CPA concentration for obtaining a beneficial effect, is far higher than other solutes in normal cell media solutions, and compromise its use by its

toxicity. In the cryopreservation process, when CPAs are added to samples, cells will initially shrink as water leaves the cells by osmosis in response to the increased solute concentration extracellularly. If permeating CPAs are employed, after the initial shrink cells will swell while CPA crosses the cell membrane. This later exchange of CPAs is due to the cells increased permeability to water rather than solutes [19]. By contrast, if only non-permeating CPAs are used, this swelling will not be occur and cells will remain dehydrated. Even though the CPAs are necessary in cryopreservation to prevent IIF, the addition and removal of CPAs could exert the above-mentioned osmotic stress on cells, which may result in excessive cell injury or death [20]. Thus, the addition of CPAs should be controlled precisely to ensure cell function and viability after the storage.

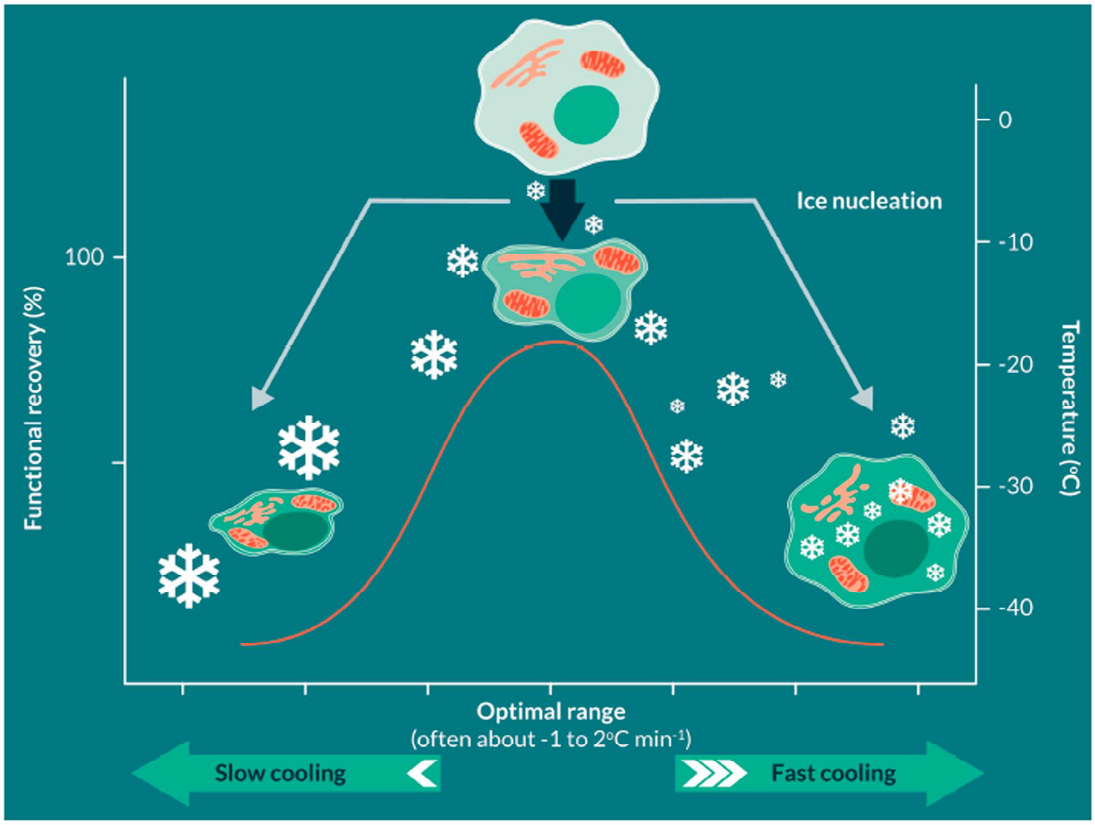


Figure 1: Schematic of Mazur´s 2-factor hypothesis. A cell with CPA protection subjected to cooling at different rates. Maximum functional recovery is achieved with an optimal cooling rate providing reversible dehydration occurring over the high subzero temperature range. If cooling is too slow, irreversible injurious dehydration can take place, for example the mitochondria and endoplasmic reticulum are structurally compromised. If cooling is too fast, cells do not have time to optimally dehydrate, and residual intracellular water can form ice, which is again injurious and can compromise structure of organelles. For many nucleated mammalian cells, 'optimal' cooling equates to rates of around -1°C min-1. Copyright of BioInsights Publishing Ltd. Cell Gene Therapy Insights 2017; 3(5), 359-378. DOI: 10.18609/cgti.2017.038.

The kinetics of cooling rates also influence the outcome of the cryopreservation. From the empirical observation of the slow cooling profiles, and their good results, Mazur and colleges proposed a hypothesis to explain the biophysical processes that occur during cryopreservation, the called Mazur's two-factor hypothesis (Figure 1). In the slow freezing preservation, cells are required to be optimally dehydrated to avoid IIF. At an optimal cooling rate, cells would be dehydrated with their sensitive molecular and ultrastructural components protected by the added CPA. However, if a too fast cooling rate is used, cells will not have time to dehydrate, and intracellular ice could form from the remaining residual intracellular water. By contrast, with a too slow cooling rate, cells will suffer an irreversible injurious dehydration from the long exposure to hypertonic solutions. This could disrupt the biochemical and physical conditions required for cell survival, and mitochondria and endoplasmatic reticulum could structurally be compromised. In this sense, the "inverted U" survival effect showed graphically by Mazur is displayed in one of the best cold biology compilations (Figure 1) [21]. In that compilation, different cell types are pulled together to demonstrate that even when the difference between the optimal cooling rates of diverse cell types is huge, this "inverted U" survival interval could be observed in all. However, this is an oversimplification of the complex biophysical processes occurring during cryopreservation, and many still remain unclear [22]. Nevertheless, the two-factor hypothesis has been observed in many other results after its proposal, and its utility to study and optimize the slow freezing protocols of different cell-based therapies is undoubted.

3. STEPS IN A SLOW FREEZING PROTOCOL

The cell-based products are subjected to different proccesses during the slow freezing cryopreservation, and each of these have to be properly performed to cryopreserve cells successfully (Figure 2). Cell-based systems have to be prepared to undergo freezing, and an appropriate CPA solution has to be chosen avoiding the mechanism that could impair the cell-based products function and integrity. Moreover, the samples should be preserved at the adequate temperatures, and although the thawing has not be given as much importance as the cooling, it deserves the same attention. The steps that should taken in consideration to achieve a successful slow freezing cryopreservation will be next summarized and discussed.

3.1.Choice of CPA solution

The CPA solution is one of the variables that is being optimized to improve the outcome of the cell-based products recovered from cryopreservation. Since the required CPA concentration to prevent IIF are not the physiological ones present in the cell media solutions,

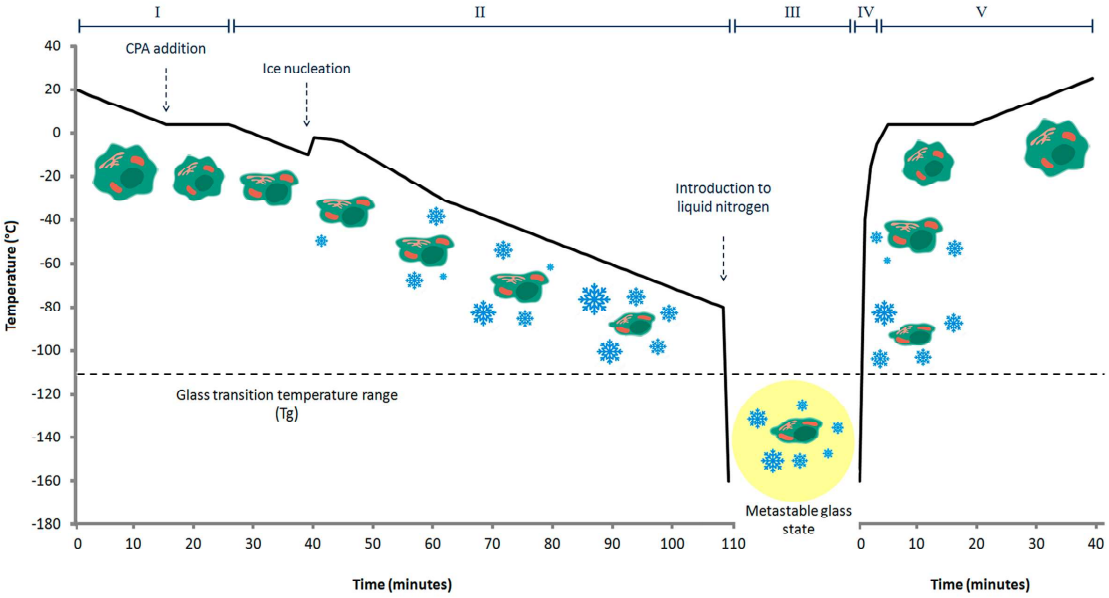
a long exposure time could lead to non reversible cell damage [17,23]. Thus, researchers always tend to reduce as much as possible the amount of CPAs in the solution, being a research line the combination of different CPAs to decrease their related toxicity [24]. It was established a distinction between CPAs, depending if were able or not to permeate the cell membrane (Table 2). Permeating CPAs such as glycerol, are molecules of low molecular weight that can go through the cell membrane by osmosis, replace the water inside the cells and hold the non-freezable water within the cells [17]. The most common is dimethylsulfoxide (DMSO), it is included in the range of 5 to 12% (v/v) in most of the CPA solutions for every cell type with excellent results [8,11,12,14,15]. For example Hemacord®, an allogeneic cord blood hematopoietic progenitor cell therapy product which is approved by the FDA, is cryopreserved with DMSO obtaining great results in clinics. However, DMSO employment was related to adverse effects after the transplantation of cryopreserved cell therapy products, and many researchers started to reduce the amount employed in their protocols, or even remove it [25,26]. After reviewing some of the clinical trials procedure that showed DMSO toxicity, it was found that DMSO was not entirely removed or that some procedures were not appropriately performed, and some experts claimed that the related adverse effect of DMSO could have been avoided [27]. In this respect, a report of an expert meeting discusses the path that research may take to make progress in successful preservation of cell therapy products, and recommends that an actual review addressing the reality of DMSO toxicity would be extremely valuable to go forward in cell-based products employment [28]. Nevertheless, other research groups are also adding non-permeating CPAs to speed up the dehydration process of the cells in supra-zero temperatures and reduce their toxicity. In this sense, sucrose and trehalose are the most used non-permeating CPAs at concentration ranges between 0.1-0.3M. They have been added to many protocols obtaining better results than with DMSO alone, and allowed in some studies the decrease of DMSO percentage in the CPA solution [24]. In the cryopreservation of human umbilical cord as source of Wharton's jelly mesenchymal stem cells, the CPA solution that combine DMSO 10% and Sucrose 0,2M displayed better results than only DMSO 10% in terms of MSCs isolation and expansion from the umbilical cord after thawing [29]. It should be borne in mind, that intracellular ice protection is required in the cryopreservation of nearly all nucleated mammalian cells, and that non-permeating CPA could provide some benefits in the extracellular environment, but not the primary ice protection inside the cells. Thus, non-permeating CPAs are usually used in addition to permeant CPAs. However, if the non-permeating CPAs, such as sucrose and trehalose were able to permeate inside the cell before cryopreservation, the use of permeating CPAs such as DMSO could be avoided entirely [30]. Zhang et al. cryopreserved fibroblasts with only trehalose in the CPA solution, inducing trehalose uptake during freezing. Although

the cell recovery was not as high as with DMSO 10%, they demonstrated that fibroblast can be cryopreserved without the use of permeant CPAs [31,32].

Other additives, which facilitate cryopreservation but have no clear primary role as permeant and non-permeant CPA and are not defined as CPAs, are usually added to the CPA solution to increase cell recovery after the cryopreservation and thawing processes. The use of antioxidants in the CPA solution has been applied for several decades in the preservation of sperm, follicles and plants [33,34]. From the above-mentioned cell injury mechanisms in the cryopreservation, reactive oxygen species are created. Reactive oxygen species have been implicated in different damages mechanisms, such as lipid peroxidation of membranes, damage in the DNA, and loss of motility and fertility in frozen and thawed sperm [33,34]. In this sense, the properties of antioxidants could reduce the impact of reactive oxygen species and cold shock damage improving preservation of cell function after thawing. Recently, the antioxidants used have been translated to the cryopreservation of other cell-based therapies, such as the hematopoietic and mesenchymal stem cells [35-37]. Although the most used antioxidants for these cell types are catalase and ascorbic acid, others such glutathione or n-acetylcysteine can also be used. As always, the used concentration is crucial and must be optimized for obtaining a positive outcome after the samples thawing [38]. Thus, these results suggest that antioxidant inclusion in CPA solution could improve the recovery for a wide range of cells, and raise the question if lots of established protocols should be modified by the inclusion of antioxidants.

Common Cryoprotectants (CPA)		
Cell permeating agents	Non-permeating agents	
	Sugars	Polymers
Dimethyl sulphoxide***	Sucrose***	Polyethylene glicol (PEG)***
Ethylene glycol***	Trehalose***	Polyvinyl pyrrolidone (PVP)***
Propylene glycol***	Raffinose**	Hydroxy ethyl starch***
Glycerol**	Mannitol**	Ficoll**
Methanol*	Glucose*	Serum proteins (mixture)**
Ethanol*	Galactose*	Milk proteins (mixture)**

Table 2: Sumary of cryoprotectants selected for cell preservation. Identified by widespread***, moderate** or infrequent* choice of agent. Particular CPA mixtures are often selected for specific cell preservation strategies. This list is not exhaustive and a wider discussion can be found on [17,23]. Oligosaccharides tend to act as non-permeating osmotically acting CPAs, whereas monosaccharides may permeate cells to a degree depending on cell type. Copyright of BioInsights Publishing Ltd. Cell Gene Therapy Insights 2017; 3(5), 359-378. DOI: 10.18609/cgti.2017.038



I Samples preparation for freezing	II Cooling	III Storage	IV Thawing	V CPA removal
•Chill samples to 0-4°C to avoid CPA toxicity •Consider adding the cooled CPA solution (4°C) gradually to prevent toxicity and osmotic changes •Let enough time to equilibrate samples with the CPA solution	•Consider measuring sample temperature •Controlled rate freezers enables further control of cooling profile •Induced ice nucleation may improve cell outcome •Slow cooling below the -60 to 100°C has no benefits	•Below the glass transition temperature (Tg) samples preservation period is extended •Storage within vapor phase or liquid nitrogen is the most common method •Consider measuring sample temperature to control and avoid temperature fluctuations during the storage	•Fast thawing is the most used method to avoid regrowth of ice nuclei •For large volumes samples, slow thawing could be interesting to prevent different post-thaw cell outcome through sample	•Remove the CPA solution gradually to avoid osmotic changes •Do not warm samples until CPA is removed to avoid CPA related cytotoxicity

Figure 2: Schematic process of slow cooling cryopreservation. Cells are usually chilled to avoid the cytotoxic effect of CPAs, and at ~ 4°C the cooled CPA solution is added, enabling CPAs to permeate the cells during a holding time. Then samples are cooled slowly, and usually around -7°C ice nucleation occurs. That could be induced by active nucleation or modulated by adding an ice nucleating agent to the CPA solution. Samples continue cooling until -80°C, where samples are cooled below the glass transition temperature by nitrogen liquid immersion, approx. -160°C, and held in the cryogenic state. The cryopreserved samples will form a solidified mixed matrix of ice, CPAs, solute and a biomass, composed of extremely dehydrated cells that contain no ice. When is required, samples could be thawed quickly, and after CPA removal at ~ 4°C the sample would be ready to use.

In the same way, antifreeze proteins have also been included to reduce the IIF. Mainly as antioxidants, their use has been focused on the preservation of reproductive cells or embryos [39]. These molecules are ice-binding proteins that prevent water from freezing by adsorbing to the ice surface and stopping the growth of diminutive ice crystals to large crystals in a non-colligative manner. They have been associated with other function such as thermal hysteresis or interaction with membranes and/or membrane proteins, and due to these characteristics they were employed in the freezing protocols of different cell lines [40-42]. The cryopreservation efficiencies have usually been enhanced regardless of cell type and freezing method, with a handful of exceptions. In spite of these effects, it is agreed that the employment of antifreeze proteins in cell storage needs a further tuning to clarify

the molecules effects on cryopreservation, as outcome depends on the antifreeze protein, preserved cell, CPA solution, and storage temperature. Also, fetal bovine serum (FBS) has been described as another additive included in several cryopreservation protocols with beneficial effect. FBS stabilizes cell membranes, decreases the extracellular ice formation, minimizes cell dehydration and prevents excessive concentration of solutes during the freezing/thawing process [43]. However, clinicians try to avoid FBS in the cryopreservation of cells such as mesenchymal stem cells (MSCs) to reduce the risk of xeno-derived infection [44,45], for example, by the use of human serum. Moreover, the variation between the used human serum batches can be significant; some reports showed beneficial effects when it was included in the CPA solution [46], and others not [47].

Lastly, ice nucleating agent inclusion in the CPA solution enhanced the freezing survival of different cell types [16]. Ice nucleating agents such as silver iodide, metallic particles or other organic compounds produced by multiple organism, are molecules that due to their structural and surface properties, facilitate the orientation of water molecules to an ice-like structure, creating active germ crystals. During cryopreservation, aqueous solutions may become super-cooled, taking place the phase transition from liquid to solid stochastically. The uncontrol of this phenomenon leads to high sample to sample variations in terms of cell viability after thawing [48]. Thus, active control of ice nucleation can lead to cryoinjury reduction during freezing [12,13,49]. However, the mechanism behind the increased cell survival is not clear yet. Some studies indicate that the membrane phase changes occurring during controlled nucleation, may reduce the incidence of IIF [50]. Moreover, if ice nucleation is closer to the melting point of the solution, the dehydration of cells would be more pronounced due to extracellular ice formation. This longer period of cell dehydration is the phenomenon that has been related to the increase of cell viability after cryopreservation [51]. Lauterboeck et al. ratified this mechanism, reporting that the active control of nucleation temperature above the spontaneous nucleation temperature leads to a higher dehydration of mesenchymal stem cells, reducing the IIF [52]. The highest ice nucleation temperature did not achieve the best results. The -10°C degrees was the best nucleation temperature for mesenchymal stem cells, which maintained a higher percentage of membrane integrity and cell recultivation when samples were frozen with 5% DMSO (Figure 3A). Also, Massie et el. demonstrated how the ice nucleation affects the post-warming viability of microencapsulated liver spheroids [12] (Figure 3B). Higher ice-nucleating temperatures, using cholesterol as a nucleating agent, resulted in doubling cell viability of the microencapsulated liver spheroids after thawing. Moreover, the ultrastructural effects of controlled ice nucleation compared to spontaneous ice nucleation were also displayed in microencapsulated liver spheroids by cryoscanning electron microscopy [12] (Figure 3C). During controlled ice nucleation slower

ice formation was created enabling cells to dehydrate appropriately (Figure 3C-1,3). By contrast, following spontaneous ice nucleation, intercellular ice was formed provoking cell injury (Figure 3C-2,4). Between the nucleation agents, there are some chemicals such as cholesterol or Icestart™, but also nucleation could be induced by a controlled metal straw placed inside the samples. These nucleating agents seed the production of an ice embryo and enable the propagation of gradual ice formation through the remainder of the sample, and as above mentioned, reduces the sample to sample variations. Thus, one should consider, as with antioxidants, the inclusion of an ice nucleator in CPA solutions to improve the cell survival after freezing, especially in large samples in which ice nucleation is more heterogeneous.

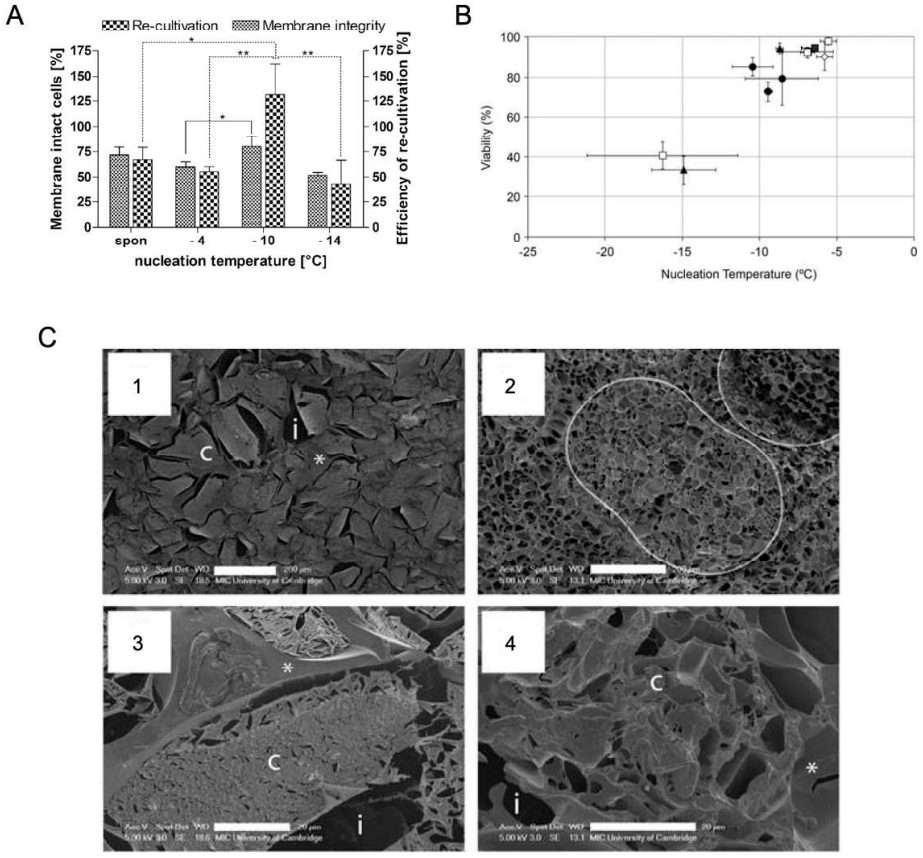


Figure 3: Ice nucleation affects on the recovery of microencapsulated cells. A) Evaluation of membrane integrity and efficiency of re-cultivation after cryomicroscopy on primate MSCs. B) Microencapsulated cell spheroids viability when cooled under different cooling protocols showing that regardless the used protocol, higher ice nucleation results in higher viabilities. C) Cryoscanning electron microscopy of fractured cryovials after cooling at a linear rate of -1°C /min. Ultrastructure resulting from controlled ice nucleation (A, C) is compared to spontaneous ice nucleation (2,4). In figure (B) sectioned alginate beads that are ~500 µm in diameter are outlined. In (1, 3, 4), cell clusters (c), ice voids (i), and smooth freeze concentrated alginate and cryoprotectant (CPA) (*) have been labeled. Scale bars on (1, 2) 200 mm, and on (3, 4) 20 mm. Notes: Spon: spontaneous *$p < 0.05$; **$p < 0.01$. (n =3). A) Reprinted from Active control of the nucleation temperature enhances freezing survival of multipotent mesenchymal stromal cells, 71(3), Lauterboeck L, Hofmann N, Mueller T, Glasmacher B, Cryobiology , 384-90, Copyright (2018), with permission from Elsevier. (B and C as originally published in [12])

3.2. Sample preparation for freezing

Before samples are loaded into freezing machines and suffer cooling, different processes are carried out, such as sample chilling and CPA solution addition. Although these processes seem simple, some variables have to be taken into account to perform them appropriately. Many CPAs, such as DMSO, are more toxic at temperatures higher than 4°C [17]. Therefore, cell-based products should be chilled to avoid CPA induced citoxicity. As mentioned before, the CPA concentration is not the one usually used in normal cell media, since it can be toxic to cells. So, chilling samples leads to a reduction in cell metabolic activity and consequently, lower CPAs toxicity [53]. However, this effect also reduces the permeability of CPAs to cells. Longer CPA exposure time are necessary if CPA addition is performed at lower temperatures, which could also increase toxicity [53]. In this case, the beneficial effect of lowering the metabolic activity is prioritized in the slow freezing cryopreservation [54]. Furthermore, in order for samples to follow the applied cooling rate and reduce variability, it is recognized that samples should be pre-chilled to around 0-4°C before freezing. The introduction of samples to the freezer or the controlled rate freezers (CRF) without chilling, could lead to differences between the real and the theoretical cooling rates of the sample. Therefore, even after applying an optimized cooling rate that reduces as much as possible IIF, samples may not recover their function after thawing. Also, there are reports that adding the chilling step before cooling improves cell recovery [55]. Furthermore, when large volume samples, such as tissues or red blood cell cryobags are frozen, this is an essential point to bear in mind [56]. Thick or large volume samples are not homogeneously cooled, and sample introduction into the cooling machine without cooling could lead to enhances sample to sample function variability after freezing and thawing.

The osmotic changes that occur when the CPA solution is added or removed, could also be detrimental to the diverse cell types. Some cells are more susceptible to osmotic changes than others, and the cooled CPA solution addition is performed gradually to decrease CPA toxicity. Moreover, it should be taken into consideration that CPAs should permeate all cells, exerting their protective effect in the entire sample. To this end, before cooling, the CPA solution should be in contact with the sample for long enough for the CPAs to permeate through all cells [57]. As with chilling, larger samples will need longer periods of exposure to CPAs, as demonstrated with the cryopreservation of intact articular cartilage [58]. Indeed, optimization of the CPA addition protocol for the cell product is required. As a take-home message, samples should be entirely chilled and permeated to CPAs before freezing, in order to prepare them appropriately for the next step in the slow freezing cryopreservation.

3.3. Controlled rate cooling protocol

The applied cooling rate should be optimized for each sample according to Mazur´s two-factor hypothesis. The cooling rate affects the rate of formation and size of both intracellular and extracellular ice crystals, and also impacts solution effects that occur during the freezing process [59]. In this sense, the -1°C per minute cooling rate is often used as an optimized rate to freeze various mammalian cell types (e.g. with Mr. Frosty™, CoolCell® or homemade freezing system passive devices) that showed repeatedly good results [14,60]. These passive cooling devices may be suitable for small volumes or sample numbers, but lack suitability for use in the cryopreservation of larger samples. By contrast, controlled cooling can be achieved by CRF based on various principles such as nitrogen vapor (e.g., Cryomed™ or Planer PLC) or electrical Stirling Engine Systems (e.g., Asymptote PLC) [12,13,55,61-63]. These technologies allow the application of different cooling rates within the same cooling protocol, providing a tool to achieve optimal cooling rates for cell survival during cryopreservation. Although the applied cooling rate is important, the control of the real cooling rate within samples is crucial. The placement of thermocouples in samples during cooling protocols enable the measurement of difference in cooling rates between different compartments of devices, samples, or even between layers of the same sample [63,64]. With these tools, the cooling rate can be corrected by changing the cooling programme if it is required. Small samples, such as cryovials, will closely match the cooling rate programmed by the machine. However, large volumes of samples, often used at clinical scale, will be more difficult to cool at the programmed cooling rate. Kilbride et al. demonstrated that the way that samples are cooled influences ice solidification within the sample [63]. Two different heat transfer modules designed for an electrical Stirling cooling machine were used to cryopreserve microencapsulated liver spheroids in cryovials. In this study, the way the ice solidified through the cryovial was studied with its effect on microencapsulated liver spheroids. When cryovials were cooled from a single surface of the cryovials, progressive ice solidification occurred in samples. By contrast, when the entire module applied the cooling to all surface of the cryovial, a dendritic network solidification was predominant. The cooling profiles were recorded, and showed that the vials that were entirely cooled (Figure 4A) matched much better the applied cooling profile those cooled just from the bottom (Figure 4B). Regarding viability and cell function, there were non-significant differences between the studied groups. By contrast, when samples larger than a few millilitres were cryopreserved, it has to consider that homogeneous cooling is a great challenge due to heat and mass transfer is slowed. This effect was demonstrated in the same study, when 2 litre of 10% aqueous glycerol solution (having the same thermal properties of microencapsulated liver spheroid)

was cryopreserved and the cooling profile of the different layers was measured. The diversity in cooling profiles in different layers of the biomass was demonstrated, confirming the difficulty in obtaining homogeneous cooling in large volumes (Figure 4C). Regarding cells, these cooling heterogeneity through the biomass will also be reflected in differences in cell dehydration, extracellular solute concentration, and ice crystal formation, that will affect enormously in cell function after cryopreservation.

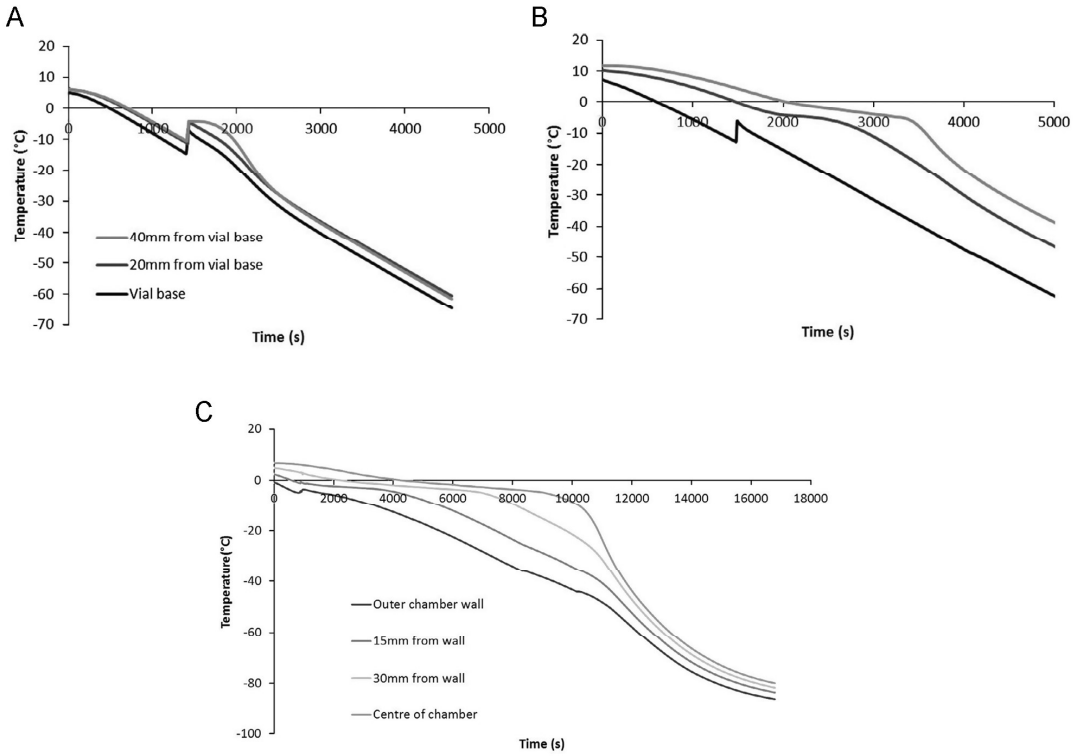


Figure 4: Differences in cooling profiles of cryovials and a 2 litre chamber. Temperature profiles in both aluminum (A) and acetal (B) heat transfer modules measured on the EF600-103 Stirling cryocooler-based controlled rate freezer. C) Measured temperature profiles inside the bioartificial liver chamber during cooling of a thermal mimic with 10% glycerol solution. (Figure as originally published in [63])

Lastly, it has been established empiricaly that the slow cooling below -60°C to -100°C has no benefits in the preservation of different systems [65]. Thus, after the cooling protocol reaches these temperatures, samples are further cooled (at faster cooling rates) and solidified into a glassy state to extend cell-based product storage life.

3.4. Storage

Depending on the desired storage time for the sample, different systems or methods

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can be used for the preservation of cell-based products. Samples preserved at -120 to -130°C are so viscous due to highly concentrated CPA in the remaining solution, that enables samples phase change into a glassy state [66]. If the slow cooling protocol is successfully applied, below those temperatures, samples will form a solidified mixed matrix of ice, CPAs, solute and a biomass, composed of extremely dehydrated cells that contain no ice [12]. The temperature in which this phase transition occurs, called the glass transition temperature (Tg), depends on the solute concentration in samples. In this glassy state, the probability of chemical reactions or molecular diffusion occurring is highly unlikely due to insufficient thermal energy. The background ionizing radiation has been suggested as the only process that could exert harmful effects on samples, and more than a century would be necessary to accumulate lethal injury. Thus, this glassy state is desired for almost all storage systems to extend sample preservation periods.

Storage at -80°C using electrical freezers has also been used for the preservation of cell-based products such as red blood cells [71-73]. It has to be taken into account that cell-based products half-life is diminished in these temperatures due to the slow and progressive recrystallization phenomenon, that will occur above the Tg, for this reason it is important to establish a storage half-life of cell-based products in these freezers. The FDA approved the storage of red blood cells in -80°C freezers for ten years for their use in clinics [10]. Moreover, a successful storage of human and porcine pluripotent stem cells in suspension at -80°C for at least one year has been achieved recently [72]. However, the detrimental effect of the storage above the Tg was reported in more complex biosystems. Cell recovery of microencapsulated liver spheroids was decreased gradually after the cryopreservation at -80°C after just few weeks. In the same work, microencapsulated liver spheroids were cryopreserved and stored at vapor phase nitrogen for a year, and high cell recoveries were achieved [74]. In this way, the emergence of -120°C freezer should have promoted new works in which their effectiveness for different cell products was assessed, however, there is still little information published for the cryopreservation of the cell-based products with these electric freezers.

3.5. Thawing

Sample thawing is as important as cooling in order to achieve a successful cryopreservation. In warming, cells can suffer the same injury they experience in cooling. The created ice nuclei during cooling could grow and reorganize to form more ice crystals extracellularly. Similarly, small ice nuclei that have been formed during cooling intracellularly and that were not big enough to exert damage, could grow and cause mechanical damage

inside cells [75,76]. Some authors have postulated that thawing processes are more crucial than the cooling itself [77]. Although this was postulated after the vitrification of oocytes, it highlights the importance of thawing for the successful recovery of cells. In this sense, as processes occurring throughout warming of slowly cooled samples are not easy to predict, fast thawing has been favored in order to prevent the feared ice crystal growth. The most used way to thaw samples is by their immersion in a water bath at 37°C obtaining good results over decades [13,14,35,36,38,64]. However, as sample contact with water could produce contamination, new machines have been developed to eliminate that risk. With their use (e.g., VIA Thaw), control of thawing rates and samples temperature end point fixing (0-4°C) are possible, which would prevent samples from reaching high temperatures and be damaged by CPA toxicity. By contrast, the fast thawing of vitrified tissues has been developed using radiofrequency-excited mesoporous silica-coated iron oxide nanoparticles. In this work, blood vessels were thawed homogenously with the inductive heating of these nanoparticles obtaining slight improvements in viability to the conventional thawed samples [78]. Regardless of the technique used for sample thawing, the monitoring of temperature by thermocouples is highly useful to assess thawing reproducibility in the cryopreservation field, and should always be measured.

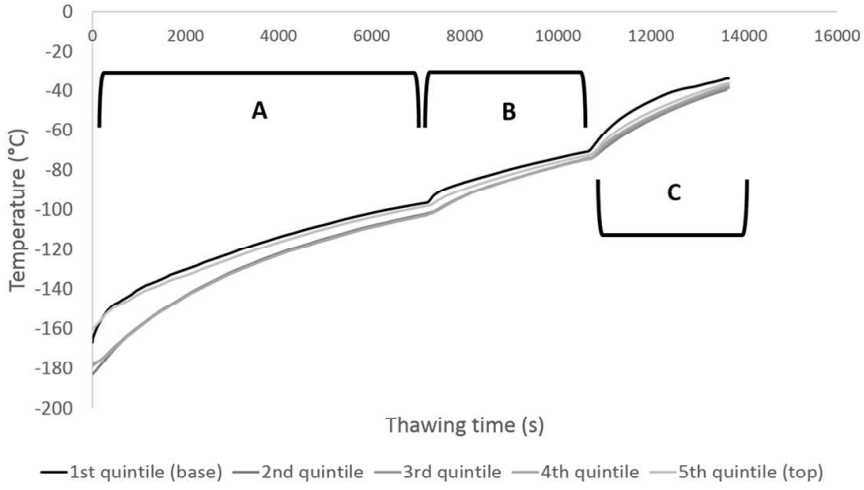

Figure 5: Warming profiles experienced during warming of the large volume cryopreservation cylindrical chamber. Thermocouples were placed at the bottom of the biomass (black) and the top of the biomass (lightest grey), as well as three others equidistant apart between the bottom and top following a straight line through the deepest part of the sample (dark to lighter grey). Section A demarks warming in the -80°C freezer, section B thawing in the -30°C freezer, and section C -10°C in the Planer controlled rate freezer. (Figure as originally published in [79])

Slow thawing of samples has also been proposed to thaw cryopreserved cell-based products. With this procedure is hypothesized that the osmotic re-equilibration mechanisms

will take place during the process, achieving beneficial effects in cell recovery after thawing. However, the longer exposure time to high CPA concentrations at high subzero temperatures could also be detrimental. Few studies thaw cell-based products slowly, but they are worth to mentioning for their positive results. Kilbride et al. cryopreserved a 2.3-litre biomass of microencapsulated liver spheroids to assess whether a bioartificial liver device could be preserved [79]. In this work, thermocouples were placed in different layers of the biomass, and a stepwise manner thawing was performed, assessing the thawing profile of the different layers of the biomass (Figure 5). Differences in cell function and cell recovery were achieved between layers, demonstrating that the achievement of homogenous heat transfer in large volumes is still challenging. However, it is remarkable that a successful and practical thawing was completed with the slow thawing procedure. Furthermore, slow thawing has been related to the improved preservation of minipig iliac arteries [80]. Microfractures and the accumulation of fluid within the arterial tissue provoked during cryopreservation were prevented with slow thawing. Even if cell recovery was not determined in this work, it indicates that slow thawing may produce less structural damage in tissues. However, although these works demonstrated that slow thawing could be an appropriate approach for the thawing of large samples or tissues, the fast thawing is the preferred method for recovering samples, reminding us that thawing is also crucial to take into consideration in slow freezing cryopreservation.

3.6. CPA removal

Lastly, after all ice crystals are thawed and before samples are ready to be used, CPAs removal is needed in order to prevent their toxic effects on cells. Moreover, if the product is implanted directly into humans without CPAs removal, it could produce adverse effects such as those produced by DMSO [26]. This step, like CPA addition, is dependent on each cell type and sample volume, and should be optimized for each sample. Normally, after thawing samples are not warmed until almost or all CPAs are removed, due to their toxic effects in cells in supra zero temperatures [17]. Also, CPA removal is performed gradually, with the addition of decreasing osmotic solutions step by step: firstly not to exert an excessive osmotic stress to cells, and secondly, to remove CPAs continuously. Lawson et al. studied a mathematical model for the addition of CPA and its removal in cell-based systems, and demonstrated that the addition and removal of CPAs should be performed at low temperatures [54]. Also, they employed gradual CPA addition and removal protocols showing the usefulness of the mathematical model for the design of CPA addition and removal protocols. As mentioned with the thawing, not optimizing removal of CPAs could negatively affect the general outcome of the slow freezing cryopreservation of cell-based

products.

4. SLOWLY CRYOPRESERVED MICROENCAPSULATED CELLS

Tissue engineering has evolved enormously in recent decades due to the technological advances and has started to be used in clinics. For this reason, several researchers require a tissue-banking system to prepare for the rising demand of tissues, cell-based products or organs. The only method to preserve cell-based systems or products for long periods is with the use of low temperatures. Thus, the efforts to cryopreserve cell-based products made of natural polymers (e.g., agarose, hyaluronic acid, fibrin or collagen) or synthetic polymers (e.g., PEG) have linearly grown with its reflection in more publications in last decades [22,30,47,79,81]. Alginate has been one of the most often used polysaccharides to form cellularized bioscaffolds due to its potential as sustained drug delivery or cell delivery systems for the treatment of different diseases (e.g., diabetes, hepatic diseases, neurodegenerative or cancer among others) [82-85]. Cells are embedded in the biocompatible matrix of alginate, and sometimes covered by a semipermeable membrane, such as poly-L-lysine. This outer membrane protects the inner cells from the host's immune system and the mechanical stress, enabling the transplantation of these cell-based products (Figure 6). However, although several in vivo experiments [86-88] and clinical trials have been investigated with alginate microcapsules for the treatment of diverse diseases such as Diabetes [89-91] or Parkinson´s [92], their cryopreservation using slow freezing still needs further research to reach the clinics, get approved by the governmental agencies or commercially available. Thus, in order to understand and facilitate the future research on the slow freezing of microencapsulated cells, the existing studies in the area will be summarized below.

4.1. Cryoprotective effect of alginate

Different studies have determined the hydrogels cryoprotective effect in the cryopreservation of microencapsulated mammalian cells [93-99], and in plant cryopreservation [100]. Different mechanisms have been related to post-thaw cell function improvement on the cryopreservation of microencapsulated cells: the influence of the polymeric matrix on extracellular ice formation [101], initiation of intracellular ice formation [102,103], buffering the CPA diffusion or protection against apoptosis [104]. For that reason, the beneficial effect of alginate encapsulation in slow freezing cryopreservation was compared to free cells after thawing (Tables 3-6).

In one of the first studies of microencapsulated and cryopreserved cells, microencapsulated rat islets were compared to free islets for their cryopreservation [93]. To that aim,

microencapsulated islets were frozen with 15.6% (v/v) DMSO using two protocols: linear cooling with a Nalgene freezing container at -1°C/min and stored in liquid nitrogen; and another linear cooling at -0.2°C/min, in which ice nucleation was induced at -7.5°C, and plunged in liquid nitrogen. After fast thawing, cryopreserved microencapsulated islets did not display differences compared to the non-cryopreserved microencapsulated islets in insulin response to high glucose. By contrast, cryopreserved free islets showed a significantly lower response to high glucose, and many islets were lost during the cryopreservation. Furthermore, the cryopreserved microencapsulated islets were able to maintain normoglycemia up to 90 days in induced diabetic mice, whereas the cryopreserved free islets maintained it for only few days. That work demonstrated for the first time that alginate encapsulation could protect cells in the freeze-thaw processes. Moreover, Matsumoto et al. tested the advantages of alginate encapsulation for the cryopreservation of rat pheochromocytoma cells [94]. In that study, the cooling and thawing rates were studied in free and microencapsulated cells. Even it was one of the first demonstrations of cryopreservation with microencapsulated cells, key variables such as the observation of ice growth on the freeze-thaw process, and the latent heat release on cooling were studied. As in Mazur´s 2-factor hypothesis, they also showed the inverted "U" effect in the post-thaw viabilities of cryopreserved microencapsulated cells with different cooling rates. In this case, slow cooling rates of 0.5 or 1°C/min demonstrated higher viabilities in terms of dopamine secretion and trypan blue exclusion assay in microencapsulated rat pheochromocytoma cells (Figure 7A-B). Interestingly, the fast cooling profiles did not prevent the ice growth within the microcapsules around -8°C, whereas the 1°C/min did not show ice growth within the microcapsules at that temperature (Figure 7C). Moreover, in slower cooling rates (0.5 or 1°C/min) less latent heat was released from cryopreserved microencapsulated cells compared to free cells (Figure 7D). Authors correlated the higher cell viabilities in cryopreserved microencapsulated cells with an increase of unfrozen water inside the microcapsule and the intracellular space, which could also explained by suitable cell and microcapsule dehydration during cooling. In another study with microencapsulated rat islets, the benefit of alginate encapsulation in cryopreservation was again confirmed [95]. Samples were cooled with 11.7% (v/v) DMSO using two different stepwise protocols in a controlled rate freezer (CRF) and stored in liquid nitrogen. The microencapsulated islets displayed similar insulin secretion as free islets, but after cryopreservation (regardless of the cooling protocol), only the microencapsulated islets were able to maintain the insulin response. It was also demonstrated that cryopreserved microencapsulated rat islets with the protocol, cooled from 0°C to -80°C at 1°C/min and stored in liquid nitrogen, normalized the metabolic blood glucose of diabetic mice for 90 days, whereas the free cryopreserved rat islets only normalized glucose for a few days. Lastly, brain cell neurospheres were also

Cell Type (Biomaterial)	CPA solution	Cryopreservation protocol	Frozen volume	Main results	Year[ref]
Rat islets (Alg-PLL-Alg)	15,6% DMSO +7.5% FCS	Two different cooling protocols: linear cooling at −1 °C/min with a Nalgene freezing container from 4 °C to −70 °C; and linear cooling from 4 °C to −45 °C at −0.2 °C/min (ice nucleation induced at −7.5 °C) and stored in LN. FT	1.8 ml	Cryopreserved encapsulated islets (with induced ice nucleation protocol) were able to maintain in vitro glucose response, and also maintained normoglycemia up to 90 days in induced diabetic mice.	1996[93]
Porcine islets (Alg-PLL-Alg)	15,6% DMSO +7.5% FBS	Two different cooling protocols: cooling with a CRF until −7.5 °C (where ice nucleation was induced), 15mins held, cooled at −0.2 °C/min to −45 °C; cooling at −1 °C/min in a Nalgene freezing container until −70 °C and stored in LN. FT	1.8 ml	Only cryopreserved encapsulated islets with induced ice nucleation were able to maintain glucose challenge, and 66% of the induced diabetic mice recovered normoglycemia for 90 days with cryopreserved encapsulated islets.	1997[95]
Rat islets (Alg-PLL-Alg)	11,7% DMSO +7,5% FBS	Two different cooling protocols: 4 °C to 0 °C at 5C°/min, hold at 0 °C 20 min, cooling from 0 °C to −80 °C at 1C°/min; 4 °C to 0 °C at a rate of 5 °C /min, hold 20 min, cooling from 0 °C to −40 °C at 0.3C°/min, and cooled from −40 °C to −80 °C at 1C°/min, stored in LN. FT	2 ml	Cryopreserved encapsulated islets (wherever the cooling protocol) were able to maintain insulin secretion. Cryopreserved encapsulated islet maintained normoglycemia in diabetic mice for 90 days.	2002[108]
Rat islets (Alg)	10% DMSO +10% FBS	Cooling with a CRF and stored in LN. FT	2 ml	Rat normoglycemia was achieved with cryopreserved encapsulated islet for 460 days. After graft retrieval similar insulin secretion in vitro of cryopreserved encapsulated islets to non-cryopreserved group.	2011[109]
Murine insulinoma βTC-tet cells (Alg)	10%DMSO	4 °C 10 min, cooling at −1 °C/min with Mr. Frosty container in a − 80 °C freezer, and stored at −150 °C. FT	2 ml	Islet metabolism maintained in encapsulated cells by either slow freezing or vitrification. Insulin secretion only maintained by slow freezing.	2012[110]

Table 3: Encapsulated and cryopreserved insulin-secreting cells. CPA solution: cryoprotectant solution; Alg: Alginate; PLL: poly-L-lysine; MCP: measured cooling profile; LN: liquid nitrogen; VN: vapor nitrogen; FT; fast thawing; DMSO: dimethylsulfoxide; FBS: fetal bovine serum; FCS: fetal calf serum

Cell Type (Biomaterial)	CPA solution	Cryopreservation protocol	Frozen volume	Main results	Year[ref]
Human MSCs (Alg)	5 or 10% DMSO +10% FCS	4 °C 15 min and 3 different profiles: 1) linear −1 °C/min to −80 °C; 2) cooled at −1 °C/min from 0 to −7 °C (where ice nucleation was induced), then cooled at −1 °C/min to −40 °C, and a − 10 °C/min until −80 °C; 3) one stage rapid cooling by direct immersion in LN. All stored in LN and FT	1 ml	10% DMSO in CPA solution displayed better results than 5% DMSO. Second protocol, which included induced ice nucleation, showed the best viability and metabolic activity after thawing.	2013[13]
Primate MSCs (Alg)	10% DMSO +17.5% FBS	4 °C 12 min, cooling at −1 °C/min with an isopropanol progressive freezing container, −80 °C overnight and stored at −150 °C. FT	2 ml	Encapsulated and cryopreserved MSCs showed better metabolic activities compared to encapsulated MSCs, cultured 24 h and cryopreserved ones.	2014[116]
Murine MSCs (Alg-PLL-Alg)	Different CPAs +10% FBS	4 °C 20 min, cooling at −1 °C/min with a Mr. Frosty freezing container, −80 °C overnight and stored in LN. FT.	2 ml	Only cryopreserved encapsulated MSCs with 10% DMSO were able to maintain metabolic activity, cell viability, EPO secretion in vitro and hematocrit levels in vivo compared to non-cryopreserved encapsulated MSCs.	2014[14]
Monkey MSCs (Alg)	10% DMSO +10% FBS	Cooling with a Mr. Frosty freezing container, −80 °C overnight and stored at −150 °C. FT	2 ml	Demonstrated that DMSO concentration could be decreased from 10 to 7.5% maintaining high cell recoveries after thawing in encapsulated MSCs.	2016[117]

Table 4: Encapsulated and cryopreserved mesenchymal stem cells. CPA solution: cryoprotectant solution; Alg: Alginate; PLL: poly-L-lysine; MCP: measured cooling profile; LN: liquid nitrogen; VN: vapor nitrogen; FT; fast thawing; DMSO: dimethylsulfoxide; FBS: fetal bovine serum; FCS: fetal calf serum; MSCs: mesenchymal stem cells.

Cell Type (Biomaterial)	CPA solution	Cryopreservation protocol	Frozen volume	Main results	Year[ref]
Rat hepatocytes (Alg-PLL-Alg)	10% DMSO +20% FBS	24 h at −70 °C, and stored in liquid LN for 7 and 30 days. FT	UNK	Cryopreserved encapsulated hepatocytes for 30 days displayed decreased cell function in vitro and in vivo	1993[120]
Murine hepatocytes (Alg-cel-PEGC)	10% DMSO	4 °C 30 min, −20 °C 2 h, −80 °C 24 h and stored in LN. FT	1 ml	No difference in albumin secretion of fresh and cryopreserved encapsulated hepatocytes.	2001[121]
Rodent & human hepatocytes (Alg-PLL-Alg)	10% DMSO	4 °C 12 min; 4.2 °C/min to −8 °C; 18 min hold, 4.2 °C/min to −100 °C and stored in LN (MCP). FT	2 ml	After xenotransplantation in FLF model mice similar survivals with fresh or cryopreserved encapsulated rat (~70%) & human (~50%) hepatocytes.	2005[122]
Porcine hepatocytes (Alg-PLL-Alg)	10% DMSO	1 °C/min from 4 to 0 °C, 0.5 °C/min to −11 °C, hold 15 min at −11 °C, 3 °C/min to −80 °C, stored in LN. FT.	2 ml	After xenotransplantation in FLF model mice similar survivals with fresh or cryopreserved encapsulated porcine hepatocytes (~75%).	2009[123]
Human hepatocytes (Alg-PLL-Alg)	10% DMSO +10% FBS	Cooling with an isopropanol progressive freezing container, −80 °C overnight and stored in LN. FT	2 ml	Urea, albumin profile secretion, and hepatocytes gene expression well maintained. Protein expression increased in CYP3A4.	2010[124]
HepG2 spheroids (Alg)	12%DMSO	4 °C 10 min,8 min held at 0 °C and main ramp of −2C/min; storage in VN (MCP). FT	1.25 ml	Same cell function recovery over 72 h using a CRF without the use of LN or with it.	2011[126]
HepG2 spheroids (Alg)	12%DMSO + Cholesterol (INA)	4 °C 10 min,8 min held at 0 °C and main ramp of −2C/min[116]; stored in VN (MCP). FT	1.25 ml	The INA addition reduced supercooling and also latent cryoinjury, improving cell numbers, viability, and function in encapsulated HepG2 spheroids over 72 h post-thawing.	2011[64]
HepG2 spheroids (Alg)	12%DMSO + Cholesterol (INA)	4 °C 10 min,8 min held at 0 °C and main ramp of -2C/min[116]; stored at −80 °C or VN (MCP). FT	1.25 ml	Rapid deterioration in functional recoveries after few weeks stored at −80 °C. VN preserve HepG2 functionality in all study (1 year)	2013[74]
HepG2 spheroids (Alg)	12%DMSO +10% FFP + Catalase & Trolox + Cholesterol (INA)	Held at 4 °C; a simple linear rate was compared with nonlinear profiles, stored in VN (MCP). FT	0.5 ml 200 ml	Similar functional recovery between small and large volume GMP cryopreservation. Large volume cryopreservation successfully scaled up.	2014[12]
HepG2 spheroids (Alg)	12%DMSO + IceStart (INA)	Held at 4 °C; 1 °C/min to −80 °C and hold 1 h; storage in −80 °C freezer for 7 days (MCP). FT.	6 ml	Small differences between progressive and network solidification in encapsulated HepG2 function.	2014[63]
HepG2 spheroids (Alg)	12%DMSO + IceStart (INA)	Held at 4 °C; 0.3 °C/min to −100 °C; stored in VN (MCP). FT.	6 ml 2 litre	Functional differences between studied layers of the large biomass. Faster thawed layers displayed higher functional recoveries.	2016[127]
HepG2 spheroids (Alg)	12%DMSO + IceStart (INA)	Held at 4 °C; 0.3 °C/min to −100 °C; stored in VN (MCP). Stepwise ST.	6 ml 2.3 litre	Functional recovery of 2.3 litre biomass after 72 h.	2017[79]
Rodent & human hepatocytes (Alg)	Different solutions with DMSO 10% + diverse additives	4 °C 10 min,8 min held at 0 °C and main ramp of −2C/min[116]; stored in VN (MCP). FT	2 ml	Best recoveries with Viaspan®, DMSO10%, glucose and an anti-apoptotic factor.	2017[81]

Table 5: Encapsulated and slowly cryopreserved hepatocytes .CPA solution: cryoprotectant solution; Alg: Alginate; Alg-Cel-PEGC: alginate-cellulose poly(methylene-co-guanidine); PLL: poly-L-lysine; MCP: measured cooling profile; LN: liquid nitrogen; VN: vapor nitrogen; ST: slow thawing; FT; fast thawing; INA: ice nucleating agent; DMSO; dimethylsulfoxide; FBS: fetal bovine serum; FFP: fresh frozen human plasma; FLF: fulminant hepatic failure; AAF: anti-apoptotic factor; UNK: unknown.

Cell Type (Biomaterial)	CPA solution	Cryopreservation protocol	Frozen volume	Main results	Year[ref]
Rat PC12 (Alg-PLL-Alg)	10% DMSO	20 °C 30 min, cooled from −20 °C to −80 °C at a cooling rate of either 0.5, 1, 5, 10 °C/min. ST	0.1 ml	Slow cooling rates of 0.5 or 1C°/min demonstrated higher viabilities in terms of dopamine secretion and tryptan blue exclusion assay.	2001[94]
Rat neurospheres (Alg-PLL-Alg)	Different solutions with 10% DMSO	4 °C 30 min, cooled at -1C°/min to -80C° and stored VN. FT	1 ml	Cryopreserved encapsulated neurospheres with Cryostor® displayed better viabilities and less cell defragmentation than cryopreserved free cells.	2010[96]
Murine C2C12 (Alg-PLL-alg)	Different % of DMSO	Slow cooling: −20 °C 1 h, transferred into a − 80 °C freezer 23 h and stored LN; slow cooling + shaking: 20 min shaking with CPA solution and previous cooling protocol applied; super cooling: −80 °C 2 h and stored LN; maxi-cooling: directly immersed in liquid nitrogen. FT	2 ml	Slow cooling demonstrated best results and 10% DMSO was the best CPA solution.	2009[129]
Murine C2C12 (Alg)	10% DMSO	4 °C 10 min, cooling at −1 °C/min with a Mr. Frosty freezing container, −80 °C overnight and stored in LN. FT	2 ml	Slow-cooled samples displayed slightly better outcome regarding metabolic activity, viability, and insulin secretion compared to the vitrified ones.	2013[128]

Table 6: Different encapsulated and cryopreserved cells . CPA solution: cryoprotectant solution; Alg: Alginate; PLL: poly-L-lysine; MCP: measured cooling profile; LN: liquid nitrogen; VN: vapor nitrogen; FT; fast thawing; ST: slow thawing; DMSO: dimethylsulfoxide; FBS: fetal bovine serum; FCS: fetal calf serum;

cryopreserved within alginate capsules to improve cell function and reduce cell defragmentation after thawing [96]. After demonstrating that neurosphere encapsulation had no impact on cell function, free and microencapsulated neurospheres were cryopreserved with a CRF at -1°C/min to -80°C and stored in the vapor phase of liquid nitrogen. Different CPA solutions were also used; a complete medium consisted of 10% (v/v) DMSO and 10% FBS, and Cryostor-CS10® solution which also contains 10% DMSO. After fast thawing, microencapsulated and cryopreserved neurospheres, independent of the CPA solution used, displayed better viabilities with less cell defragmentation compared to cryopreserved free cells. Moreover, Cryostor-CS10® provided further cryoprotection for both free and microencapsulated neurospheres compared to the other CPA solutions. Altogether these studies, demonstrated that alginate encapsulation could have a protective effect in the cryopreservation of diverse cell types displaying fewer defragmented cells and enhancing cell function after thawing.

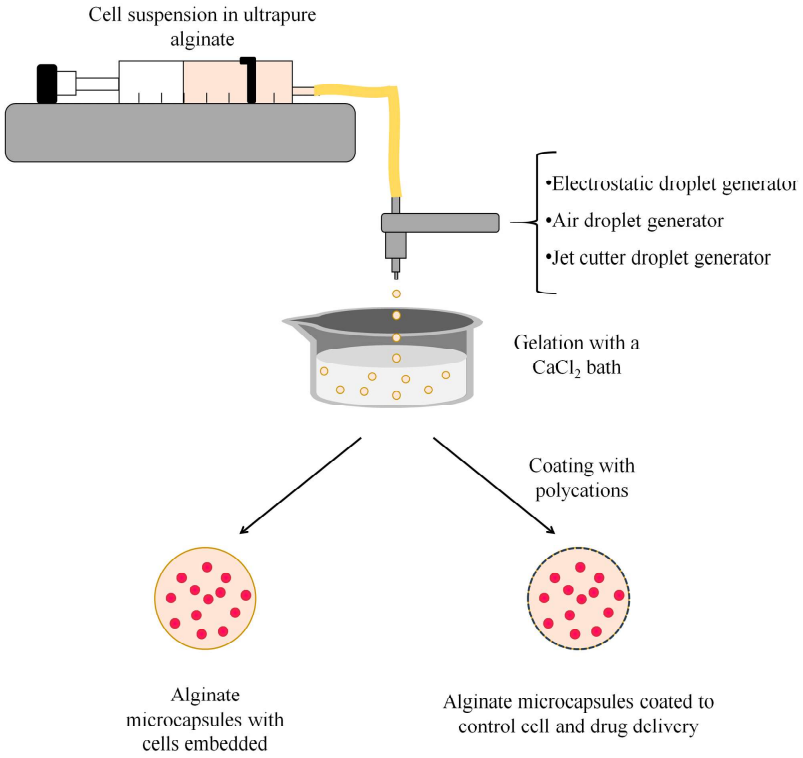

Figure 6: Scheme of the microencapsulation process. Cells are suspended in ultrapure alginate (1-3%(w/v)) obtaining a viscous solution with variable cell densities depending on cell type and therapeutic application (1-5x10^6 cells/mL alginate). This cell suspension is next extruded through a needle in which suspensions droplets can be generated by different encapsulation technologies (electrostatic, air or jet cutter) which are solidified in a bivalent cations bath, such as calcium chloride. The generated microcapsules can be directly used for therapeutic applications, or coated with polycationic molecules, such as poly-L-lysine.

4.2. Islets and insulin-secreting β-cells

Most efforts of cell encapsulation are aimed at treating type I diabetes, a disease that develops as a consequence of the destruction of pancreatic islets including the insulin-secreting β-cells. Cell encapsulation research has focused on restoring and regulating the insulin supply, and many clinical trials were performed to translate the technology to the clinic [105-107]. In order to reduce cost and provide cell-based product banks, the preservation of microencapsulated islets and β-cells have also been investigated with the slow freezing in the last two decades [93,95,108-110] (Table 3).

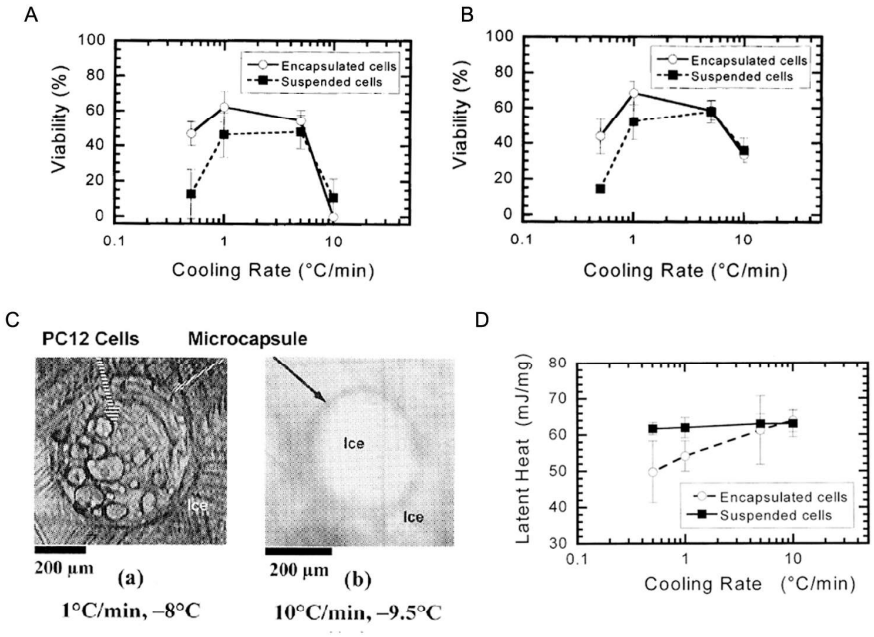

Figure 7: Cooling rate effects on cell function after thawing and ice nucleation temperatures. Viability and cooling rate relationship determined by dopamine secretion (A) or trypan blue exclusion assay (B). C) Cryomicroscopy images of microencapsulated P12 cells with slow (a) and fast (b) cooling rates showing differences in ice nucleation temperatures. Scale bar: 200 µm. Reprinted from Improvement in the Viability of Cryopreserved Cells By Microencapsulation, Matsumoto Y, Morinaga Y, Ujihira M, Oka K, Tanishita K, JSME international Journal Series C, 384-90, Copyright (2018), with permission from JSME.

In the first study performed with cryopreserved microencapsulated islets, Inaba et al. demonstrated the benefits of alginate encapsulation in islets storage [93]. Next, the same group studied the slow freezing of porcine microencapsulated islets with 15.6% (v/v) DMSO in two diverse cooling protocols [108]. After fast thawing, only the batches frozen with induced ice nucleation were able to respond to static high glucose and isobutylmethylxanthine challenge, demonstrating the importance of inducing ice nucleation. The intraperitoneal transplantation into diabetic mice of cryopreserved microencapsulated islets was performed

only with the frozen batches in which ice nucleation was induced and, 60% of recipients recovered normoglycemia compared to the 91% of the non-cryopreserved group showing that cryopreservation was effective. Moreover, freezing procedure did not alter capsule surface, and did not display fibrotic overgrowth during transplantation. In another study, the microencapsulated and cryopreserved islet function was assessed 460 days after the transplantation [109]. Rat normoglycemia was assured with the microencapsulated and cryopreserved islets with similar insulin secretion after graft retrieval compared to the microencapsulated and non-cryopreserved group. Cryopreservation of a pancreatic substitute consisted of microencapsulated murine insulinoma βTC-tet cells was also performed comparing slow freezing to vitrification [110]. For the slow freezing, microencapsulated cells were cooled at -1°C/min with a Mr. Frosty Freezing container, and stored in liquid nitrogen. After fast thawing of samples, and using a C13 nuclear magnetic resonance and isotopomer analysis, the metabolism was maintained in microencapsulated βTC-tet cells by either slow freezing or vitrification. By contrast, insulin secretion was maintained only after slow freezing, displaying the advantages of slow freezing compared to vitrification in the storage of microencapsulated βTC-tet cells.

4.3. Mesenchymal stem cells (MSCs)

MSCs are being studied intensively due to their advantageous properties for cell-based therapies. MSCs can differentiate into three different lineages (e.g., osteogenic, adipogenic and chondrogenic) making them appropriate for regenerative medicine. Moreover, this cell type can immune-modulate the environment, avoiding the immune system reaction, which is desirable for cellular implants [111,112]. Their encapsulation in hydrogels or alginate capsules has been studied extensively [84,113-115], and their storage also investigated in the last few years (Table 4).

In the first study with slow cryopreservation of microencapsulated MSCs, Pravdyuk et al. considered the influence of DMSO concentration (5-10% v/v) and three cooling protocols (e.g., two slow and a rapid cooling protocols) in the post-thaw outcome of microencapsulated human MSCs [13]. It was demonstrated that DMSO at 10% was the optimal concentration maintaining viability and metabolic activity in microencapsulated MSCs after thawing. The second cooling protocol, with induced ice nucleation, displayed better viability and metabolic activity, showing the positive effect of induced ice nucleation. Afterwards, Grishkov et al. compared the influence of culture time in microencapsulated primate MSCs before cryopreservation, cryopreserving immediately or after 24hour incubation of the cell-based products [116]. In this regard, microencapsulated cells were cryopreserved with 10%

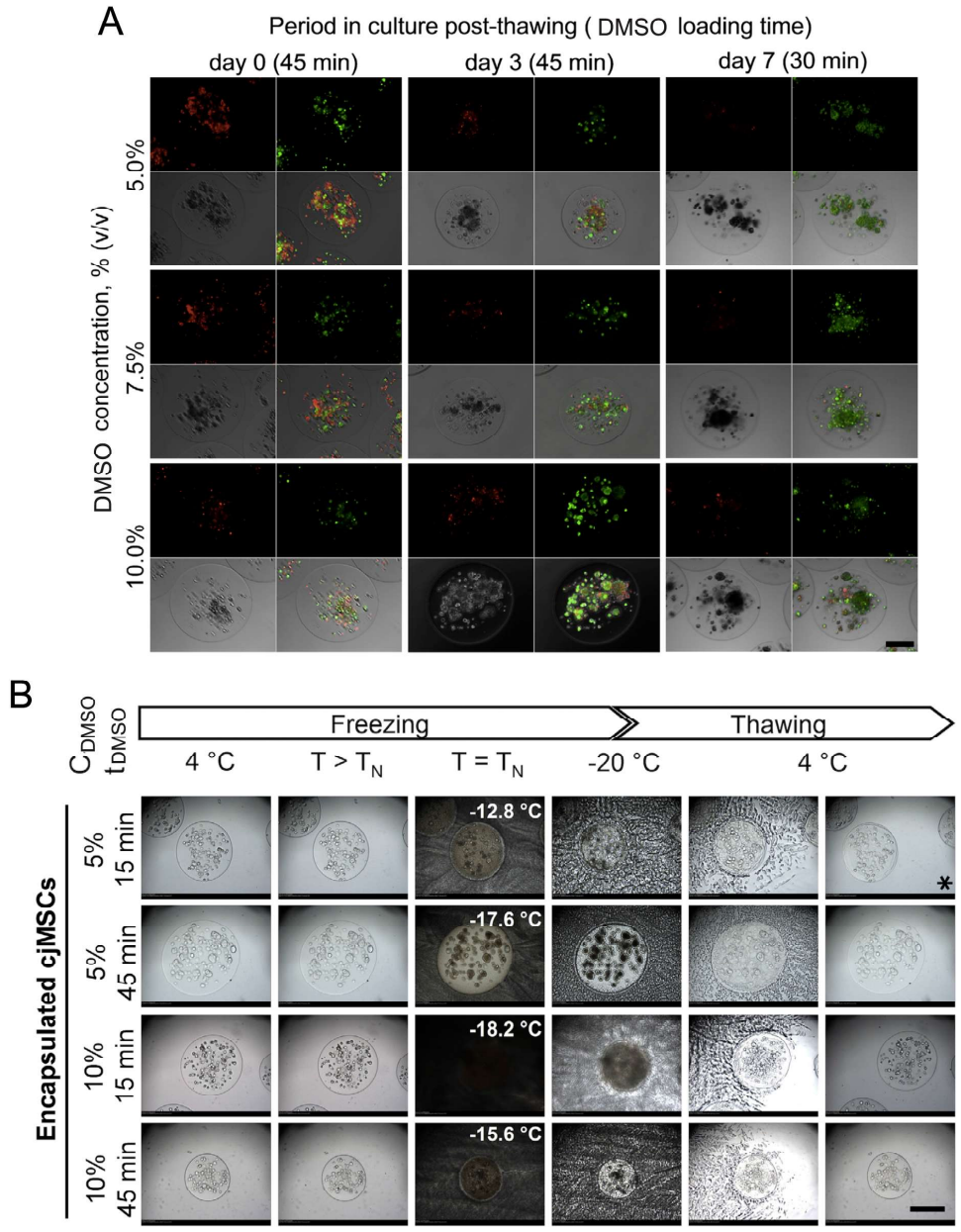

Figure 8: Evaluation of monkey MSCs microencapsulated in alginate beads. A) Live (green)-dead (red) staining of microencapsulated monkey MSCs after thawing following seven days in culture (day 0, day 3, day 7) utilizing 5%, 7.5% and 10% (v/v) DMSO with 30 min and 45 min of pre-loading interval. Freezing was performed using 2.5%, 5%, 7.5% and 10% (v/v) DMSO with respective pre-freeze loading interval. B) Evaluation of microencapsulated monkey MSCs during freezing/thawing using cryomicroscopy. The temperature value at T = TN represents the temperature of ice formation. TDMSO represents the DMSO pre-loading interval. The structural damage of the alginate beads rises with rising temperatures of ice formation. Stars depict the process settings that noticeably impaired the homogeneity and/or structure of alginate beads. Scale bars represent 250 µm. Scale bar: 200 µm. Reprinted from Multipotent stromal cells derived from common marmoset Callithrix jacchus within alginate 3D environment: Effect of cryopreservation procedures, 71 , O. Gryshkov, N. Hofmann, L. Lauterboeck, D. Pogozhykh, T. Mueller, B. Glasmacher, Cryobiology, 103-111, Copyright (2018), with permission from Elsevier.

(v/v) DMSO and 17.5% FBS, cooled at -1°C/min with a Mr. Frosty Freezing container and stored at -150°C. After fast thawing and five days culture, the microencapsulated MSCs cryopreserved just after their encapsulation, showed better metabolic activities, and demonstrated that microencapsulated MSC culture time before cryopreservation can impact cell function after thawing. Our group also investigated the cryoprotective effect of different CPAs in the cryopreservation of microencapsulated erythropoietin secreting murine MSCs [14]. Different CPA solutions combining DMSO, trehalose, and glycerol were studied to reduce the concentration of DMSO in cryopreservation. Similar to the previous study, microencapsulated MSCs were cooled at -1°C/min with a Mr. Frosty Freezing container to -80°C, and stored in liquid nitrogen. After sample fast thawing, only cryopreserved microencapsulated MSCs with 10% (v/v) DMSO were able to maintain metabolic activity, cell viability and erythropoietin secretion compared to non-cryopreserved microencapsulated MSCs. Furthermore, only the 10% DMSO group was able to maintain the erythropoietin increase in transplanted mice compared to non-cryopreserved microencapsulated group. These findings suggest that further investigation should be assessed in the cryopreservation protocol to reduce the used DMSO in the CPA solution. Following this research line, Grishkov et al. studied the DMSO concentration (2.5-10% v/v) in CPA solution and the pre-freezing DMSO loading time (0, 15, 30 and 45 minutes) of microencapsulated monkey MSCs [117], with similar cooling and thawing processes to their previous study [116]. Independent of the pre-freezing DMSO loading times used, better cell viability (Figure 8A), metabolic activity and membrane integrity were obtained with 7.5% and 10% DMSO concentration in the CPA solution. Moreover, also the ice nucleation temperature was studied using cryomicroscopy, demonstrating that different DMSO loading times, and DMSO concentrations in CPA solutions could lead to differences in ice nucleation temperatures (Figure 8B). Interestingly, it was also shown that higher ice-nucleation temperatures (-12.8°C) impaired the structure of alginate beads after thawing. Altogether, it was demonstrated that DMSO concentration could be decreased from 10 to 7.5% maintaining high cell recoveries after thawing in microencapsulated monkey MSCs.

4.4. Hepatocytes for liver diseases

The encapsulation of hepatocytes has been proposed for supplementing essential functions lacking in some disorders such as fulminant liver failure (FLF). Diverse reports in vivo demonstrated the potential of hepatocyte encapsulation for the FLF treatment [83,88,118,119]. Since in FLF the treatment need is immediate, and thus, the banking of these cell-products for the “on demand” access of patients has been extensively studied for

two decades mostly with the use of slow freezing cryopreservation (Table 5).

In the first study that was performed with hepatocytes, rat hepatocytes were microencapsulated in alginate-poly-L-lysine-alginate capsules [120]. Microencapsulated hepatocytes were cryopreserved with 10% (v/v) DMSO keeping cryovials 24 hours at -70°C, and next stored in liquid nitrogen for 7 and 30 days. After fast thawing, the cryopreserved microencapsulated hepatocytes displayed similar urea synthesis to the non-cryopreserved microencapsulated group. By contrast, cryopreserved microencapsulated hepatocytes for 30 days showed a decrease in protein synthesis. Furthermore, in 30 days cryopreserved microencapsulated hepatocytes, the serum bilirubin levels of transplanted Gunn rats, which have a genetic deficiency of bilirubin glucuronidation, did not decrease as much as in other cryopreserved groups. In this study, the storage time of microencapsulated rat hepatocytes could have affected the post-thaw cell function, indicating that the cryopreservation process requires further. In the early 2000s, murine hepatocytes in alginate-cellulose poly(methylene-co-guanidine) were cryopreserved [121]. The microencapsulated hepatocytes were cooled with 10% (v/v) DMSO following a stepwise protocol: 30mins at 4°C, 2 hours at -20°C, 24 hours at -80°C and stored in liquid nitrogen. After fast thawing, only albumin secretion was determined, and there were no differences between the non-cryopreserved and cryopreserved microencapsulated hepatocytes. Next, the slow freezing cryopreservation of microencapsulated hepatocytes was studied in vivo. Before starting the xenotransplantation studies, Mai et al. investigated the cryopreservation influence on primary rat and immortalized human microencapsulated hepatocytes [122]. In this case, the cryopreservation protocol was performed with a CRF and sample temperature was measured: 12 min hold at 4°C, 4.2°C/min to -8°C, 18 min hold, 4.2°C/min to -100°C and stored in liquid nitrogen. After fast thawing in a 37°C water bath, both microencapsulated and cryopreserved cell types showed similar albumin secretion profiles over 7 days compared to the non-cryopreserved group. In the xenotransplantation study, the cryopreserved microencapsulated rat or immortalized human hepatocytes showed similar survival rates in mice with induced FLF compared with non-cryopreserved microencapsulated hepatocytes (~70% and ~50% respectively). A similar study using cryopreserved microencapsulated porcine hepatocytes in FLF induced mice was also performed [123]. Biological functions were determined in vitro in terms of urea and albumin synthesis, and lidocaine, ammonium and diazepam metabolism. Similar cell function profiles were displayed between the cryopreserved microencapsulated hepatocytes and non-cryopreserved microencapsulated hepatocytes, without differences in the survival rates of FLF induced mice after xenotransplantation. The authors claimed that the cryopreserved microencapsulated xenogeneic hepatocytes showed life supporting liver-specific metabolic functions in vivo, and suggested that these findings had great potential for clinical use. In the

same way, Hang et al. studied the cryopreservation of microencapsulated human hepatocytes, additionally determining their gene and protein expression [124]. In this case, 2 ml cryovials were cooled with a progressive isopropanol freezing container in a -80°C freezer, and stored in liquid nitrogen. After sample fast thawing, it was demonstrated that the urea and albumin secretion, and gene expression of the hepatocytes were maintained compared with non-cryopreserved group. In contrast, the cytochrome P450 (CYP3A4) expression was incremented in the cryopreserved microencapsulated hepatocytes compared to the cryopreserved free hepatocytes. Recently, Jitraruch et al. tested the effect of commercial modified CPA solutions on the cryopreserved microencapsulated rat and human hepatocytes [81]. Viaspan® (with glucose and DMSO 10% (v/v)) and the Cryostor® solution were compared in terms of metabolic activity, urea synthesis, albumin production and cytochrome P450 activity for the cryopreservation of microencapsulated hepatocytes. Slow freezing was performed stepwise with a CRF, and after fast sample thawing, the modified Viaspan® had the best protective effect in the cryopreservation of microencapsulated rat and human hepatocytes, maintaining cell function for 7 days. Also, it was determined that the addition of an antiapoptotic factor, the benzyloxycarbonyl-Val-Ala-DL-Asp-fluoromethylketone (ZVAD), has a positive effect on cell function of cryopreserved microencapsulated rat hepatocytes, especially in reducing apoptosis. This is the first study using an antiapoptotic factor in the CPA solution for the cryopreservation of microencapsulated hepatocytes, opening another research line for improving the post-thaw cell function.

The Liver Group, from University College of London (UCL), is developing a bioartificial liver ($7x10^{10}$cells biomass) for the treatment of FLF [83,88,125], studying for that aim, the slow freezing cryopreservation of alginate microencapsulated liver HepG2 spheroids [12,63,64,74,79,126,127]. To reduce contamination risks in the process, they compared a CRF without the use of liquid coolants with another CRF that use liquid nitrogen for slow cooling cryopreservation [126]. The 1.25ml sample cooling was performed with a multi-step protocol and stored in the vapor phase of liquid nitrogen. After fast thawing, both groups were compared for 3 days to non-cryopreserved groups in terms of viability, cell counts and albumin secretion. Cell function decreased similarly in both cryopreserved groups at day 3 (90% for viability, 70% for cell count and 65% for albumin secretion), certifying the use of a CRF that enables to work without nitrogen infection risk. Some studies have demonstrated that the active control of ice nucleation temperature could increase the cell recovery after thawing [51,52]. In this regard, the addition of cholesterol, as an ice nucleating agent, in the CPA solution was studied [64]. The cholesterol reduced supercooling and latent cryoinjury during cryopreservation, and consequently almost doubled the cell number after thawing. Moreover, the protein and detoxification function, as well as the oxidative metabolism of

cryopreserved microencapsulated liver spheroids were improved when cholesterol was present in the cryopreservation, demonstrating its benefitial effects. Furthermore, the influence of the storage temperature in the microencapsulated liver spheroids was investigated up to a year storing them in a -80°C freezer or in vapor phase nitrogen [74]. The preservation of cell-based products above the Tg was harmful to cell function after thawing. A rapid deterioration in functional recoveries of microencapsulated liver spheroids was displayed after only a few weeks of storage at -80°C. However, storage in vapor phase nitrogen maintained the cell function of microencapsulated liver spheroids more than a year. This study demonstrated the importance of banking cell-based products below the Tg and ensure their appropriate long-term storage. The cryopreservation protocol developed for small volumes (0.5ml) was scaled up to larger volumes (~200ml) in a liquid nitrogen-free CRF [12]. Since the CPA excess will limit the cooling and warming rate, and possibly influence cell recovery, the microencapsulated liver spheroids and CPA ratio in cryovials was studied with the aim of reducing the amount of excess CPA volume during cryopreservation. Removal of the CPA excess had no significant impact on cell function after thawing with the biomass to CPA ratio 1:0. Moreover, fast thawing (37°C water bath) was the best way to recover the cell-based products. Also, the influence of the following cooling rates was investigated in cell function of cryopreserved microencapsulated liver spheroids in cryovials (0.5ml) and bags (200ml): a linear cooling rate of 1°C/min, and two non-linear profiles that produced an average cooling rate of -1°C/min from -8°C to -60°C. The first non-linear cooling profile, which has an extended holding period in the temperature zone following ice nucleation and enables more time to be established for osmotic equilibrium between the cells and the external environment at high subzero temperatures, displayed the best results with highest viable cell numbers and high alpha-1-fetoprotein production, either in cryovials or bags 24 hours after thawing.

Interestingly, with the aim of cryopreserving a whole bioartificial liver (2.3 litre biomass) the process of ice formation and propagation was investigated in the slow freezing cryopreservation [63]. In this scenario, the water-ice phase change can be dominated by the network solidification process, which is typically present in small samples such as cryostraws or cryovials, or progressive solidification, which is more often observed in large volumes or environmental freezing. Thus, even the progressive solidification will be the most probable in larger volumes, its effect compared with network solidification was studied in the post-thaw recovery of microencapsulated liver spheroids in cryovials. Two different heat transfer modules were used in an electrical Stirling CRF to freeze 6 ml cryovials, modifying slightly the CPA solution and changing the cholesterol for another ice nucleating agent (Icestart®). The freezing protocol consisted on cooling at -1°C/min to -80°C and storage in a -80°C freezer for 7 days. After fast thawing of samples non-significant differences in viable cell

number or function between the two solidification processes was demonstrated. By contrast, the ultrastructural effects of the two processes displayed by electron scanning microscopy, showed different behaviors of ice formation. For that reason, the authors suggested that both ice formation processes should be further investigated, focusing especially on progressive solidification that is unavoidable in large volume cryopreservation. Following this research line, the effects of progressive solidification were investigated in the recovery of microencapsulated liver spheroids in the different regions across the frozen cores of 6mL cryovials and a 2 litre cylindrical chamber [127]. It has to be noted that whereas in cryovials only microencapsulated liver spheroids were placed, the 2 litre biomass, within the cylindrical chamber, consisted mainly of non-cellularized alginate microcapsules, in which microencapsulated liver spheroids pouches were placed in different areas to study the freezing effect in every region (Figure 9A). The microencapsulated liver spheroids proportion, CPA solution, cooling, storage and thawing protocol used for the cryovials were the same as in the previous study. Even though complex manipulations were performed for the cooling and thawing of the microencapsulated liver spheroids pouches in the 2 litre biomass, a similar cooling and fast thawing profile was achieved to study the progressive ice solidification (Figure 9B). The first solidified microencapsulated livers spheroids in the bottom of cryovials showed significantly higher viable cell number and function compared to last solidified on day 3. By contrast, microencapsulated liver spheroids frozen in the middle of the cryovials displayed higher alpha-fetoprotein production and glucose consumption compared to microencapsulated liver spheroids at the cryovial edges on day 2. On the other hand, cryopreserved microencapsulated liver spheroids in the 2 litre chamber displayed a different behavior after thawing. In the middle of the biomass, the metabolic activity and viable cell number of microencapsulated liver spheroids were higher immediately after thawing. In summary, spatial considerations of microencapsulated liver spheroids had a great impact on the cell recovery either in cryovials or the 2 litre chamber, and these results demonstrated that spatial considerations are important to consider when large volumes are cryopreserved with progressive solidification. To approach the clinic, the cryopreservation of a whole bioartificial liver (2.3 litre microencapsulated liver spheroids biomass) was investigated [79]. In this case, the 2.3 litre biomass was frozen with the same CPA solution and protocol. By contrast, as the fast thawing of a whole 2.3 litre microencapsulated liver spheroids biomass was difficult to achieve, a stepwise slow thawing consisted on -80°C 2 hours, -30°C 1 hour, -10°C 1 hour and final fast thawing with a saline solution was used successfully (Figure 5). Microencapsulated liver spheroids viable cell number, glucose consumption, lactate production and alpha-fetoprotein production recovered to pre-freeze values by day 3 after thawing, and comparing the 2.3 litre biomass results to the microencapsulated liver spheroids

cryopreserved in cryovials, longer recovery times were needed in the biomass for recovering pre-freeze cell function values. For that reason, even the 2.3 litre biomass was successfully cryopreserved, authors suggested that further improvements in thawing would increase the post-thaw cell function of microencapsulated liver spheroids.

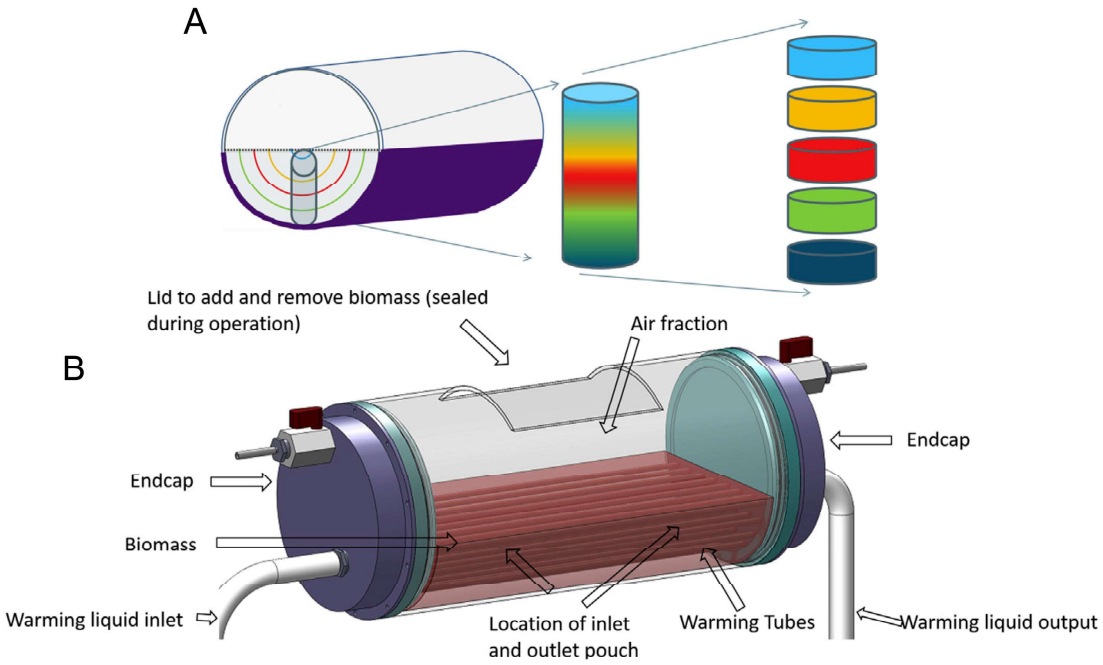

Figure 9: A schematic of the bioartificial liver chamber. The bioartificial liver chamber contained pouches (with microencapsulated liver spheroids) of nylon mesh, permeable to culture medium and ice but impermeable to microencapsulated liver spheroids. This chamber was cooled from the edges (as indicated in purple), and ice developed radially to the central semi-circle, with the semicircles representing areas that solidified at the same time (A). The biomass fill is represented by the dotted black line. This pouch was extracted and dissected into 5 as shown on the right of the figure. These sections were thawed consistently to determine spatial differences in damage on cooling. (B) 25 warming tubes were passed through the biomass (indicated in red), with ethanol equally distributed through each tube using larger endcaps. Pouches were placed within 5 cm of either the inlet or outlet tubes, with pouches of cells nearer the warming inlet thawing more rapidly. (Figure as originally published in [127])

4.5. Myoblasts

Besides the microencapsulated cell types mentioned, others have been cryopreserved for sustained therapeutic molecules delivery [128,129]. Murua et al. investigated various cooling protocols and DMSO concentrations for the cryopreservation of microencapsulated erythropoietin-secreting C2C12 cells (Table 6)[128]. After fast thawing, the erythropoietin reduction was determined, and the slow cooling protocol showed less erythropoietin

reduction compared to non-cryopreserved microencapsulated C2C12. With this cooling protocol, DMSO concentration was studied (1, 5, 10, 20 and 30% v/v) in CPA solutions. As in other studies, 10% DMSO appeared to be optimal in terms of erythropoietin secretion after thawing. Moreover, cryopreserved microencapsulated cells with 10% DMSO were able to maintain erythropoietin increase in transplanted mice compared to non-cryopreserved microencapsulated cells. Another group also investigated the cryopreservation of microencapsulated genetically modified C2C12 myoblasts to secrete insulin comparing slow cooling to vitrification [129]. In the slow cooling, insulin-secreting microencapsulated C2C12 were cooled with 10% (v/v) DMSO at -1°C/min in a Mr. Frosty Freezing container to -80°C, and stored in liquid nitrogen. After fast thawing, the slow-cooled samples indicated slightly better outcome regarding metabolic activity, viability, and insulin secretion compared to those that had been vitrified. Due to the procedure simplicity authors concluded that slow freezing was superior to vitrification for the cryopreservation of microencapsulated insulin-secreting C2C12. As in other cell types, it seems that the 10% DMSO is the best CPA solution for the cryopreservation of microencapsulated C2C12 cells.

5. EXPERT OPINION AND FUTURE DIRECTIONS

Cell microencapsulation represents a promising technology for the treatment of different diseases; however, it needs further improvements to optimize this cell-based therapy and reach the clinics. In this sense, the cell-based product banking is one of the processes that will help achieving the desired goal, and many works have been performed in the cryopreservation of microencapsulated cells in the last two decades, illustrating different protocols and results. In this regard, there are several aspects in cryopreservation that still have to be optimized to improve cell outcome and capsule integrity, to guarantee the reproducibility and quality of the cell-based products.

Even though DMSO replacement in CPA solution has been a subject of research for the last two decades, DMSO removal seems improbable in this field so far. Every study performed in the slow freezing cryopreservation of microencapsulated cells used DMSO with good results [12,14,79,96]. For that reason, another aspect should be also investigated in the slow freezing of microencapsulated cells, such as the appropriate DMSO removal after thawing [27]. In this respect, different methods have been developed to remove CPAs from the cell-based products (e.g, dilution-centrifugation, mass trasnsfer in microfluidical devices or dilution-filtration with hollow fibers), being dilution-centrifugation the most widely used method [130]. In the reviewed studies, no one determined if the applied post-thaw recovery method gets rid of the DMSO completely from microcapsules. Although, they

are not normally employed or added in cryopreservation studies, with the aim of measuring the residual CPA after thawing, capillary zone electrophoresis [131], high-performance liquid chromatography (HPLC) [132], gas chromatography [133] or electrical conductivity [130] methods have been proposed. These techniques, determing DMSO removal, would be very useful, especially when the cell-based products are implanted in patients. In this way, the DMSO adverse effects will not take down the future use of these microencapsulated cells in the clinic. The ice-nucleating agent addition in CPA solution raised the ice nucleation temperature in cooling and demonstrated its benefits in the post-thaw microencapsulated liver spheroids cell function [64]. However, the ice nucleation temperature increment could also impair capsules (Figure 8B)[116]. In this respect, it has to be taken into account that capsules damage could alter the biomechanical properties, changing their behavior (e.g., as sustained drug delivery or cell delivery system) and affect the quality of the cell-based products. In this sense, light microscopy observation has been widely performed to determine general microcapsules appearance in terms of circularity and broken capsules after thawing [116,128]. Those observations are insufficient to determine capsule stiffness and pore size, and assure the capsules mechanical stability. For that reason, even ice-nucleating agents should be used to improve cell function, the capsules mechanical integrity should also be investigated with other assays (e.g., capsule swelling [14]) after their cryopreservation. In any case, the capsules biomechanical properties is a key aspect that must always be studied to guarantee the post-thaw quality of the cell-based products. Lastly, different additives have been included in CPA solution to reduce cell apoptosis and death by other mechanisms after cryopreservation. Often cells look healthy and viable immediately after thawing, but after some hours a significant portion of cells are lost due to the overall mechanisms involved in cell apoptosis and necrosis after cryopreservation, the called cryopreservation induced onset cell death (CIOCD)[134]. Mainly this phenomena occurs when cryopreservation processes may be optimized to protect cells structurally, but fails to adequately manage the other stresses associated with this process (e.g., reactive oxygen species creation). With the scope of reducing the CIOCD phenomena, antioxidants and anti-apoptotic factors were included in CPA solutions for the cryopreservation of microencapsulated cells. On the one hand, the antioxidants (trolox and catalase) were used in the cryopreservation of microencapsulated liver spheroids, but as their effect was not compared to cryopreserved microencapsulated liver spheroids without the antioxidants, their efficiency could be questioned [12]. On the other hand, anti-apoptotic factor effects in CPA solution were investigated with hepatocytes reducing apoptosis significantly in the first 24 hours after thawing [81]. In this regard, cell death and apoptosis behavior within microcapsules have not been carefully studied after cryopreservation. However, the post-thaw cell loss profile was determined in highly dense

microencapsulated liver spheroids [12,64,79,127]. Cell death and proliferation mechanisms were balanced towards cell death in the first two days, displaying up to 40% fewer cells compared to pre-freezing cell numbers. After the second day, when apoptosis and cell death diminished, microencapsulated liver spheroids behavior changed increasing the viable cell number and recovering pre-freezing cell functions. The displayed microencapsulated liver spheroids cell loss behavior makes sense due to CIOCD phenomena that are normally observed within 6 to 48 h after post-thaw culture [134,135]. Any improvement made by the antioxidants or anti-apoptotic factors in microencapsulated liver spheroids cell function recovery time will enable significant progress in the development of the bioartificial liver for the clinic. For that reason, even though there are few studies performed with these molecules, the antioxidants and anti-apoptotic factor addition could inhibit the CIOCD phenomena and improve overall cryopreservation efficiency in microencapsulated cells.

CPA addition and removal have also changed over the last two decades. In the first studies, the CPA solutions were not added chilled without avoiding the CPA related cytotoxicity [94,95,108,121], but rapidly protocols changed and CPA solutions were added chilled to samples [123,128]. In the same way, samples were not cooled before cooling, but nowadays all microencapsulated cells are chilled adding a holding step at 4°C prior to freezing [64,126]. Moreover, CPA removal after sample recovery has not been described; it has usually been performed by the slow drop by drop dilution of the microencapsulated cells in the CPA solution. Although this method works for the thawing of microencapsulated cells, more complex investigations of CPA dilution after thawing should be investigated to improve cell function and diminish the CIOCD phenomena. In this regard, slow freezing could take advantage of vitrification, in which the thawing protocols have been deeply investigated to upgrade the thawing process [54]. Moving forward, cooling has been described extensively in the reviewed studies, showing that diverse protocols were used independently of the cell type. In this sense, cooling protocols (e.g., non-linear or linear, with induced ice nucleation) have been investigated commonly with a CRF to reduce the IIF and optimize post-thaw cell outcome. They have changed from non-linear to linear profiles due to their simplicity and high-quality results obtained either with small or large volumes [12]. Although CRF enables the optimization of the cooling process preventing sub-optimal cell functions and CIOCD phenomena after thawing [94], passive progressive freezing containers were also used for the cryopreservation of different microencapsulated cells, displaying good results [14,117,128]. In any case, if something has been common in all the described studies is that microencapsulated cells were cooled until the range of -80 to -100°C and then immersed in liquid or vapor phase nitrogen to store the cell-based products below the Tg temperature. It has to be noted that few studies measured or displayed the temperature profiles in the

freeze-thaw process. Importantly, the temperature measurement during cryopreservation would probably explain the difference obtained between some of the reviewed studies, as ice nucleation temperature and the real cooling profile would be showed giving more information to the field. We believe that this is one of the aspects that being applied will significantly improve the overall clarity and understanding of the studies. Lastly, for the thawing of microencapsulated cells, the fast thawing procedure have been extensively used by sample immersion in a water bath at 37°C. Slow thawing was studied when the large samples size made impossible the fast thawing. In this sense, a 2.3 litre litremicroencapsulated liver spheroids biomass, which works as a bioartificial liver, was thawed in a stepwise manner [79]. Even though good results were obtained with functional recovery of the biomass at 72hours, further improvements in slow thawing would reduce the post-thaw recovery time of the bioartificial liver. In this regard, among the reviewed studies, the bioartificial liver is the only microencapsulated cell-based product that seems to have the potential to reach the clinic. However, the bioartificial liver biomass has to be manufactured under GMPs in all process, assuring the reproducibility and quality of the biosystem to reach the clinics [136]. On this regard, taken into considreation the quality controls that are used on the handling, processing and disposal of stem cells, which are the most used cryopreserved cell therapies around the world [137], we have enumerated the aspects that should be followed on the quality control of the microencapsulated cells from the elaboration to the use in clinics (Figure 10). First, the function of the encapsulated cells (cell viability, menbrane integrity, metabolic activity and therapuetic molecule release quantification among others) as well as the integrity of the microcapsules (swelling assay) should be assesed always before cryopreservation, being mandatory the microcapsules integrity assesment when the goal is the implantation. Next, during the freeze-thaw process the temperature must be tracked to ensure no thermal flucutations that could affect product stability. Finally, after thawing the following two main aspects should be considered: the microencapsulated cell function and microcapsules integrity maintainance should be assesed, and residual CPA after removal should be quantified with the aformentioned analytical techniques to prevent CPA related toxicity. In addition, clinicians training with microencapsulated cells result an important factor to avoid handling errors that can take down the effectiveness of the cell therapy product. Considering all these quality control steps, currently a clinical scale bioartificial liver has been developed for GMP [83], showing its potential in vivo and demonstrating that a big part of the path has been done for the treatment of FLF patients.

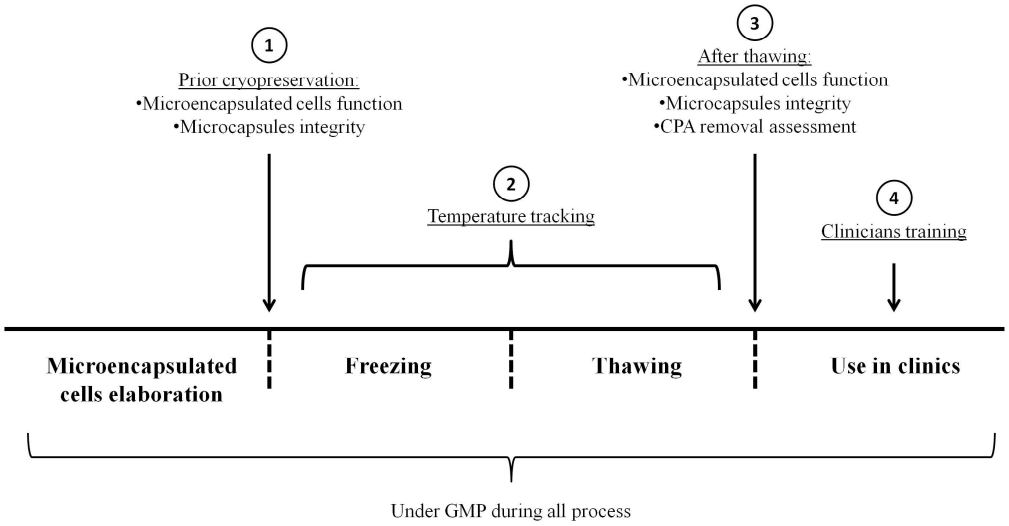

Figure 10: Quality control steps of microencapsulated cells from elaboration to clinics: 1) Assessment of microencapsulated cell function and microcapsules integrity (mandatory for implantation purposes before cryopreservation) 2) Temperature tracking during freeze-thaw process; 3) Quantification of microencapsulated cells function and microcapsules integrity maintenance after thawing, and determination of residual CPA after complete removal. 4) Specific clinicians training with microencapsulated cells handling. All the steps should follow GMP procedures.

6. CONCLUSIONS

Slow freezing cryopreservation has been widely studied and developed over recent years for the preservation of many cells, tissues or cell-based products. In this sense, microencapsulated cells are one of the biosystems that has been extensively studied to be cryopreserved with the aim of providing a cell-based product bank that facilitates the "on demand" access of patients to the treatments in a clinic far from the manufacturer. Although many studies have been performed with microencapsulated cells displaying successful results, there are many aspects in the slow freezing procedure that should be optimized, such as the CPA solution and the freeze-thaw process. Thus, further research of the slow freezing in these cell-based products is needed for this technology to the reach the desired goal, the clinical translation.

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2

Objectives

2. OBJECTIVES

The use of cell therapies for the treatment of diverse diseases has increased enormously in the last decades, promoting the research of the areas that will enable the advance and spread of these 3D cell-based products. For example, cell microencapsulation in which cells are immobilized in biocompatible matrix, is a technology that is being used as a sustained drug or cell delivery system to treat diseases such as diabetes, cancer, neurodegenerative and hepatic diseases. However, diverse aspects should be still optimized for the future translation from bench to the bedside of these cell-based products, being the process that will allow correct storage, shipment and delivery of cell-based products one of them. On this regard, slow freezing cryopreservation has been widely used for many years to preserve cells and tissues, but this technology is still not well understood and applied. 3D cell-based products cryopreservation, such as microencapsulated cells, would approach this therapy to clinics, facilitating the "on demand" access of patients to the treatments in a clinic far from the manufacturer, reducing its cost. Among the different factors to be optimized in cryopreservation, one of the hot topics is the cryoprotectant agent (CPA) election. Thus, in order to give a boost to cell encapsulation translation we intended to determine an optimal natural non-toxic CPA for 3D cell-based products, developing the following specific goals:

1. To determine if there is a current cryoprotectant agent (CPA) that provide to 3D cell-based products better integrity and cell function after thawing than dimethylsulfoxide (DMSO), using alginate microcapsules as model of study.

2. To investigate hyaluronan as an alternative natural CPA for slow freezing cryopreservation of 3D cell-based products, using alginate microcapsules as model of study.

3. To study a real clinical 3D cell-based product where hyaluronan can act as a natural CPA.

3

Cryopreservation of microencapsulated murine mesenchymal stem cells genetically engineered to secrete erythropoietin

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Cryopreservation of microencapsulated murine mesenchymal stem cells genetically engineered to secrete erythropoietins

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ABSTRACT

The ability to cryopreserve and store for long term the structure and function of therapeutic cells and tissues plays a pivotal role in clinical medicine. In fact, it is an essential pre-requisite for the commercial and clinical application of stem cells since preserves cells at low temperature and creates a reserve for future uses. This requisite may also affect to encapsulated stem cells. Several parameters should be considered on encapsulated cell cryopreservation such as the time and temperature during the cryopreservation process, or the cryoprotectant solutions used. In this study, we have compared the influence of penetrating and non penetrating cryoprotectants on the viability and functionality of encapsulated mesenchymal stem cells genetically modified to secrete erythropoeitin. Several cryoprotectant solutions combining DMSO, glycerol and trehalose at different concentrations were studied. Although almost no differences among the studied cryoprotectant solutions were observed on the differentiation potential of encapsulated mesenchymal stem cells, the penetrating cryoprotectant DMSO at a concentration of 10% displayed the best viability and erythropoietin secretion profile compared to the other cryoprotectant solutions. These results were confirmed after subcutaneous implantation of thawed encapsulated mesenchymal stem cells secreting erythropoeitin on Balb/c mice. The hematocrit levels of these animals increased to similar levels of those detected on animals transplanted with non cryopreserved encapsulated cells. Therefore, DMSO 10% represents the most suitable cryoprotectant solution among the solutions here studied, for encapsulated mesenchymal stem cells cryopreservation and its translation into the clinic. Similar studies should be performed for the encapsulation of other cell types before they can be translated into the clinic.

Keywords: Cryopreservation, Mesenchymal stem cells, Microencapsulation, Nanotechnology

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1. INTRODUCTION

Cell microencapsulation is extensively used to enclose cells allowing the exchange of nutrients between the environment and the core of the microcapsule containing the cells [1]. In vivo, microcapsules protect the cells from the immune system while allowing the release of therapeutic molecules by the entrapped cells [1]. Although several hydrogels have been used for microencapsulation including agarose [2], chitosan [3] and hyaluronic acid [4], alginate is by far the most frequently employed biomaterial. Alginate microcapsules have shown to be safer and to work in physiological conditions such as physiological temperature, pH and isotonic solutions instead of cytotoxic solvents [5,6]. In addition, different polycations such as poly-L-lysine (PLL), poly-D-lysine (PDL) and poly-L-ornithine (PLO) have also been evaluated for coating alginate microcapsules, showing that PLL coating provides the highest resistance [7]. Pancreatic islets are the most widely cell type studied for cell encapsulation and alginate microcapsules have shown promising results. Thus, the intraperitoneal transplantation of encapsulated pancreatic islets can alleviate the dependency of diabetic patients from insulin injections [8,9]. But alginate cell encapsulation has also been applied to other diseases. For example, brain Aß burden, hyperphosphorylated-tau and cognitive impairment are attenuated in an Alzheimer's disease mice model after the implantation of encapsulated VEGF-secreting cells [10]. Our group has previously studied the microencapsulation of genetically engineered C2C12 myoblasts to secrete erythropoietin (EPO), showing that encapsulation allows long term expression after allogeneic transplantation, up to 210 days, as well as in xenogeneic transplantation, up to 98 days [11-14].

In addition to foreign body reaction to the biomaterial [15], the prevention of immune rejection for the entrapped cells offered by alginate microcapsules is not always enough to completely bypass the immune system. CD4+ T cells, B cells and macrophages can reject encapsulated cells in xenograft transplants by the secretion of immune molecules and complement that can traverse the microcapsule membrane destroying the inner cells [16]. In this sense, mesenchymal stem cells (MSCs) are able to secrete the desired transfected therapeutic molecules, while immune-modulating the microenvironment and avoiding the immune responses, representing a good candidate cell type to circumvent the immune system [17]. Moreover, MSCs exhibit pleitropic immune regulatory activities that inhibit the function of different immune cell subpopulations of the innate and adaptive immunity [18]. MSCs are able to suppress T cell proliferation through the secretion of PGE-2, IDO and TGF-B[19], induce CD4+CD25+FoxP3+ and CD8+ regulatory cells [20], block B cells proliferation in presence of IFN-γ [17] and down-regulate the co-stimulatory molecules CD40, CD80 and CD86 expression on dendritic cells [21]. MSCs´ immuno-modulatory properties and their

potential to be genetically modified for therapeutic applications make them an attractive candidate for cell microencapsulation [22].

Cryopreservation could be an attractive system for the long-term storage of microencapsulated MSCs that would simplify their clinical application. Liquid nitrogen freezing represents one of the oldest and the most common storage technique for cell lines [23]. Among the most important factors for a successful cryopreservation is the nature of the cryoprotectant [24,25]. Several cryoprotectants are currently being used at the clinical settings, in order to avoid the damage caused by the ice formation on the cell freezing. Cryoprotectants can be classified into two groups: penetrating and non-penetrating. Penetrating cryoprotectants are low molecular weight and permeable chemical compounds, such as dimethyl sulfoxide (DMSO) or glycerol [26,27]. They are able to displace the inner water from the cell, avoiding intracellular ice crystal formation [28]. Non-penetrating cryoprotectants are non-permeable high molecular weight chemical compounds, incluiding poly-ethylene-glycol (PEG), poly-vinyl-pyrrolidone (PVP) or trehalose [29-31]. They act from the cell outside promoting a quick dehydratation of the cell [32,33]. Although DMSO is currently the most widely used cryoprotectant, good results have also been shown with other cryoprotectants or their combination with DMSO [6,31,34]. For example, the combination of DMSO and trehalose resulted in more than 50% increase on the recovery of cryopreserved pancreatic islets and 14 fold more insulin release in comparison to those cryopreserved without trehalose [31]. Glycerol has shown better cryoprotectant properties on bull sperm cryopreservation than DMSO or ethylene glycol (EG), with greater values of preserved membrane integrity and lower chromatin damage [34]. However, these different cryoprotectants have not been compared between each other with the same encapsulated cell type.

Our group has previously tested several cryopreservation protocols at different concentrations of DMSO with microencapsulated genetically engineered myoblasts to secrete EPO [35]. We showed that a slow-cooling protocol (1 hour at -20ºC, 23 hours at -80ºC and finally at liquid N2) with 10% DMSO was a suitable strategy for long term storage of therapeutic microencapsulated myoblasts. At the present work, we apply these cryopreservation conditions focusing in the long term storage of MSCs genetically modified to secrete EPO, a cell type with higher potential than myoblasts for clinical translation. Our goal is to compare several penetrating and non-penetrating cryoprotectants at different concentrations to elucidate the optimal cryoprotectant solution for MSCs cryopreservation.

2 MATERIAL AND METHODS

2.1. MSCs culture and differentiation

D1-MSCs were purchased from ATCC (at passage 20) and routinely cultured at subconfluency in maintenance medium consisting on high glucose DMEM (ATCC) supplemented with 10% heat- non-inactivated fetal calf serum (FCS,Gibco) and 1 unit/ml of penicillin-streptomycin (P/S,Gibco). Resistance of D1MSCs to puromycin was assessed by plating 10^5 D1MSCs into six-well plates with media at serial 2 fold dilutions of puromycin (higher dose 50 μg/ml). Cells were observed over time until the completed detachment of wells, and the lowest dose that completely detached cells from the well was chosen (6.25 μg/ml).

To induce osteogenic differentiation, six-well plates with 10^5 D1-MSCs cells/well were cultured in DMEM supplemented with 10% FCS, 1% P/S, 0.05 mM L-ascorbic acid, 20 mM β-glycerophosphate and 100nM dexamethasone (Sigma Aldrich). Culture medium was replaced every 2-3 days. At 1, 2, and 3 weeks post-differentiation, cells were fixed in formaline 10% (Sigma Aldrich) for one hour, rinsed with water and stained with Alizarin RedS (Sigma Aldrich). To induce adipogenic differentiation, six-well plates with 10^5 D1-MSCs cells/well were cultured in DMEM supplemented with 10% FCS, 1% P/S, 0.5 μM dexamethasone, 0.5 μM 3-isobutyl-1-methylxanthine, and 50 μM indomethacin (Sigma Aldrich). Culture medium was replaced every 2-3 days. At 1, 2, and 3 weeks post-differentiation, cells were fixed in formaline 10% (Sigma Aldrich) for one hour, rinsed with PBS and stained with Oil Red O (Sigma Aldrich). To induce chondrogenic differentiation, $8x10^5$ D1-MSCs cells were cultured on 15 ml conical tubes with 0.5 ml of DMEM supplemented with 10% FCS, 1%P/S, 50 nM L-ascorbic acid, 6.25 μg/ml bovine insulin, and 10ng/ml transforming growth factor-β (TGF-β) (Peprotech Inc,). Culture medium was replaced every 2-3 days. After 3 weeks post-differentiation, pellets were fixed in formaline 10% (Sigma Aldrich) for one hour, rinsed with PBS, stained with 1% Alcian Blue (Sigma Aldrich) and rinsed with 0.1% HCl. Similar osteogenic and adipogenic differentiation protocols were followed for microencapsulated MSCs, staining the cells attached to the wells after 1, 2 and 3 weeks.

2.2. Cloning of lentivirus containing Epo

The cDNA of mus musculus Epo was cloned from the plasmid EX-Mm02357-M02 containing Epo (GeneCopoeia™), using the following primers: SpeI.EPO_F, aattactagtccaccATGggggtgcccgaacgt and EcoRI.EPO_R, aattgaattctcacctgtcccctctcctgc, which included the Kozak sequence (bold) before the starting codon (capitals) and the SpeI and EcoRI restriction sites (italic and underlined) at the 5`and 3`end respectively. PCR amplification was performed with 1U of Platinum® Taq DNA polymerase High Fidelity (Invitrogen) in 60 mM Tris-SO4 (pH 8.49), 18 mM ammonium sulfate, 2 mM MgCl2, 0.2

mM dNTPs and 0.2 µM of each primer. The PCR consisted of a first step at 94°C for 2 min followed by 35 cycles of amplification (15 seconds at 94°C, 15 seconds at 55°C and 1 min at 68°C). Amplified fragments were resolved on a 2% agarose gel containing 0.5 µg/ml ethidium bromide and purified using the QIAquick® Gel Extraction Kit following manufacturer's recommendations (Qiagen). Gel extracted DNA fragments were cloned using the pGEM®–T Easy Vector system as described by manufacturer (Promega). Bacterial clones were grown overnight and plasmids were extracted using QIAprep® Spin Miniprep Kit (Qiagen). Presence of the appropriate DNA fragment was confirmed by EcoRI and SpeI restriction enzyme digestion (Promega). Plasmids were quantified using a Nanodrop ND-1000 Spectrophotometer (NanoDrop Technologies, Inc) and sequenced to confirm the correct reading frame of modified Epo.

Modified Epo sequence was next cloned on pSIN lentiviral vector. The vectors pSIN-EF2-Nanog-Pur (Addgene plasmid 16578) [36] and pGEM containing the modified Epo were digested with EcoRI and SpeI restriction enzymes and the products loaded on a 2% agarose gel containing 0.5 µg/ml ethidium bromide. Linearized pSIN-EF2-Nanog-Pur without Nanog fragment and modified Epo fragment were purified with the QIAquick®Gel Extraction Kit. Purified vector and insert fragment were incubated for one hour at room temperature with 1 U of T4 DNA Ligase (Invitrogene) in 50mM Tris-HCl (pH 7.6), 10 mM MgCl2, 1 mM DTT and 5% (w/v) polyethylene glycol-8000. The ligation product was transformed into One Shot® Stbl3™ Chemically Competent E. coli (Invitrogen). Presence of Epo fragment was confirmed by EcoRI and SpeI restriction enzyme digestion. Clones with Epo fragment (pSIN-EF2-Epo-Pur) were quantified with Nanodrop ND-1000 Spectrophotometer and sequenced.

2.3. Lentivirus production and MSCs infection

pSIN-EF2-Epo-Pur was transfected into One Shot® Stbl3™ Chemically Competent E. coli, extracted with QIAprep® Spin Maxiprep Kit (Qiagen) and quantified with Nanodrop ND-1000 Spectrophotometer. 2.5x106 293T cells were cultured with DMEM, 10% FCS and 1% P/S overnight and further transfected with 9 µg of pSIN-EF2-Epo-Pur, 3 µg of pMD2.G (Addgene plasmid 12259), 6 µg of psPAX2 (Addgene plasmid 12260) and 45 µl of lipofectamine® 2000 in OptiMEM medium following provider´s recommendations. After three days at 37ºC, supernatants from transfected 293T cells were collected, spun up for 10 minutes at 2000xg, filtered with a 0.45 µm PVDF low retention Millex® filter (EDM Millipore), and stored in liquid nitrogen on 1 ml aliquots until further use.

D1-MSCs cells were plated in 6 well-plates at a density of $5x10^4$ cells/well. A final volume of 2 ml with 1800 µl of stored supernatant containing viruses codifying Epo-Pur and

4 μg/ml of polibrene were added next day to D1-MSCs. Cells were incubated for 48 hours and supernatant was replaced with new maintenance medium containing puromycin 12.5 μg/ml. D1-MSCs were cultured and expanded with medium and 12.5 μg/ml puromycin for 1 month to ensure all cells were infected.

For assessing lack of virus production by infected cells, supernatant from infected cells cultures was transferred to 10^5 virus-free D1-MSCs and incubated for 3 days. "Re-infected" cells were collected and their genomic DNA extracted with QIAamp DNA Mini Kit (Qiagen). Insertion of Epo cDNA sequence into the genome was assessed by PCR following the same parameters than for Epo cloning.

2.4. Microencapsulation

D1-MSCs genetically engineered to release EPO (MSCs-Epo) were immobilized into alginate-poly-L-lysine-alginate (APA) microcapsules using a pneumatic atomization generator (bioencapsulation portable platform Cellena®). Cells were suspended in 1.5% (w/v) low viscosity and high guluronic (LVG) alginate sterile solution, obtaining a cell density of $5x10^6$ cells/mL alginate. This suspension was extruded through a disposable nebulizer for 160-190 μm particle size using a 5 mL sterile syringe from a peristaltic pump. The resulting alginate beads were maintained in agitation for 15 min in a $CaCl_2$ solution (55 mM) for complete ionic gelation. Afterwards, they were ionically linked with 0.05% (w/v) PLL for 5 min, followed by a second coating with 0.1% alginate for another 5 min. Microcapsules were prepared at room temperature, under aseptic conditions and were cultured in complete medium. The diameter (160 μm) and overall morphology of the mcirocapsules were characterized using an inverted optical microscopy (Nikon TSM).

2.5. Criopreservation

All criopreservant solutions were fresh prepared by diluting the cryoprotectant agent in maintenance medium. Eight different cryopreservative solutions were prepared by the combination of three cryoprotectants: DMSO (ATCC), glycerol (Sigma Aldrich) and trehalose (Sigma Aldrich). A volume of 200 μl of microcapsules containing MSCs-Epo were resuspended in cryovials (Nalgene) with 1 ml of the different cryoprotective solutions previously prepared. The following solutions were tested: DMSO10%, DMSO5%, glycerol 10%, trehalose 10%, trehalose 5% + DMSO5%, trehalose 5% + DMSO 2.5%, trehalose 2.5% + DMSO2.5% and trehalose 2.5% + DMSO5%. Cryovials were frozen at -80ºC for one day on a Nalgene Cryo container and stored later on liquid N2 tanks for 2 weeks before performing any assay. Cryovials were thawed quickly at 37ºC until no ice was observed in the

solution. Cryopreserved microcapsules were rinsed three times with 10 ml of maintenance medium to remove the remaining cryoprotectant solution.

2.6. Osmotic resistance test

The swelling behavior of the microcapsules was determined after 1% citrate solution (w/v) treatment. Briefly, 100 µL of microcapsule suspension were mixed with 900 µL of PBS and placed in a 24-well plate. The 24-well cell culture plate was put in a shaker at 500 rpm and 37 °C (heater) for one hour. Subsequently, PBS supernatants were eliminated and 800 µL of sodium citrate solution was added. The 24-well plate was maintained at static conditions at 37°C for 24 h. The next day, the diameters of 20 microcapsules of each group were measured. The washing and shaking step with PBS were repeated at day 3 and 5. Meanwhile the encapsulated cells were maintained at static conditions at 37°C in the heater.

2.7. Viability assays

Two assays were performed to evaluate the viability of the cells inside the microcapsules: calcein/ethidium and CCK8 assay. For calcein/ethidium assay, ten microliters of microcapsules containing MSCs-Epo were rinsed three times with DPBS and plated into six 96 well-plates/ group. Samples were stained with LIVE/DEAD® Viability/Cytotoxicity Kit (Invitrogene ™). Briefly, 0.5 µM calcein AM and 0.5 µM ethidium homodimer-1 in DPBS were added to each well and incubated at room temperature for 40 minutes. All samples were observed under an Nikon TMS confocal microscope with the excitation/emission settings for calcein AM (excitation 495 nm, emission 515 nm) and ethidium homodimer (excitation 495 nm, emission 635 nm). At least three independent experiments were analyzed for each cryopreservant. Microscopy analysis was performed with Eclipse Net software, version 1.20.0. Representative micrographs were chosen randomly from all samples in each experiment.

For CCK8 assay, seven 96 single well-plate for each group of study were plated with a total of twenty microliters of microcapsules containing MSCs-Epo and ten microliters/ well of Cell Counting Kit-8 CCK-8 (Sigma Aldrich) were added to a final volume of 30 microliters. Plates were incubated inside a wet chamber for 4 hours at 37ºC and read on an Infinite M200 TECAN plate reader at 450 nm with reference wavelength at 650 nm. At least three independent experiments were analyzed for each cryopreservant.

2.8. ELISA Epo

Epo secretion was quantified in vitro prior- and post- cryopreservation. Six well

plates were plated with 106 MSC-Epo cells or 200 microliters of microcapsules containing MSC-Epo. Supernatants were collected at several timepoints (24 hours, and 1, 2, 3 and 4 weeks) and Epo concentration was assayed with the Quantikine IVD Epo ELISA kit (R&DSystems). All samples and standards were measured at least in duplicate. At least three independent experiments were analyzed for each cryopreservant and controls.

2.9. In vivo experiments

Syngenic six weeks old Balb/c mice were purchased from Janvier Labs and housed with sterile feed and autoclaved water. Six animals/cryopreservant or control were implanted subcutaneously with 300 μl of microcapsules containing MSC-Epo cells and suspended in Hank´s Balanced Salt Solution in a final volume of 1 ml using a 18-gauge catheter. During the procedure all animals were maintained under anesthesia by isofluorane inhalation. Cryopreserved microcapsules thawing and control non-cryopreserved microcapsules preparation was performed the same day of implantation. Blood samples were collected weekly by facial vein puncture with heparinized capillary tubes (Deltalab) and capillaries were spun down at 760xG for 15 minutes. Hematocrits were determined using a standard microhematocrit method and expressed as mean + standard desviation. All the experimental procedures were performed in compliance with protocols approved by the institutional animal care and use committee of the University of Basque Country UPV/EHU (Permit Number: CEEA/303/2013/HERNANDEZ MARTIN).

2.10. Statistics

Statistical analysis was performed using SPSS software, version 21.00.1. Data are expressed as means + standard deviation. $p< 0.05$ and $p < 0.001$ were considered significant for comparison of groups using ANOVA, Tukey's Post Hoc Test.

3. RESULTS

3.1. Characterization of genetically engineered murine MSCs-Epo

D1-MSCs were transduced with the lentiviral (LV) construct SIN-EF2-Epo-Pur as described above (see Material en Methods). SIN-EF2-Epo-Pur viral vector codifies Epo murine protein under EF1α promoter, linked to puromycin resistance through an IRES motif (Figure 1A). After one-month selection of MSCs-Epo with puromycin, cells were visualized and compared with D1-MSCs under inverted microscope. No morphological differences were detected between both cell types, showing similar growth patterns (Figure 1B). Next, MSCs-Epo were characterized by their ability to differentiate into the 3 mesoderm lineages,

osteoblasts, adypocytes and chondrocytes. Similarly to D1MSCs, two weeks in contact with osteogenic and adipogenic differentiation media were enough to identify osteoblasts and adipoblasts on the MSCs-Epo cultures (Figure 1C). Chondrocytes differentiation required 3 weeks in presence of chondrogenic medium, not detecting differences in the differentiation potential between D1MSCs and MSCs-Epo (Figure 1C). Secretion of Epo by MSCs-Epo was quantified and compared to D1MSCs after 24 hours, showing Epo levels of 2.8 + 0.16 UI/ml from 10^6 MSCs-Epo which confirms the insertion of the lentivirus codifying Epo at the correct reading frame (Figure 1D).

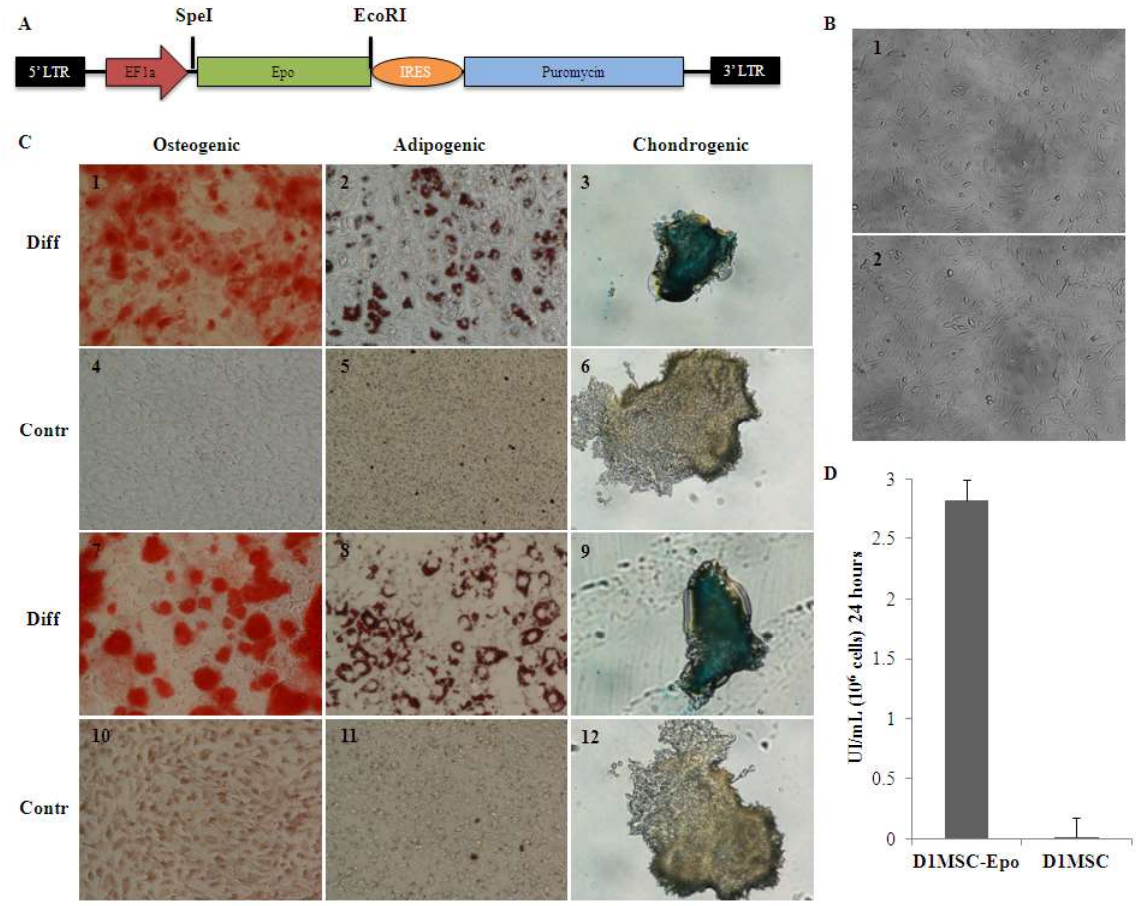

Figure 1: Characterization of engineered modified mesenchymal stem cells to express erythropoietin (Epo): A) Schematic lentiviral construct. B) Microscopic images of D1-MSCs (1) and MSCs-Epo (2). C) Lineage differentiation of D1 MSCs (1-6) and MSCs-Epo (7-12): Osteogenic differentiation images at 2 weeks and 20x amplification, adipogenic differentiation images at 2 weeks and 10x amplification and chondroigenic differentiation at 3 weeks and 10x amplification. D) Epo expression of 106 cells after 24 hours expressed in UI/ml. Note: Contr: cells with no differentiation medium; Diff: cells with the respective differentiation medium.

3.2. Effects of different cryoprotectants in morphology of alginate microcapsules

Eight formulations of cryoprotectants were tested with alginate microcapsules, following a slow-cooling freezing protocol (-80ºC /1 day and liquid N2): DMSO10%, DMSO5%, glycerol 10%, trehalose 10%, trehalose 5% + DMSO5%, trehalose 5% + DMSO 2.5%, trehalose 2.5% + DMSO2.5% and trehalose 2.5% + DMSO5%. The cryopreserved alginate microcapsules with the solutions containing trehalose 10%, trehalose 5% + DMSO 2.5%, trehalose 2.5% + DMSO2.5% and trehalose 2.5% + DMSO5% did not recover properly their original size and shape after thawing, showing the formation of wrinkles in the outer surface of the particles (Figure 2.F-H). In contrast, alginate microcapsules cryopreserved with DMSO10%, DMSO5%, glycerol 10% and trehalose 5% + DMSO5% recovered their original shape after rinsing and exposure to maintenance medium (Figure 2B-E). Next, we studied the mechanical stability of the microcapsules with a recovery like their original shape. Thus, we compared microcapsules stored in DMSO10%, DMSO5%, glycerol 10% and trehalose 5% + DMSO5% to those stored with trehalose 10% or not cryopreserved (Figure 2J) by means of a osmotic resistance test. The cumulative percentage of broken microcapsules cryopreserved with DMSO 10% was the same than those non cryopreserved while higher rates of broken microcapsules were observed with the remaining cryoprotectants. However, cryopreservation with trehalose 10% provided the group with higher rates of broken microcapsules under swelling test. The comparison between the initial diameters from microcapsules and the diameters after 3 days exposed to sodium citrate did not yield differences among the different cryoprotectants tested and the microcapsules non cryoprotected.

3.3. Cell viability and metabolic activity of cryoprotected microencapsulated cells

Cryoprotected microcapsules containing MSCs-Epo recovered similarly to empty microcapsules after thawing at the respective cryoprotectant solutions tested. DMSO 10% and trehalose 5% + DMSO5% formulations showed a good recovery acquiring the original shape after rinsing and exposure to maintenance medium. No differences were observed with the aforementioned cryoprotectant solutions when compared with non cryopreserved microcapsules containing cells. However, the cryopreservation with DMSO 5%, glycerol 10% and trehalose 10% did not maintain the original shape of the microcapsules after thawing, rinsing and exposure to maintenance medium (Figure 3A). Microencapsulated cells viability was tested by means of calcein/ethidium assay at 4 hours, 2 days and 1 week after thawing. DMSO10%, DMSO5%, and trehalose 5% + DMSO5% stained similarly to non cryoprotected microcapsules (control) (Figure 3B). However, the microencapsulated cells stored with glycerol 10% or trehalose 10% showed lower calcein and higher ethidium

staining, indicating a decrease on the cell viability compared to the non-cryopreserved microencapsulated cells.

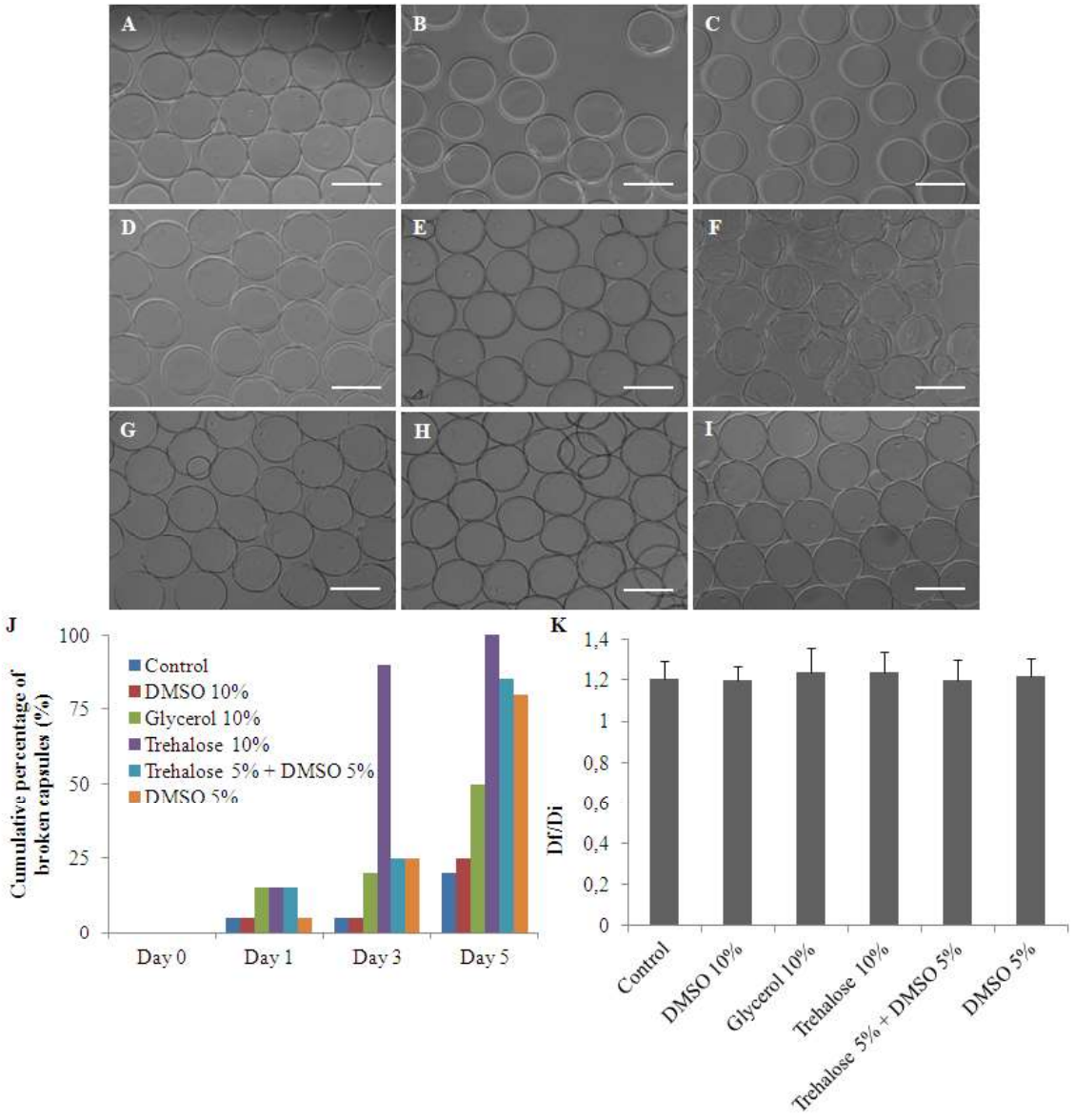

Figure 2: Microcapsules recovery after thawing with several cryoprotectants formulations: Microscopy images after thawing of cryopreserved microcapsules with: A) Non cryopreserved (control), B) DMSO10%, C) DMSO5%, D) Glycerol 10%, E) Trehalose 5% DMSO5% and F) Trehalose 10% G) Trehalose 5% DMSO 2.5%, H) Trehalose 2.5% DMSO2.5% and I) Trehalose 2.5% DMSO5%. Scale bar: 200 um. J) Cumulative percentage of broken microcapsules after 5 days of citrate treatment (swelling assay). K) Ratio (final diameter/initial diameter) from non broken microcapsules after 3 days exposed to sodium citrate.

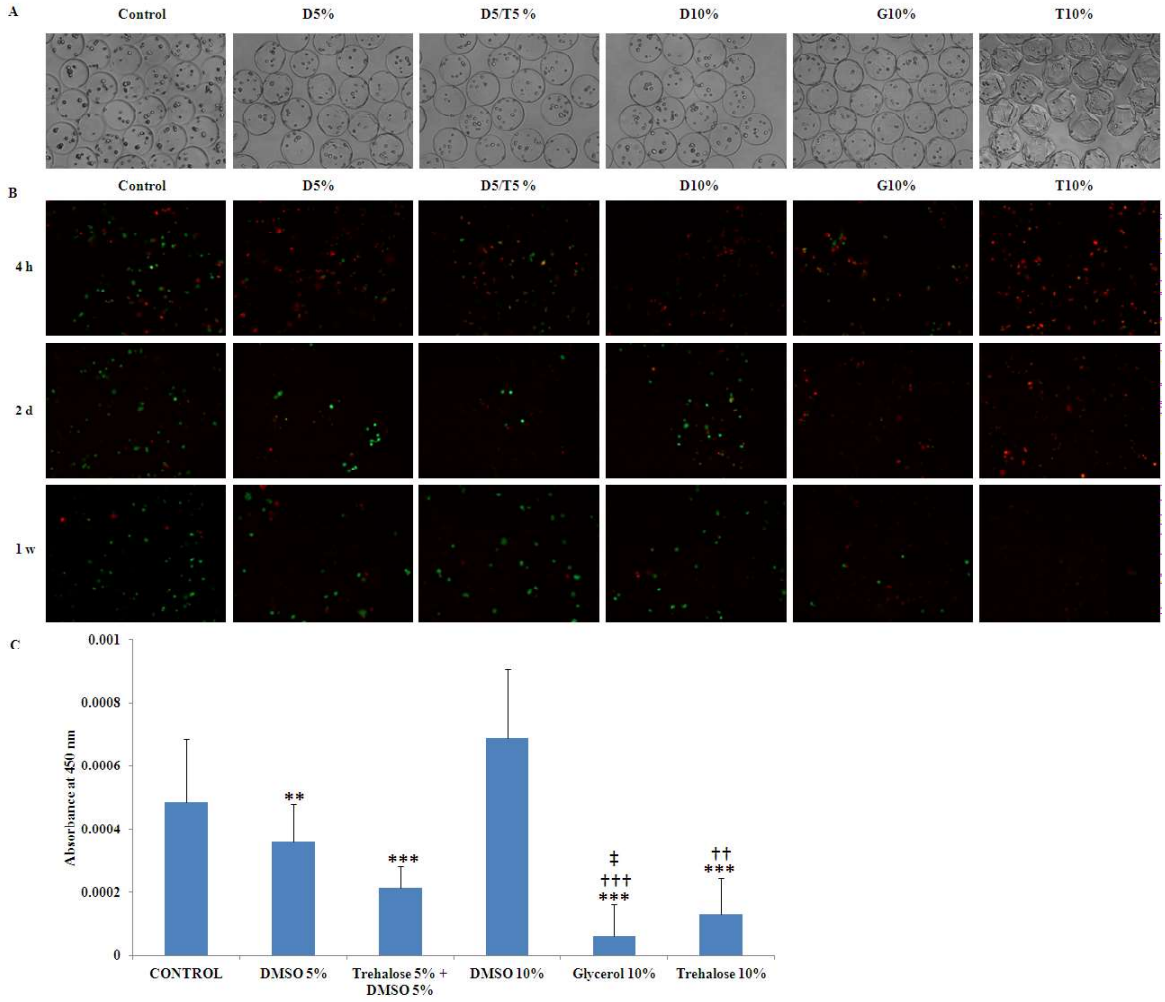

Figure 3: Cryopreserved microencapsulated MSCs Epo with different cryoprotectants formulations viability and metabolic activity after thawing. A) Microscopy images of capsules after thawing. B) Calcein/ethidium microscopy images at 4 hours (4h), 2 days (2d) and 1 week (1w) after thawing. Note: Control: non cryopreserved microcapsules; D5%: DMSO5%; D5/T5%: DMSO 5% + trehalose 5%; D10%: DMSO 10%; G10%: glycerol 10% and T10%: trehalose 10%. C) Graphical representation of metabolic activity measured by means of CCK8 assay for the different cryoprotectants formulations 1 week after thawing. Note: *: compared to DMSO 10%, †: compared to control and ‡ compared to DMSO 5%. ***: $p<0.001$, **: $p< 0.01$ and *: $p< 0.05$.

Metabolic activity was quantified by CCK8 assay 1 week post-thawing. The highest metabolic activity values were detected on the non-cryopreserved microencapsulated cells and the ones stored with DMSO 10% with no significant statistical differences ($p>0.1$) (Figure 3C). The microencapsulated cells cryopreserved with the remaining cryoprotectant formulations showed significantly lower metabolic activity than DMSO10% ($p<0.05$) at one week. The cryoprotectant solutions trehalose 10% and glycerol 10% displayed significant differences ($p<0.05$) compared to non-cryoprotected microencapsulated cells (controls), but no compared to DMSO 5% or trehalose 5% + DMSO 5%. Finally, microencapsulated cells

cryopreserved with DMSO5% exhibited significant higher metabolic activity ($p<0.05$) than those cryopreserved with glycerol 10%.

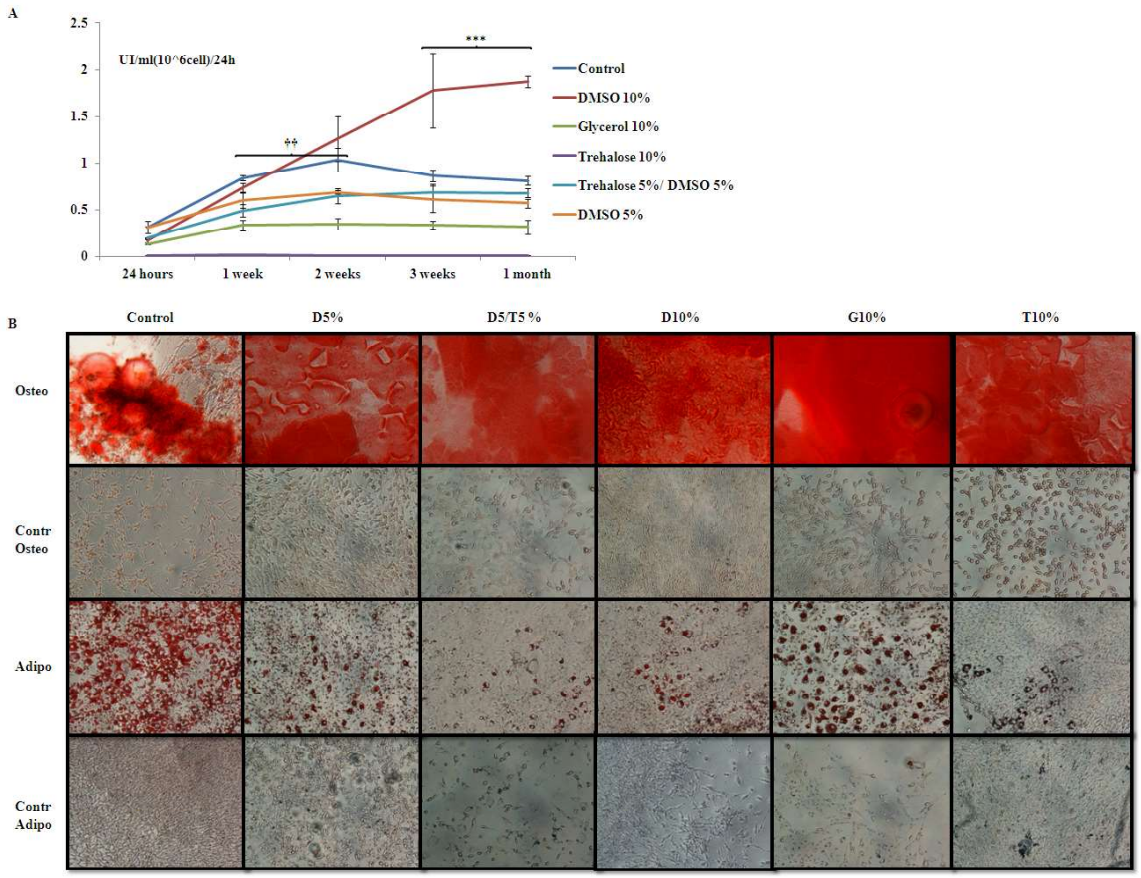

Figure 4: Transgene expression and secretion, and differentiation potential of cryopreserved microencapsulated MSCs-Epo after thawing. A) Epo expression and secretion during time of cryopreserved microencapsulated MSCs-Epo with different cryoprotectant formulations after thawing. Note: ***: $p<0.001$ compared to DMSO 10% and ††: $p< 0.01$ compared to control. B) Differentiation potential of cryopreserved microencapsulated MSCs-Epo with different cryoprotectant formulations after thawing. Microscopic images at 10 x amplification 3 weeks after differentiation. Note: Control: non cryopreserved microcapsules; D5%: DMSO5%; D5/T5%: DMSO 5% + trehalose 5%; D10%: DMSO 10%; G10%: glycerol 10% and T10%: trehalose 10%. Osteo: osteogenic differentiation; Contr Osteo: non osteogenic differentiation (staining control); Adipo: adipogenic differentiation and Contr Adipo: non adipogenic differentiation (staining control).

3.4. Epo secretion and differentiation potential of cryoprotected microencapsulated cells

Secretion of Epo by cryopreserved encapsulated MSCs-Epo was quantified at several time points after thawing (Figure 4A.). Non cryopreserved encapsulated MSCs-Epo showed higher Epo secretion ($p<0.001$) 24 hours after thawing than all the cryopreserved samples except those cryopreserved with DMSO5%. However, differences between samples

widened 1 week after thawing. Microencapsulated cells cryopreserved with trehalose 10% and glycerol 10% did not increase Epo secretion at the same rate, showing significant differences with the control and the remaining cryopreserved samples ($p<0.01$). Although cryopreserved samples increased Epo secretion 1 week after thawing, non cryopreserved microcapsules still released higher concentrations of Epo ($p<0.01$). However, 2 weeks after thawing, those microcapsules cryopreserved with DMSO 10% doubled their Epo release showing significant differences compared to the remaining cryopreserved samples ($p<0.001$), except with the non cryopreserved. These control samples still showed higher Epo secretion when compared to the remaining cryopreserved samples ($p<0.01$). Three and four weeks after thawing, microencapsulated MSCs-Epo and those cryopreserved with DMSO 10% increased Epo released showing significant differences even with the control samples ($p<0.001$) and reaching values of 1.87 + 0.06 UI/ml (106 cells)/24 hours. Microencapsulated MSCs-Epo and those cryopreserved with DMSO 5% or trehalose 5% + DMSO 5% did not show significant differences ($p>0.05$) with the non cryopreserved samples three weeks after thawing, while those cryopreserved with glycerol 10% or trehalose 10% showed significant differences ($p<0.01$), following the same trend one week later.

The mesenchymal potential of cryopreserved and microencapsulated cells was tested after thawing and culturing with osteogenic and adipogenic differentiation media for 3 weeks. After three weeks with osteogenic differentiation medium, a calcified matrix was observed at the bottom of the wells with all the cryoprotectants solutions assessed (Figure 4B). With adipogenic differentiation medium, the presence of vacuoles were detected after staining with almost no qualitative differences among all the cryoprotectants solutions tested (Figure 4B).

3.5. Hematocrit levels of syngeneic mice after cyroprotected microencapsulated MSCs-Epo subcutaneous implantation

Based on in vitro viability, metabolic activity and Epo production, we compared the subcutaneous implantation of cryoprotected encapsulated MSCs-Epo with the following formulations: DMSO 10%, DMSO 5% and DMSO5%+ trehalose 5% (Figure 5). Cryoprotected encapsulated MSCs-Epo with these cryoprotectant formulations were compared to non cryoprotected encapsulated MSCs-Epo. After one week, the hematocrit increased in mice implanted with non cryoprotected encapsulated MSCs-Epo and cryoprotected encapsulated MSCs-Epo with DMSO 10%. The aforementioned formulations showed significant differences on the hematocrit compared to the mice implanted with cryoprotected encapsulated MSCs-Epo with either DMSO 5% or DMSO5% + trehalose 5%

($p<0.001$). The significant differences between these groups extended up to 6 weeks when mice were euthanized. Mice implanted with DMSO 5% or DMSO5% + trehalose 5% did not show any increase on hematocrit levels compared with sham controls.

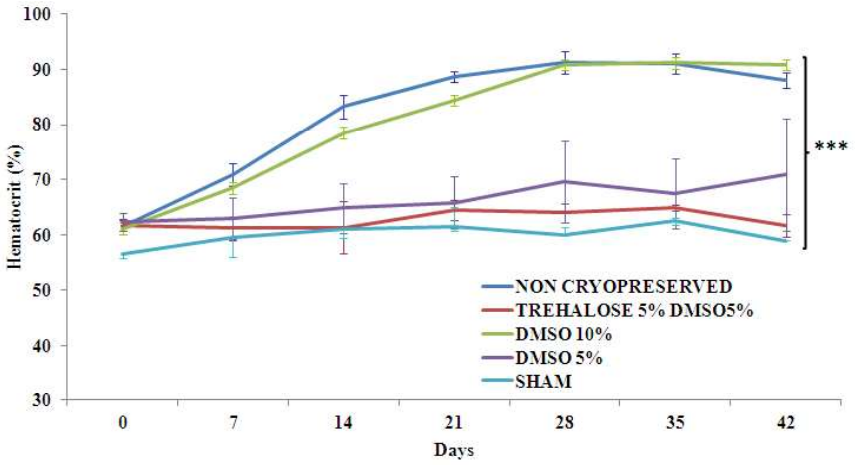

Figure 5: Hematocrit levels of implanted Balb/c mice with cryopreserved microencapsulated MSCs-Epo. Evaluation of non-cyropreserved microcapsule implantation vs cryopreserved microcapsules for 45 days. Sham control: PBS. Values represent mean + SD. Note: ***, $p<0.001$.

4. DISCUSSION

Compared to traditional pharmacological therapies, encapsulated cells provide the advantage of being a source of sustained continuous release of "de novo" therapeutic products. This capacity avoids the need of repeated drugs administration. As a consequence, microencapsulated cells are being evaluated in a wide range of pathologies. However, bearing in mind clinical translation, efficient storage procedures are needed in order to get therapeutic encapsulated cells on demand. Cryopreservation represents currently the best method for long-term cell preservation and it has been extensively used on research labs as well as for several clinical therapies. Cryopreservation has been applied to many cell types including different stem cell types such as human embryonic stem cells, induced pluripotent stem cells, hematopoietic stem cells or mesenchymal stem cells [37], representing an important step in the translation of cell therapies into the clinic within the regenerative medicine field. Cryopreservation methods reach low temperatures in order to avoid the damage caused by the ice formation during freezing. However, they are still associated with some cell membrane damage and induction of apoptosis if they are applied directly without any additional agent [38,39]. Hence, cryopreservation has relied on coating cells with agents, termed cryoprotectants, in order to maintain their viability. The mechanisms through which

these agents act are not well known and they are classified based on their cell permeability as penetrating or non-penetrating cryoprotectants. Since the election of the most appropriate cryoprotectant for each cell type needs to be empirically studied, similar studies should be done when cryopreservation is applied to microencapsulated cells.

In this study, we transfected mesenchymal stem cells to permanently express the therapeutic protein erythropoietin generating a suitable cell model for the determination of the best cryoprotectant solution on the storage of this cell type after microencapsulation. Our group has previously used transfected C2C12 myoblasts expressing erythropoietin and determined that slow cooling is the best freezing protocol for microencapsulated myoblasts long-term storage [35]. After having determined the best protocol for cryopreserving encapsulated cells in terms of time and temperature, the study of other parameters influencing the viability and metabolic activity of the microencapsulated cells, such as the effect of different cryoprotectant solutions, still remains unclear. Therefore, we moved one step forward and compared the most often used cryoprotectants in clinical studies (DMSO, glycerol and trehalose) on microencapsulated cells. We chose to study the best cryoprotectants for microencapsulated mesenchymal stem cells due to their advantages on immunomodulation after transplantation and their potential future in clinical translation. Other groups have confirmed that slow cooling protocols with encapsulated mesenchymal cells combined with DMSO provides viabilities and metabolic rates after thawing similar to non cryopreserved cells [40], but they failed to compare the effect of different cryoprotectant solutions.

In our study, the non-penetrating cryoprotectant trehalose did not provide appropriate physical recovery of empty alginate microcapsules. Instead, microcapsules presented wrinkles formation similarly to studies where microcapsules containing human retinal pigment epithelial cells ARPE-19 and stored with trehalose by freeze drying [41]. Even, microcapsules stored with trehalose 10% were broken after exposition to the extreme conditions at the swelling test although intact capsules did not showed differences from the initial diameter after the test. The surface wrinkle formation after thawing could be due to the described interaction between trehalose and poly-L-lysine (PLL) that modifies the original secondary structure of PLL and it is reflected on changes of the trehalose FT-IR bands [42]. However, 5% trehalose combined with 5% of the penetrating cryoprotectant DMSO improved the recovery and the osmotic resistance after thawing. In fact, wrinkles were no detected on capsule surface. Also, other penetrating cryoprotectants, such as glycerol, formed wrinkles on microcapsules after thawing, similar to the results reported with alginate poly-l-lysine microcapsules loaded with bifidobacteria [43]. However, even when wrinkles are formed in the microcapsules surface freeze dried with trehalose, the viability of loaded ARPE-19 cells was not affected after reconstitution [41]. Therefore, we tested trehalose effect on

cryopreserved encapsulated MSCs-Epo viability, metabolic activity, transgene expression and secretion, and differentiation potential after thawing.

It has been previously reported that MSCs keep their differentiation potential to osteogenic, adipogenic and chondrogenic lineages after microencapsulation [44], even when they are cryopreserved with DMSO 10% [40]. In this sense, we studied other formulations (DMSO 5%, DMSO 5% + trehalose 5%, glycerol 10% or trehalose 10%,) not observing almost differences on the differentiation capacity of MSCs-Epo although less differentiation potential could be detected with some formulations. Therefore, these results indicate that the study of the differentiation potential of microencapsulated MSCs might not be the best assay to identify the most accurate cryoprotectant solution. However, cell viability and metabolic activity do depend on the cryoprotectant used. Formulations of trehalose 10% or glycerol 10% dramatically decreased encapsulated MSCs-Epo viability, maybe due to the formation of wrinkles in the microcapsule surface which could affect the exchange of oxygen, nutrients or cell waste with the medium. However, the combination of trehalose 5% with DMSO 5% is able to increase cell viability and metabolic activity compared to the aforementioned formulations, showing that the presence of the penetrating cryoprotectant DMSO improves the]recovery of the encapsulated MSCs-Epo. It should also be taken into account the concentration of the cryoprotectant agent, since a reduction from 10% to 5% of DMSO results in lower viability and metabolic activity on encapsulated MSCs-Epo [40]. Our research group has previously observed a 40% decrease on Epo release when encapsulated C2C12 myoblasts secreting Epo were cryopreserved with DMSO 10%, and a 80% decrease when were cryopreserved with DMSO 5% [35]. Similarly, two weeks after thawing, we have detected a 50% reduction on Epo release from cryopreserved encapsulated MSCs-Epo with DMSO 10% compared to those cryopreserved with DMSO 5%. This difference on Epo secretion reduction amongst these formulations increased over the time after 2 weeks post-thawing.

We have observed how the secretion of the therapeutic molecule from MSCs-Epo decreased after encapsulation since this glycoprotein needs to go across the alginate matrix to be completely released. Similar results were described after the encapsulation of MSCs expressing the therapeutic molecule hemopexin-like protein (PEX). However, the reduction on PEX secretion by the implanted encapsulated MSCs did not alter their capacity to reduce the sizes of the tumors in the animal models tested [44]. In this study, we have detected a reduction from 2,8 ± 0.16 UI/ ml (106 cells)/ 24hours to 0.30 ± 0.06 UI/ml (106 cells)/24hours after MSCs-Epo encapsulation. Similarly to PEX, this reduction in Epo release did not affect the ability of encapsulated MSCs-Epo to increase the hematocrit of mice after their implantation. However, the outcomes in vivo due to the reduction on the release of other

therapeutic agents after cell microencapsulation should be specifically assessed. Moreover, cell proliferation seems to enhance over the time after cryopreservation with DMSO 10% compared to non cryopreserved and, therefore, the release Epo reaches higher rates than non cryopreserved microcapsules. These results go in accordance to studies with non encapsulated MSCs which have shown an enhanced proliferation rate of MSCs cryopreserved with DMSO compared to non cryopreserved MSC. In these studies, authors showed that the proliferation rate of post-cryopreserved MSC after 1 month storage was significantly higher than that of unfrozen control cells, despite less adhesion after plating of cryopreserved cells compared to unfrozen cells the next day after plating. Authors proposed a cell selection of ''stronger'' cells after cryopreservation [45], a hypothesis that can explain the results described on this paper. Moreover, authors indicated that future clinical studies, when MSC transplantation is planned immediately tests of cell viability should be previously performed.

Our in vitro results can be extrapolated to in vivo outcomes on hematocrit percentage when cryopreserved microencapsulated MSCs-Epo are implanted. In fact, we have shown that encapsulated MSCs-Epo cryopreserved with DMSO 10% are able to increase the hematocrit similarly to no cryopreserved MSCs-Epo during the time studied, while those ones cryopreserved with DMSO 5% are not. However, since the Epo amount secreted by our non cryopreserved microcapsules is enough to increase up to the highest murine hematocrit levels, we were not able to observe differences on the hematocrit values between mice implanted with non cryopreserved microcapsules and cryopreserved with DMSO10%, even when there is a twofold increment on in vitro Epo release. Similar studies should be performed for the cryopreservation of different microencapsulated cell types. Moreover, in order to translate cell microencapsulation into the clinic, long term (from months to years) studies covering two different aspects will be needed: first, studying the effect on microencapsulated cells of longer periods of storage than the assessed on this paper and second, assessing the maintenance of the biological properties of microencapsulated cells during a longer period of time (more than one month) after thawing. This might be one way that microencapsulated cells can be ready on demand for clinical treatment.

5. CONCLUSION

The present research work suggests that DMSO 10% is the most suitable cryoprotectant solution, among the assessed ones, for the slow cooling cryopreservation of microencapsulated mesenchymal stem cells. Moreover, it has been demonstrated that storage with non-penetrating cryoprotectants such as trehalose do not provide an appropriate recovery for microencapsulated cells. Altogether, this work indicates the importance of the proper selection of the most suitable cryoprotectant solution and its concentration before the

use of microencapsulated cells can be translated into the clinic.

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Low molecular-weight hyaluronan as a cryoprotectant for the storage of microencapsulated cells


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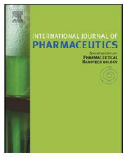

Low molecular-weight hyaluronan as a cryoprotectant for the storage of microencapsulated cells

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ABSTRACT

The low-temperature storage of therapeutic cell-based products plays a crucial role in their clinical translation for the treatment of diverse diseases. Although dimethylsulfoxide (DMSO) is the most successful cryoprotectant in slow freezing of microencapsulated cells, it has shown adverse effects after cryopreserved cell-based products implantation. Therefore, the search of alternative non-toxic cryoprotectants for encapsulated cells is continuously investigated to move from bench to the clinic. In this work, we investigated the low molecular-weight hyaluronan (low MW-HA), a natural non-toxic and non-sulfated glycosaminoglycan, as an alternative non-permeant cryoprotectant for the slow freezing cryopreservation of encapsulated cells. Cryopreservation with low MW-HA provided similar metabolic activity, cell dead and early apoptotic cell percentage and membrane integrity after thawing, than encapsulated cells stored with either DMSO 10% or Cryostor 10. However, the beneficial outcomes with low MW-HA were not comparable to DMSO with some encapsulated cell types, such as the human insulin secreting cell line, 1.1B4, maybe explained by the different expression of the CD44 surface receptor. Altogether, we can conclude that low MW-HA represents a non-toxic natural alternative cryoprotectant to DMSO for the cryopreservation of encapsulated cells.

Keywords: Dimethylsulfoxide (DMSO), hyaluronan, cryoprotectant agent (CPA), slow freezing, cell encapsulation, CD44 surface receptor

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1. INTRODUCTION

Cell encapsulation technology is being used as a sustained drug or cell delivery system to treat diseases such as diabetes, cancer, neurodegenerative and hepatic diseases [1-4]. The 3D design of microcapsules permits the exchange of nutrients between the environment and the core, allowing the release of de "novo" produced therapeutic molecules by the entrapped cells. Moreover, the outer layer of the microcapsules functions as a barrier to the recognition by the immune system, preventing the entrance of immunoglobulins and thus, avoiding the immune rejection after encapsulated cells implantation [5]. Although diverse clinical trials have been performed with the implantation of encapsulated cells [6,7], there are still several aspects that should be optimized to reach the clinics [8]. One of the aspects that need improvement is a correct storage, shipment and delivery of encapsulated cells, facilitating the "on demand" access of patients in the clinics far from manufacturer and providing a bank of regulatory-tested cells for future applications [9,10] .

The preservation at deep cryogenic temperatures (ranging from -196°C to approximately -150°C) in which cell-based products are mixed with one or more cryoprotectant agent (CPAs), is so far the only technology that enables the storage of encapsulated cells. Currently, the most used method is the slow freezing [11], in which samples are controlled cooled (for mammalian cells -1˚C/minute) using low concentrations of CPAs (5-20%). In slow freezing, several factors result critical for optimal outcomes, such as samples preparation to freezing, cooling protocol, storage, thawing and CPAs nature, among others. Regarding CPAs, they can be classified into permeant and non-permeant. Permeant CPAs displace the inner water from the cell and prevent intracellular ice crystal formation. On the other hand, non-permeant CPAs speed up the dehydration process of the cells in supra-zero temperatures, reducing the CPA toxicity during the slow freezing [12]. Up to now, the most used CPA is dimethylsulfoxide (DMSO). Many studies have shown good results with DMSO in the cryopreservation of encapsulated cells, restoring cell function efficiently after thawing [3,13,14]. Our group has previously demonstrated that the slow freezing procedure with DMSO 10% is a suitable strategy for long-term storage of microencapsulated C2C12 [14] and D1 mesenchymal stem cells (D1MSCs) [15]. However, DMSO toxicity can be harmful to some cell types [16,17], and cryopreservation of cells with DMSO causes post-transplantation complications [18,19], questioning its clinical use [20]. For that reason, many research groups are combining DMSO with non-permeating CPAs, such as sucrose or trehalose, enabling the decrease of DMSO percentage in the CPA solution [21,22]. Other CPAs combinations without DMSO have also been used in different cell types, demonstrating similar cryoprotective properties to DMSO [23,24].

Hyaluronan (HA) has been postulated as a natural non-toxic CPA. HA is an anionic non-sulfated glycosaminoglycan, with molecular weight (MW) ranging from 10^4 to 10^6 Da, distributed widely throughout several tissues in mammalians. It is integrated by the repetition of a disaccharide unit of a N-acetylglucosamine and a β-glucuronic acid, and interacts with cells through the CD44 surface receptor [25]. HA has been described for being involved in a wide variety of biological procedures (e.g., cell signaling mediation, regulation of cell adhesion and proliferation) [26], and due to its favorable mechanical properties, its use has been extensively promoted in tissue engineering over the last decades [27-29]. Moreover, HA possess low cytotoxicity and high hydration capability, which makes it a good CPA candidate. In fact, low MW HA (MW $3x10^4$-$5x10^4$ Da) is able to preserve cell viability of several cells after thawing, thanks to its extracellular and intracellular protective effect. Two different approaches have been described using HA in slow freezing. On the one hand, HA has been combined with DMSO showing an improvement of the cell outcome after thawing. Thus, pre-incubation of human dermal fibroblast with low MW-HA 0,5%, followed by freezing with DMSO 10%, displayed better cell viability than cells cryopreserved only with DMSO 10% [30]. Similarly, the viability and cell proliferation of human MSCs were improved after cryopreservation when HA was mixed with DMSO in the CPA solution, ratifying its possible use as a CPA [31]. On the other hand, pre-incubation with low MW-HA 0,5% followed by freezing with low MW-HA 5%, also showed a cryoprotective effect reducing cell damage after thawing [32].

Based on the aforementioned cryoprotective outcomes of low MW-HA as a natural non-toxic CPA for cells, we hypothesized that it could represent a good CPA candidate for the cryopreservation of microencapsulated cells. In the present work, first we optimized the conditions of encapsulated cells cryopreservation with low MW-HA, assessing the optimal pre-incubation equilibrium time of low MW-HA with cells or encapsulated cells. Next, we describe the beneficial outcomes of cryopreserving several encapsulated cell types with low MW-HA, following the optimal conditions assessed. Finally, we correlate the outcomes displayed by certain encapsulated cell types with their surface receptor CD44 expression.

2. MATHERIAL AND METHODS

2.1. Cell culture

D1-mesenchymal stem cells (D1MSCs) were purchased from ATCC (ATCC CRL12424) and grown in Dulbecco's modified Eagle's medium (DMEM) (ATCC 30−2002) supplemented with 10% fetal bovine serum (FBS), 1% penicillin/streptomycin, and 12.5 μg/mL puromycin. Murine C2C12 myoblasts were grown in T-flasks with Dulbecco´s

modified Eagles´s medium supplemented with inactivated 10% FBS, 2 mM L-glutamine and 1% antibiotic/antimycotic solution. The human insulin-secreting cell line 1.1B4 was purchased from Sigma Aldrich and grown in T-flasks with RPMI 1640 culture medium supplemented with 10% FBS, and 1% penicillin/streptomycin. The human retinal pigment epithelial cell line Arpe-19 was purchased from ATCC (ATCC®CRL-2302) and grown in T-flask with DMEM:F12 (ATCC 30-2006) supplemented with 10% FBS, and 1% penicillin/ streptomycin. All the cell lines were plated in T-flasks, maintained at 37°C in a 5% CO2/ 95% air atmosphere, and passaged every 2−3 days. Unless other company is indicated, all reagents were purchased from Gibco (Life Technologies, Spain).

2.2. Cell microencapsulation

All cell lines were immobilized into alginate microcapsules using an electrostatic droplet generator (Nisco ®). Briefly, cells were suspended in 1.5% (w/v) low viscosity and high guluronic (LVG) alginate sterile solution (Novamatrix®), obtaining a cell density of $5x10^6$ cells/mL alginate. Suspensions were extruded in a sterile syringe through a 0.17 mm needle at a 5.9 mL/h flow rate, using a peristaltic pump. The resulting alginate beads were maintained in agitation for 10 min in a CaCl2 solution (55 mM) for complete ionic gelation. Microcapsules were prepared at room temperature, under aseptic conditions and cultured in complete medium. The diameter (380 μm) and overall morphology of the microcapsules were assessed using an inverted optical microscope (Nikon TSM).

2.3. Cryopreservation

Three different CPA solutions were employed in this study: Cryostor10® (CS10), DMSO 10% and Sodium Hyaluronate (Low MW-HA, mean MW: $3,5x10^4$-$5x10^4$ Da (Contipro)) at 5%, freshly dissolving the last two CPAs in the respective cell line maintenance medium. A volume of 200 μL of microencapsulated cells (10^6 cells) was resuspended in 1 mL of each cold CPA solutions within cryovials (Nalgene). The cryopreservation procedure consisted on 20 minutes on ice at 0-4 Cº, overnight at -80ºC within a CoolCell (Biocision) container and a final storage in liquid N2 tanks for three weeks.

The influence of low MW-HA 0,5% was studied in attached D1MSC and C2C12 cells before encapsulation, incubating cells with HA in each maintenance cell line medium for 45 minutes, 2, 6 and 24 hours. Next, they were cryopreserved in cryovials with DMSO 10% or HA 5% CPA solutions following the aforementioned slow freezing protocol.

Before performing any assay, cryovials were thawed quickly at 37 °C until no ice was observed in the solution. For CPA solution removal, fresh medium was added slowly to

inhibit osmotic damage, allowing microcapsules to decant by gravity. Next, microencapsulated cells were rinsed twice with 10 ml of maintenance medium to remove any remaining CPA solution.

2.4. Cell viability and metabolic activity assessment

Metabolic activity from thawed encapsulated cells was quantified by means of Cell Counting Kit-8 (CCK-8) (Sigma-Aldrich). Briefly, 6 µl of microcapsules per well were placed in 100 µl of medium on 96-well plates. Afterward, 10 µl of Cell Counting Kit-8 (CCK-8) solution (Sigma-Aldrich) were added to each well and incubated for 4 hours at 37°C. Samples were read on an Infinite M200 (TECAN Trading AG, Switzerland) microplate reader at 450 nm, with reference wavelength set at 650 nm. At least five wells per sample and three independent experiments were analyzed for each condition.

Cell membrane integrity was assessed by quantifying the release of lactic dehydrogenase (LDH) with the In Vitro Toxicology LDH based Assay Kit (Sigma-Aldrich) from supernatants 1 and 7 days after thawing cryopreserved encapsulated cells. Briefly, 50 µl of microcapsules were incubated with 500 µl of medium for 24 hours, next collecting supernatants to determine the released LDH. Simultaneously, other 50 µl of microcapsules were incubated with 500 µl of medium for 24h hours and next lysed to determine the total LDH activity. All supernatants were placed in 96 well plates and subjected to the reduction of NAD by LDH and further reaction with tetrazolium dye following manufacturer's recommendations. The resulting colored compound absorbance was read out on an Infinite M200 microplate reader at a wavelength of 490 nm, with absorbance reading at 690 nm as background. All measurements were conducted in triplicates, with at least three independent experiments per condition.

Early apoptosis of microencapsulated cells was quantified by flow cytometry with the Annexin-V-FITC Apoptosis Detection Kit (Sigma-Aldrich) at day 1 and 7 after thawing. Briefly, cells were released by incubation with sodium citrate 1% in mannitol for 5 minutes, next, rinsing twice with DPBS and resuspending in 10 mM HEPES/NaOH containing 0.14 M NaCl and 2.5 mM CaCl2 (binding buffer, pH 7.5). Cell suspension was stained with annexin V-FITC and propidium iodide for 10 minutes at room temperature protected from light. Fluorescence was quantified immediately with a BD FACS CaliburTM flow cytometer (BD Biosciences). The appropriate acquisition parameters were established with unstained samples or samples stained only with annexin V-FITC or propidium iodide. All quantifications were conducted in triplicates, with at least three independent experiments.

The cell viability of cryopreserved microencapsulated cells was quantified by flow

cytometry, with the LIVE/DEAD® Viability/Cytotoxicity Kit (Life Technologies) at day 1 and 7 after thawing. Cells were released again with sodium citrate at 1% in mannitol for 5 minutes and stained with 100 nM calcein AM and 8 μM ethidium homodimer-1 solution for 20 minutes at room temperature, protected from light. Fluorescence was determined immediately in a BD FACS Calibur flow cytometerTM with unstained samples or samples stained only with 100 nM calcein AM or 8 μM ethidium homodimer-1 as controls establishing the appropriate acquisition parameters. All the quantifications were conducted in triplicates, with at least three independent experiments.

Cell viability was confirmed by fluorescence microscopy. Cryopreserved microcapsules were rinsed three times with DPBS and mixed with an optimal previously determined concentration of dies (0.5μM calcein AM and 0.5μM ethidium homodimer-1 in DPBS). After incubation at room temperature for 40 minutes in the dark, samples were observed under a Nikon TMS microscope with excitation/emission settings for calcein AM of 495/515 nm and ethidium homodimer of 495/635 nm. At least three independent experiments were analyzed for each condition.

2.5. CD44 expression analysis

CD44 expression was quantified by flow cytometry from single cell suspensions. After blocking FcγII/III receptors with CD16/32 antibody, cells were incubated with CD44-PECy5 (clone BJ18, Biolegend) for 15 minutes at 4°C protected from light. After rinsing, cells were resuspended in 6μM DAPI to exclude dead cells. Unstained cells were used to evaluate autofluorescence, while cells stained with mouse IgG1, K-PE-Cy5 were used as isotype control. Fluorescence was quantified by means of BD FACS CaliburTM flow cytometer (BD). At least three independent experiments were performed for each quantification.

2.6. Statistics

Statistical analysis was performed using SPSS software, version 21.00.1. Data are expressed as means standard deviation. $p < 0.05$ and $p < 0.001$ were considered significant for comparison of groups using ANOVA, Tukey's Post Hoc and Kruskal-Wallis H test.

3.RESULTS AND DISCUSSION

3.1. Low MW-HA displays similar cryoprotective effect than DMSO.

DMSO is a suitable CPA in the cryopreservation of microencapsulated cells [14,15], but its adverse effects after transplantation encouraged us to continue in the research of novel CPAs for the cryopreservation of encapsulated cells. Low MW-HA is able to preserve human

dermal fibrotic monolayers [30,32], and provide substitute structure-stabilizing H-bonds useful in the lyophilization of biological materials, such as liposomes [33]. Based on these evidences, we aimed to assess if low MW-HA displays similar cryoprotective effects than DMSO with microencapsulated D1 mesenchymal stem cells (D1MSCs) or myoblasts (C2C12). To that end, we incubated on ice with low MW-HA both encapsulated cell lines for 20 minutes, 1, 2 and 4 hours before their slow freezing. Then, we quantified their metabolic activity in culture, 1 and 7 days after thawing.

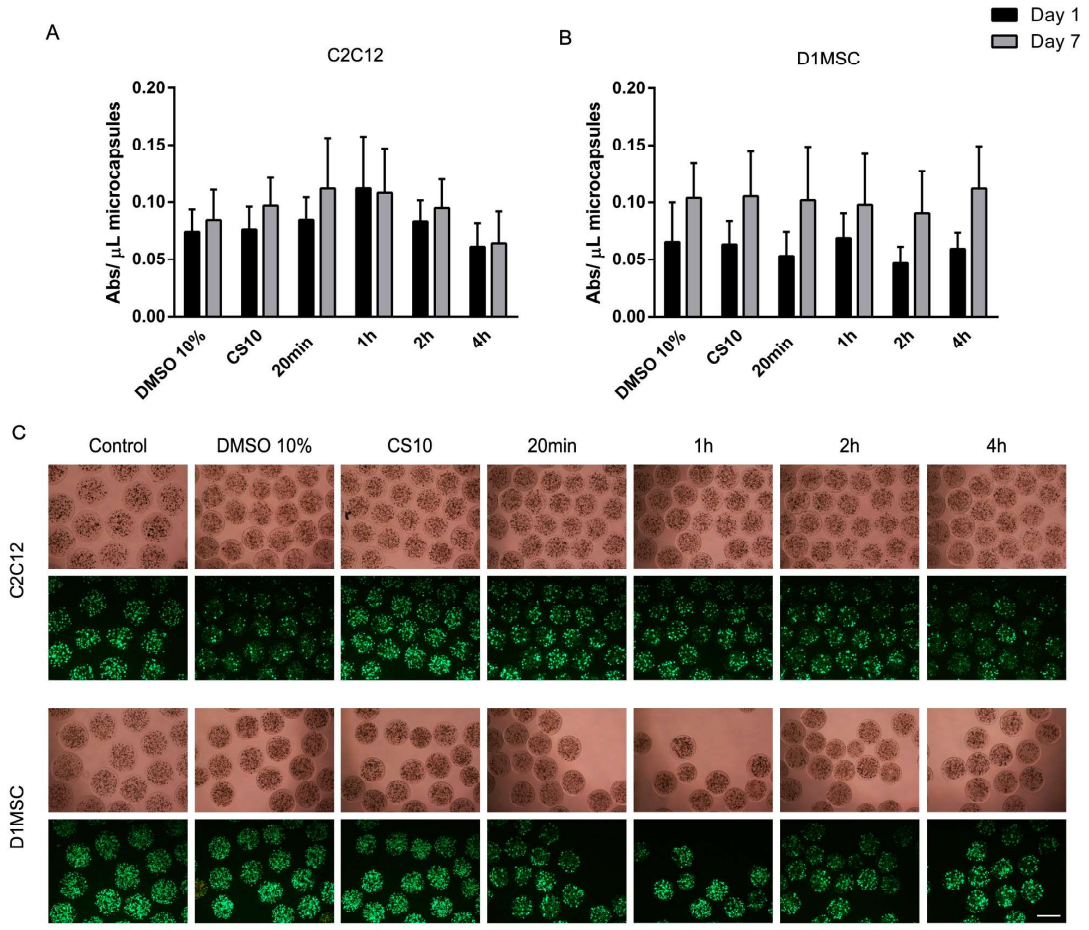

Figure 1. Determination of low MW-HA equilibration time before cryopreservation by comparison with DMSO and CS10. Metabolic activity 1 and 7 days in culture after thawing of microencapsulated C2C12 (A) and D1MSC (B) cells, cryopreserved by pre-incubation at 4ºC for 20 minutes, 1, 2 and 4 hours. C) Micrographs of calcein/ethidium stained cryopreserved microencapsulated C2C12 and D1MSC cells 7 days in culture after thawing. Note: Control: non-cryopreserved bioscaffolds. Values represent mean ± SD. Scale bar: 400µm

Encapsulated C2C12 stored with low MW-HA 5% did not show statistical metabolic activity differences 1 and 7 days in culture after thawing, independently of the incubation time at 4ºC, when zcompared with encapsulated C2C12 cryopreserved with DMSO 10% or

the commercial CPA solution called CS10, an anmal componet-free that contains also DMSO 10% (Figure 1 A). Similarly, encapsulated D1MSCs did not show statistical differences with DMSO 10% and CS10 after following the same study than encapsulated C2C12 cells (Figure 1 B). Interestingly, although without statistically significances, the metabolic activity decreased in encapsulated C2C12 from 1 hour to 4 hours of equilibration with low MW HA 5% at 4ºC, reflected in the number of viable cells stained by calcein/ethidium (Figure 1 C). With these results, we first demonstrated that low MW-HA retains the same metabolic activity than DMSO 10% or CS10 after thawing in both cryopreserved encapsulated cells, and second, that this effect is achieved with just 20 minutes equilibration time on ice. In this sense, this short period of incubation time indicates that low MW-HA acts outside the cell since biological mechanisms require longer periods than 20 minutes to internalize HA, especially when the cell metabolic activity is slowed down by ice. This hypothesis is supported by the fact that periods longer than 20 minutes are required for the internalization of HA into B16-F10 melanoma cells at 37°C, and prolonged even more with incubations at 4°C [34]. On this regard, low MW-HA could exert its extracellular cryoprotective effect by acting as a non-permeating CPA, such as sucrose or trehalose, stabilizing biological membranes and proteins by direct interaction of HA with polar residues through hydrogen bonding, and forming a metastable glass upon water cell dehydration [35]. Although non-permeant CPAs, such as low MW-HA, are usually used in combination to permeant CPAs to avoid the intracellular ice formation [36,37], good cell outcome after cryopreservation with only non-permeant CPAs have also been reported. For example, cryopreserved rat hepatocytes with 0.2M trehalose secreted more albumin and showed better viability after thawing than cryopreserved cells with DMSO 5% [38].

Our results also showed that the cryopreservation with low MW-HA was able to maintain the spherical structure of microcapsules after thawing, displaying similar round smooth surfaces than non-cryopreserved microcapsules (Figure 1 C). This is a critic parameter in cell encapsulation, since the implantation of capsules and devices with wrinkles, acute angles or sharp edges, induce higher foreign body reaction after implantation [39-41]. Therefore, we concluded that low MW-HA 5% is able to preserve the metabolic activity of encapsulated cells, similarly to DMSO 10% or CS10, maintaining the original shape of the beads with an equilibration time no longer than 20 minutes at 4ºC before the slow freezing procedure.

3.2. Cells preincubation with low MW-HA does not improve cryopreservation outcomes of encapsulated cells

Since the preincubation at 37°C with low MW-HA, followed by cryopreservation with either with DMSO [30] or low MW-HA [32], can improve the viability of human dermal fibroblasts cells after thawing, we studied if this preincubation could improve the outcomes of cryopreserved encapsulated cells with both CPAs. Thus, we incubated D1MSCs and C2C12 cells with low MW-HA 0.5% at 37ºC for 45 minutes and 2, 6 and 24 hours. Next, we encapsulated those cells and froze them with either low MW-HA 5% or DMSO 10%.

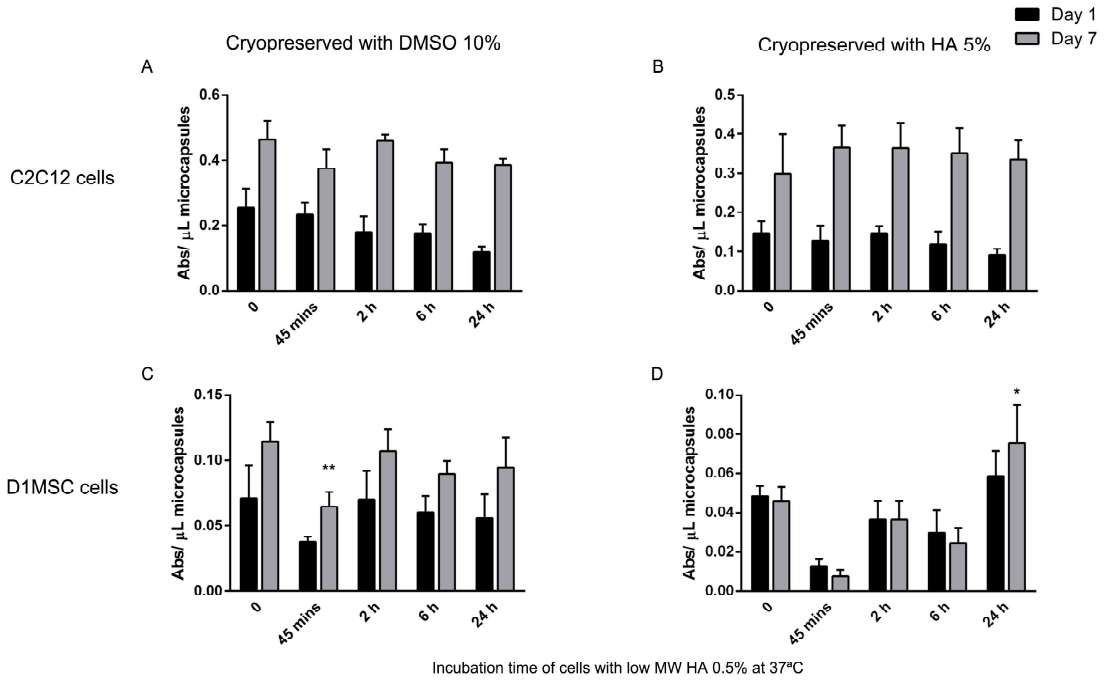

Figure 2. Effect of preincubation with low MW-HA 0,5% on metabolic activity of cryopreserved encapsulated cells after thawing. Metabolic activity 1 and 7 days in culture after thawing of C2C12 cells incubated for 45 minutes, 2, 6 and 24 hours with low MW HA 0,5% at 37ºC, next encapsulated and cryopreserved with (A) DMSO 10% or (B) low MW-HA 5%. Metabolic activity 1 and 7 days in culture after thawing of D1MSC cells incubated for 45 minutes, 2, 6 and 24 hours with low MW HA 0,5% at 37ºC, next encapsulated and cryopreserved with (C) DMSO 10% or (D) low MW-HA 5%. Note: Values represent mean ± SD. *: $p < 0.05$ and **: $p < 0.01$ compared to the group with no incubation of low MW HA 0,5%.

Thawed encapsulated C2C12 maintained the same metabolic activity after 1 and 7 days in culture, independently of the incubation time with low MW-HA 0.5% or the CPA used (Figure 2 A-B). Similarly, encapsulated D1MSCs following the same procedure with DMSO 10% as CPA, did not show any metabolic activity enhancement after thawing. Instead, some significant metabolic activity decrease was quantified in encapsulated D1MSCs preincubated for 45 minutes with low MW-HA 0.5% at 37ºC (Figure 2 C). This contrasts with the reported viability enhancement displayed by human dermal fibroblast monolayers [30], or the proliferation improvement in human MSCs [31]. However, the metabolic

activity of encapsulated D1MSCs previously preincubated with low MW-HA 0.5% for 24 hours, and cryopreserved with low MW-HA 5%, significantly enhanced ($p<0.05$) 7 days after thawing in culture (Figure 2 D), in agreement with the synergistic effect described in human dermal fibroblast monolayers [32]. The discordance with non-permeating CPAs has also been described with trehalose. Preincubation of either suspended or attached fibroblast with trehalose at 37oC showed different membrane integrity depending on the trehalose concentration in the CPA solution [42]. CPA solutions with low trehalose concentrations (25-50mM) showed a slight enhancement of membrane integrity after thawing the trehalose-preincubated fibroblasts, whereas the cells cryopreserved with CPA solutions with higher trehalose concentrations (100-500mM) diminished the viability after cryopreservation. Therefore, after the lack of concordance in the results preincubating with low MW-HA, and in order to standardize the cryopreservation protocol of encapsulated cells with the natural non-toxic HA, we followed studying the cryoprotective effect of low MW-HA in different encapsulated cell types without preincubating cells with low MW-HA 0,5% at 37^{o}C, avoiding complicacies in the overall procedure.

3.3. Beneficial outcomes of cryopreserving several encapsulated cell types with low MW-HA

Based in the good cryoprotective properties shown by non-toxic low MW-HA we decided to study more in deep its effects following the procedure described above without preincubation at 37^{o}C in the murine cell lines, C2C12 and D1MSC, and the human cell lines Arpe-19 (human retinal pigment epithelial cell line) and 1.1B4 (human insulin-secreting cell line).

First, we confirmed the metabolic activity of thawed encapsulated C2C12 after cryopreservation with low MW-HA 5% for 1 and 7 days in culture. No differences in metabolic activity were quantified when low MW-HA was compared to DMSO 10% and CS10 (Figure 3 A). Similarly, no differences in the membrane integrity assay were detected among the three CPA solutions after thawing the encapsulated cells (Figure 3 B). However, statistically significant lower apoptotic cell percentage ($p<0.001$) was quantified only after 1 day in culture in encapsulated myoblasts cryopreserved with CS10 than with low MW-HA, but higher protection was observed by low MW-HA 7 days after thawing, indicating that low MW-HA protects more than DMSO 10% against apoptosis induced by cryoinjury (Figure 3 C). Surprisingly, low MW-HA displayed the highest dead cell percentage ($p<0.001$) quantified after 7 days in culture of encapsulated C2C12 cells cryopreserved with low MW-HA (Figure 3 D). The fluorescence micrographs of calcein/ethidium stained encapsulated

C2C12 cells cultured 7 days after thawing confirmed these data (Figure 3 E).

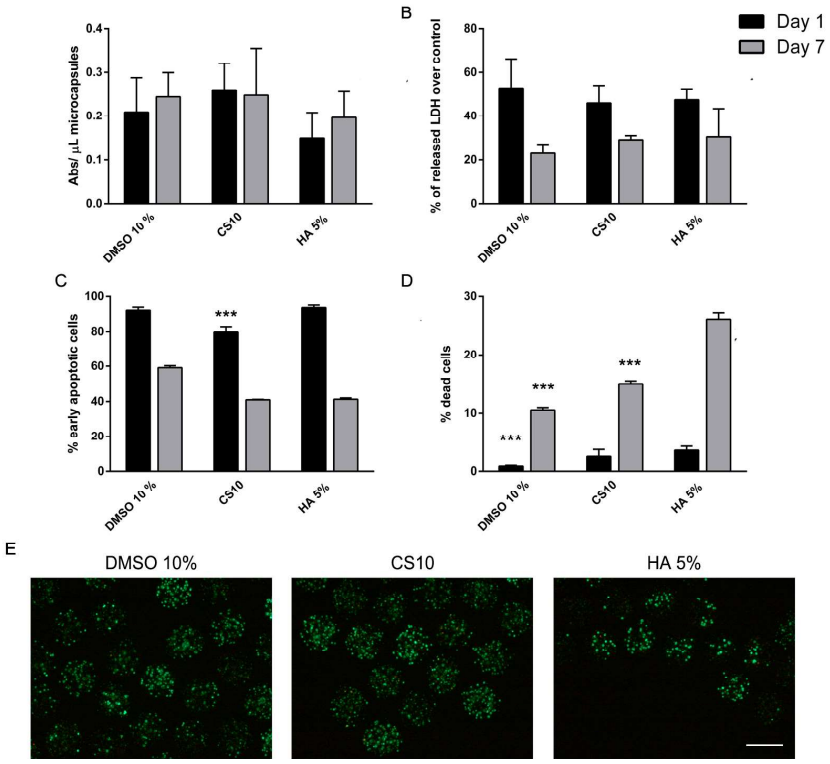

Figure 3. Assesment of cryopreserved encapsulated C2C12 cells with the studied CPAs. Quantification 1 and 7 days after thawing of A) metabolic activity, B) membrane integrity, C) early apoptotic cell percentage, and D) dead cell percentage. E) Micrographs of calcein/ethidium stained cryopreserved microencapsulated cells 7 days in culture after thawing. Note: Values represent mean ± SD. **: $p < 0.01$ and ***: $p < 0.001$ compared to low MW HA 5%. Scale bar: 400µm

We next studied more in deep the outcomes of cryopreserving encapsulated D1MSCs with low MW-HA. Encapsulated cells cryopreserved with low MW-HA displayed similar metabolic activity than encapsulated cells cryopreserved with both DMSO containing CPA solutions (Figure 4 A). However, membrane integrity of encapsulated cells cryopreserved with low MW-HA was higher after 1 and 7 ($p<0.001$) days in culture, as reflected by their lowest released of LDH (Figure 4 B). Moreover, the percentage of apoptotic cells was significantly the lowest in encapsulated cells cryopreserved with low MW-HA after 1 day in culture ($p<0.01$), although these differences were not reflected 6 days later (Figure 4 C). The good outcomes of cryopreservation with low MW-HA of encapsulated D1MSCs were also identified by a significant ($p<0.01$) lower dead cell percentage at both 1 and 7 days in culture when compared with both DMSO containing CPA solutions (Figure 4 D). Micrographs from fluorescence microscopy displayed similar viabilities between the cryopreserved groups

(Figure 4 E).

To determine if the cryoprotective effect of low MW-HA is also beneficial in human cell lines, the human retinal pigment epithelial cell line Arpe-19 was assessed. Metabolic activity was significantly the lowest in cryopreserved encapsulated Arpe-19 cells with low MW-HA compared to DMSO10% and CS10 ($p<0.01$) (Figure 5 A). However, membrane integrity was higher only on encapsulated cells cryopreserved with low MW-HA and cultured for 7 days compared to DMSO 10% ($p<0.01$) (Figure 5 B). No differences among all cryopreserved encapsulated cells were detected when apoptotic cells were quantified at any time in culture (Figure 5 C), but cryopreserved encapsulated Arpe-19 cells with low MW-HA displayed lower dead cell percentages ($p<0.001$) 1 day in culture after thawing compared to CS10 cryopreserved group (Figure 5 D). No significant differences were displayed in the fluorescence micrographs of calcein/ethidium stained encapsulated cells 7 days after thawing (Figure 5 E).

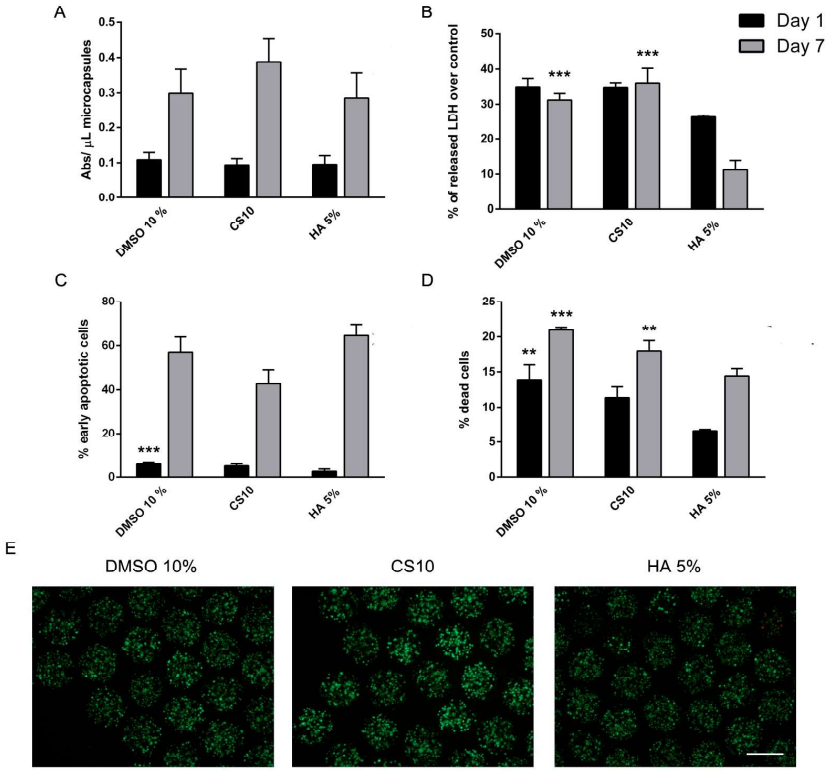

Figure 4. Assesment of cryopreserved encapsulated D1MSC cells with the studied CPAs. Quantification 1 and 7 days after thawing of A) metabolic activity, B) membrane integrity, C) early apoptotic cell percentage, and D) dead cell percentage. E) Micrographs of calcein/ethidium stained cryopreserved microencapsulated cells 7 days in culture after thawing. Note: Values represent mean ± SD. **: $p < 0.01$ and ***: $p < 0.001$ compared to low MW HA 5%. Scale bar: 400µm

The cryopreservation with low MW-HA of the human insulin-secreting cell line 1.1B4 was also assessed. The metabolic activity of cryopreserved encapsulated cells with low MW-HA was the lowest compared to DMSO10% and CS10 ($p < 0.001$) 1 and 7 days in culture after thawing (Figure 6 A). Also, the worst membrane integrity was showed with low MW-HA both days, especially the next day after thawing compared to CS10 ($p<0.01$) (Figure 6 B). The highest apoptotic cell percentages were also quantified in cryopreserved encapsulated cells with low MW-HA at day 7 in culture, significantly compared to CS10 ($p<0.001$) (Figure 6 C). The worst outcomes shown by cryopreservation with low MW-HA of encapsulated 1.1B4 cells were also quantified in cell death percentages ($p<0.001$) (Figure 6 D), also evidenced by the fluorescence micrographs (Figure 6 E).

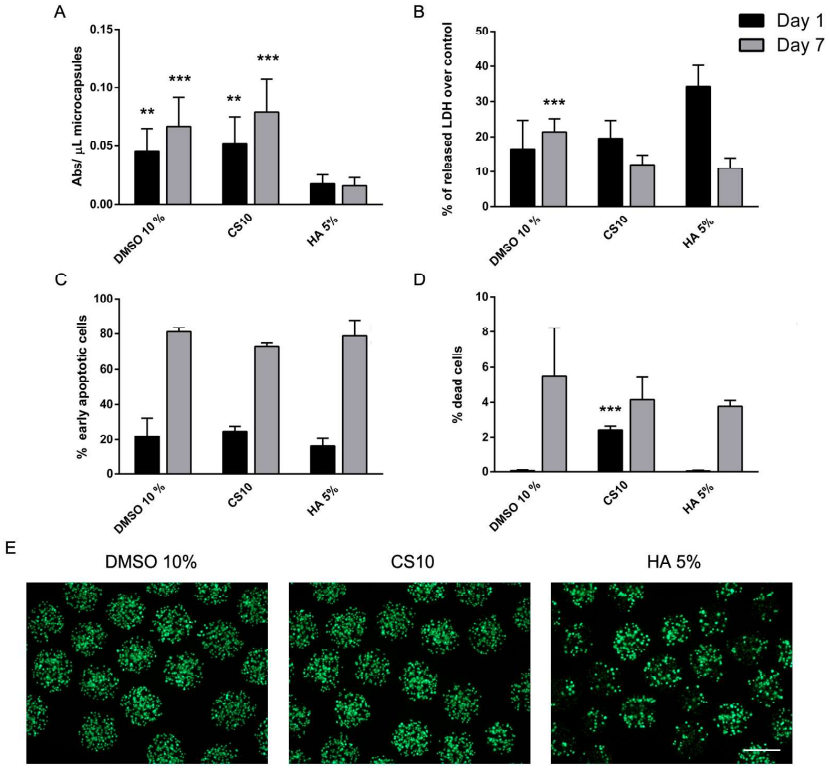

Figure 5. Assesment of cryopreserved encapsulated Arpe-19 cells with the studied CPAs. Quantification 1 and 7 days after thawing of A) metabolic activity, B) membrane integrity, C) early apoptotic cell percentage, and D) dead cell percentage. E) Micrographs of calcein/ethidium stained cryopreserved microencapsulated cells 7 days in culture after thawing. Note: Values represent mean ± SD. **: $p < 0.01$ and ***: $p < 0.001$ compared to low MW HA 5%. Scale bar: 400µm

We compiled the quantified results from each assay in the different cryopreserved encapsulated cell types in one chart, considering a positive result when low MW-HA provided similar or better outcome than the DMSO containing CPA solutions. When at least

three assays displayed positive results, overall, we concluded that low MW-HA represents an alternative as a DMSO free CPA solution for that concrete encapsulated cell line (Figure 7A). Altogether, we can conclude that low MW-HA 5% acts as a good cryoprotectant for encapsulated C2C12, D1MSCs and Arpe-19, with no good outcomes in 1.1B4. In order to discern the causes of the different responses by the encapsulated cell lines, we quantified the single cell expression of the transmembrane CD44 receptor by flow cytometry, since CD44 is the primary receptor that interacts with HA [26,43]. As expected, all cell lines expressed CD44 (Figure 7B) [44-46]. However, only C2C12, D1MSC and Arpe-19 cells extensively expressed (>90%) the receptor. In contrast, the 1.1B4 cell line did not homogeneously express CD44 (<70%), correlating with the worst cryoprotective effect of low MW-HA. Therefore, the cryoprotective effect of low MW-HA might not only be performed following the mechanisms of a non-permeant CPA, but also with an interaction between the CD44 receptor and the natural non-toxic CPA.

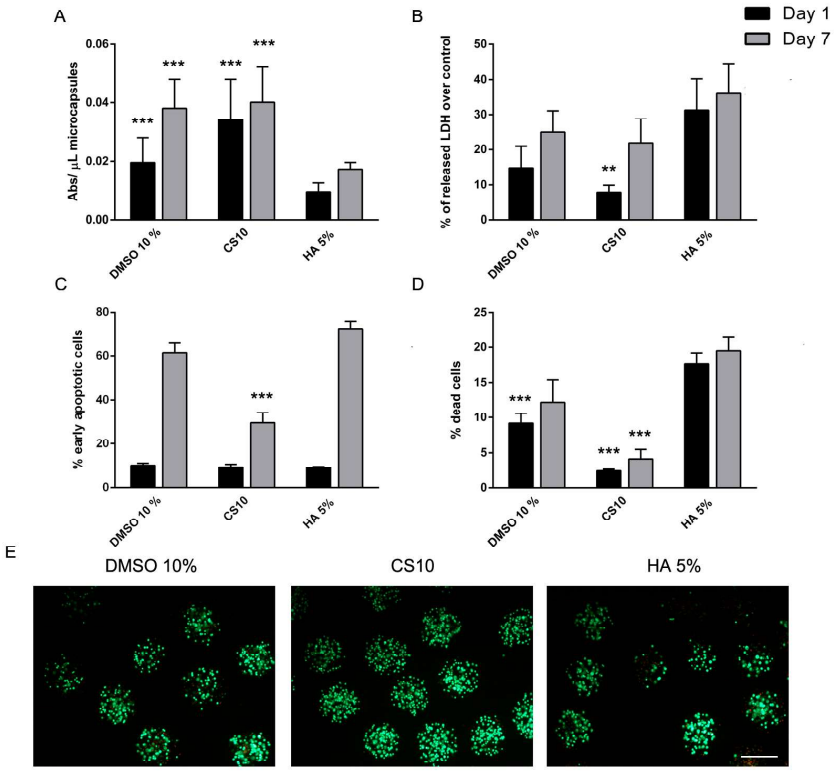

Figure 6. Assesment of cryopreserved encapsulated 1.1B4 cells with the studied CPAs. Quantification 1 and 7 days after thawing of A) metabolic activity, B) membrane integrity, C) early apoptotic cell percentage, and D) dead cell percentage. E) Micrographs of calcein/ethidium stained cryopreserved microencapsulated cells 7 days in culture after thawing. Note: Values represent mean ± SD. **: $p < 0.01$ and ***: $p < 0.001$ compared to low MW HA 5%. Scale bar: 400µm

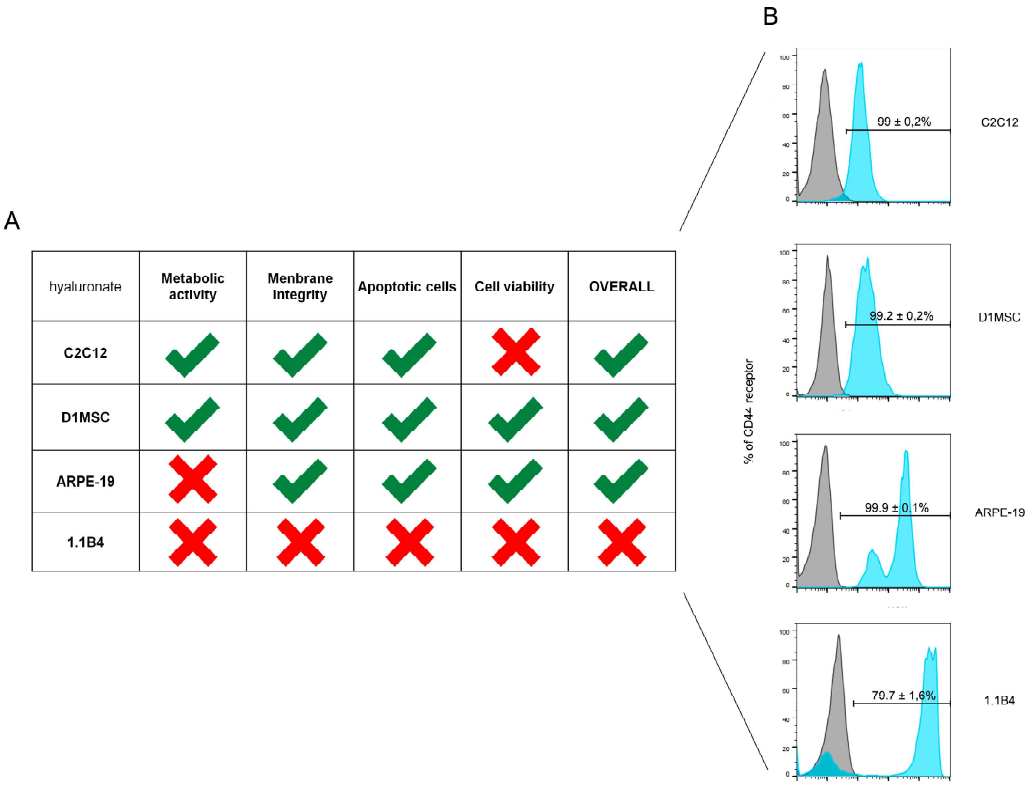

Figure 7: Overall effect of cryopreservation with low MW-HA 5%. A) Chart describing the outcomes from each assay performed in the different cryopreserved encapsulated cell types. Green tick: similar or better outcome than the DMSO containing CPA solutions; red X: lower outcome than the DMSO containing CPA solutions. B) Flow cytometry quantification of CD44 receptor in the studied cell lines.

Interestingly, complexes formed by the HA-CD44 interaction activate cell survival anti-apoptotic proteins [25,47-49]. In fact, this HA-receptor complex interacts with multiple tyrosine kinase receptors, such as ErbB2, ErbB3, or EGFR, and, consequently, activates intracellular pathways that lead to alterations in cytoskeleton and regulate certain gene expression promoting cell survival. For example, the recruitment of ErbB2 by HA-CD44 enhances the transcription of cyclooxygenase-2 through PI3K-Akt and β-catenin, further strengthening the apoptosis resistance through the increase of prostaglandin E2 expression [49,50]. The expression of some CD44 isoforms has also been correlated with the apoptosis resistance in human colon cancer cells. For example, cancer cells that do not express CD44 present high apoptosis after the exposure to a cytotoxic cancer drug, whereas cells that express the CD44 receptor upregulate Akt-phosphorylation levels and downregulate the pRb tumor suppressor, presenting lower apoptosis percentages [51]. On this regard, several CPA solutions include anti-apoptotic factors in order to downregulate the overall mechanisms involved in cell apoptosis and necrosis after cryopreservation, known as “cryopreservation-induced onset cell death.” For example, benzyloxycarbonyl-Val-Ala-DL-Asp-fluoromethylketone, a

pan-caspase inhibitor, is able to enhance hepatocyte-specific functions and reduce apoptosis of microencapsulated rat hepatocytes significantly 24 hours after thawing [52]. Altogether, it can be concluded that low MW-HA might act as an anti-apoptotic factor interacting with the CD44 transmembrane receptor, reducing apoptosis and enhancing cell survival after thawing, explaining the beneficial outcomes observed with low MW-HA in cells that homogeneously express CD44.

This is not the first description of cell survival enhancement by HA addition after oxidative cell injury. The interaction of CD44 and low MW-HA is able to significantly increase cell survival and restore cell viability of cultured human chondrocytes damaged by hypoxanthine and xanthine oxidase [53]. Interestingly, low MW-HA has also demonstrated high antioxidant properties [54,55]. Low MW-HA exhibites significant inhibitory effects on lipid peroxidation and glycation end products formation, and also enhances 2,2-diphenyl-1-picrylhydrazyl and nitrogen oxide radical-scavenging capacity. In this sense, the addition of antioxidants in CPA solutions is being also investigated for optimizing cryopreservation procedures [56,57]. Antioxidants would block the reactive oxygen species created during the freeze-thaw processes, and would decrese the induced onset cell death. Therefore, it could be postulated that HA can enhance cell recovery after cryopreservation by inhibiting the reactive oxygen species created in the freezing process.

4. CONCLUSIONS

Low MW-HA 5% represents an alternative CPA to DMSO in the slow freezing of encapsulated cells, avoiding the post-transplantation complications derived from the toxicity of DMSO. However, further investigations will reveal the mechanisms underlying the cryopreservation with low MW-HA and will shed light on the better suitability of DMSO for the cryopreservation of some encapsulated cells. On this regard, interactions between CD44 and HA could explain these differences.

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SCIENTIFIC REPORTS

Cryopreservation of Human Mesenchymal Stem Cells in an Allogeneic Bioscaffold based on Platelet Rich Plasma and Synovial Fluid

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ABSTRACT

Transplantation of mesenchymal stem cells (MSCs) has emerged as an alternative strategy to treat knee osteoarthritis. In this context, MSCs derived from synovial fluid could provide higher chondrogenic and cartilage regeneration, presenting synovial fluid as an appropriate MSCs source. An allogeneic and biomimetic bioscaffold composed of Platelet Rich Plasma and synovial fluid that preserve and mimics the natural environment of MSCs isolated from knee has also been developed. We have optimized the cryopreservation of knee-isolated MSCs embedded within the aforementioned biomimetic scaffold, in order to create a reserve of young autologous embedded knee MSCs for future clinical applications. We have tested several cryoprotectant solutions combining dimethyl sulfoxide (DMSO), sucrose and human serum and quantifying the viability and functionality of the embedded MSCs after thawing. MSCs embedded in bioscaffolds cryopreserved with DMSO 10% or the combination of DMSO 10% and Sucrose 0,2 M displayed the best cell viabilities maintaining the multilineage differentiation potential of MSCs after thawing. In conclusion, embedded young MSCs within allogeneic biomimetic bioscaffold can be cryopreserved with the cryoprotectant solutions described in this work, allowing their future clinical use in patients with cartilage defects.

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1. INTRODUCTION

Osteoarthritis (OA) is a highly prevalent degenerative joint disease, that involves the cartilage and the surrounding tissue, with the pain as the clinical disease hallmark. Its incidence is increasing and prevalence grows with age, especially after the age of 50. Currently, 46 million patients suffer OA in the developed countries and this pathology might reach 70 millions by 2030 [1]. In the treatment of knee OA, the implantation of autologous mesenchymal stem cells (MSCs) has emerged as an alternative to conventional therapies. Nowadays, MSCs from bone marrow are being used in the knee OA for cartilage repair, showing good safety profiles and similar effectivity than chondrocytes in the improvement of patients´ symptomatology, without major adverse effects [2-4]. However, chondrogenically induced bone marrow MSCs have the inherent risk of forming defective tissues, such as transient fibrocartilaginous tissue, calcifying cartilage and subchondral bone overgrowth [5]. Subsequently, other MSC types are actively investigated [6]. Interestingly, MSCs derived from the synovial joint tissues, such as synovial fluid (SF), synovial membrane and articular cartilage, have been proposed as alternatives due to their higher chondrogenic capacity and cartilage regeneration than bone marrow MSCs [7,8]. For example, magnetic resonance imaging, qualitative histology and Lysholm scores results from a 3-year follow-up clinical study, showed the improvement in patients with symptomatic single cartilage lesion of the femoral condyle and transplanted with MSCs derived from synovial membrane [9]. Because MSCs from the SF have similar gene expression and surface antigens profiles to MSCs from synovial membrane, with the advantage that are easier to obtain [10], MSCs from SF might result more appropriate in the treatment of cartilage tissue.

SF is a viscous liquid composed of lubricin, hyaluronan (HA), growth factors and cytokines, mainly derivated from plasma and secretions of synoviocytes and chondrocytes [11]. Moreover, SF sometimes contains a minor presence of cells, such as MSCs, whose origin is still debated between the subchondral bone, the synovial membrane and the breakdown zone of the articular cartilage [12]. However, the migration of MSCs to the SF is enhanced while SF volume is increased, when the articular cartilage, synovial membrane, subchondral bone or the knee joint are affected, with inflammation and aggression of the intra-articular tissues [13,14]. SF is routinely extracted without harming other tissues when inflammation occurs, providing large quantities of SF from each patient. Therefore, as SF volume and MSCs number are incremented in patients suffering OA, SF could be a viable and adequate MSCs source from these patients, for their future use in the treatment of the disease.

We have developed an allogeneic and biomimetic scaffold, composed of SF and blood plasma enriched with platelets, hereafter called Platelet Rich Plasma (PRP). The

mixture of PRP and SF permits the formation of an autologous bioscaffold (PRP-SF) due to the synthesis of a fibrin structure after plasma activation [15]. Our group have optimised a PRP-SF bioscaffold with an appropriate structure that shows high viabilities of embedded MSCs extracted from SF [16]. This bioscaffold can be formed during SF extraction, allowing a short preservation of embedded MSCs without the need of cell attachment and culture, and therefore, simplying the labor of the clinician in terms of time and cost [17]. Moreover, our easy and economical PRP-SF bioscaffold provides other advantages. On one hand, its size can be modulated by modifing the volume of SF and on the other hand, PRP-SF bioscaffold provides a closer environment to MSCs since it contains hyaluronic acid, growth factors and cytokines among others. Therefore, PRP-SF bioscaffold with embedded MSCs represent an alternative to the standard procedure of isolation and preservation of MSCs from SF.

Patients with OA or other cartilage defects, usually need to be treated several times during their lifes. However, while the age of the patient increases, the number, the growth potential and the replicative capacity of the MSCs from the patient decrease [18,19]. This reduction in the number and replicative potential is also reflected in the embedded MSCs within PRP-SF bioscaffold obtained from the patients. Therefore, the long time preservation of MSCs from SF or embedded MSCs within PRP-SF bioscaffold would be extremely convenient for their future clinical translation. However, to the best of our knowledge, PRP-SF has not been cryopreserved yet.

The cryopreservation of cell lines is extensively and successfully used in cell culture laboratories, but the cryopreservation of embedded primary cells within complex tissues or structures is still challenging [20-23]. The size of the structures represents an obstacle for the penetration of cryoprotectants (CPAs), provoking different exposition of the embedded cells to the CPA depending on the cell location in the structure, and therefore, leading to different cell viabilities throughout the structure [24,25]. Currently, two procedures are used for cryopreservation of embedded cells in 3D scaffolds: slow freezing [26] and vitrification [21]. Vitrification has theoretical advantages over slow freezing as no ice is created in the process. However, the high CPAs concentrations required to achieve the vitreous state are toxic to cells [27]. Slow freezing is a simpler procedure with lower CPAs concentrations than vitrification [28]. Moreover, slow freezing do not need advanced equipment and can be worldwide applied in any laboratory.

In this manuscript, we have determined that DMSO 10% or the combination of DMSO 10% and sucrose 0,2M are required for the slow freezing cryopreservation of embedded MSCs within PRP-SF bioscaffolds, allowing to move this biosystem from the bench to the clinic.

2. MATERIAL AND METHODS

2.1. Samples isolation and bioscaffold formation

2.1.1. MSCs Isolation from Knee Synovial Fluid (SF)

A SF sample from a 48-year-old female knee with patellar chondropathy was harvested by a syringe aspiration before intraarticular infiltration of PRP. The collected SF was diluted in phosphate buffer saline (PBS) and spin down to isolate the cellular content. Cells were seeded in a T175 flask (Corning), and cultured with Dulbecco's Modified Eagle Medium (DMEM; Lonza) supplemented with 10% human serum (HS) in a humidified incubator at 37°C in the presence of 5% CO2. Following overnight incubation, non-adherent cells were removed by replacement with fresh culture medium. Every 2-3 days, medium was removed, cells were washed with PBS (Gibco) and fresh medium was added. All the experiments, were performed with cells between 2-6 passages.

2.1.2. PRP-SF bioscaffold formation

Knee SF samples were obtained by syringe aspiration from patients before intra-articular PRP infiltration. Moreover, blood was drawn from different patients and centrifuged at 1200 g for 8 minutes at room temperature. After centrifugation, PRP was collected from the plasma fraction located above the sedimented red blood cells, but not including the buffy coat [54]. For the formation of PRP-SF bioscaffold, 450µL of SF, 5x104 cells (Passage 2-6) and 450µL of PRP were mixed in a cryovial (Corning). Next, the addition of 20 µL of calcium chloride activated PRP triggering the subsequent formation of the bioscaffold. Bioscaffolds were cultured overnight in a humified incubator in the cryovials until their cryopreservation.

2.1.3. PRP bioscaffold formation

For PRP bioscaffold formation 900 µL of collected PRP and $5x10^4$ cells (Passage 2-6) in suspension were mixed into a cryovial, next adding 20 µL of calcium chloride to activate PRP and trigger the subsequent formation of the bioscaffold. Bioscaffolds were cultured overnight in a humified incubator in the cryovials until their cryopreservation.

The institutional review board named "Comité ético de investigación clínica (CEIC) del Hospital universitario de Araba" approved the harvest of all the samples (Code: UCA-04/EE/15/CAR), obtaining informed consents from every patient to whom biological samples were extracted. All methods were performed in accordance with the relevant guidelines and regulations.

2.2. MSCs characterization

MSCs derived from SF were cultured and phenotyped with an 8-color direct immunofluorescence flow cytometry (Passage 2 and 6). Cell were stained with the following combination of labelled monoclonal antibodies: phycoerythrin (PE)-CD105 (clone 1G2 from Beckman Coulter), orange chrome (OC) 515-CD45 (clone GA90 from Citognos), fluorescein isothiocyanate (FITC)-CD73 (clone AD2 from BD), peridinin chlorophyll protein-cyanin 5.5 (PerCP-Cy5.5)-CD271 (clone ME204 from Biolegend), allophycocyanin (APC)-CD34 (clone 8G12 from BD), PE-cyanin 7 (PECy7)-CD13 (clone L138 from BD), Brilliant violet (BV) 421-CD90 (clone 5E10 from BD), APCH7-CD44 (clone G44-26 from BD). Negative controls of fluorescence were defined using unstained MSCs measured under the same cytometer settings as the (8-color) stained MSCs.

MSCs were also differentiated into osteoblasts, adipocytes and chondrocytes lineages to determine its multipotency (Passage 2). To induce osteogenic and adipogenic differentiation $5x10^5$ cells were seeded and cultured into 6-well culture plates until achievement of 70-80% confluency. Adipogenic differentiation was induced culturing with growth medium supplemented with 10% FBS (Gibco), 0.5µM dexamethasone, 0.5 µM 3-isobutyl-1-methylxanthine, and 50 µM indomethacin. For osteogenic differentiation growth medium was supplemented with 10% FBS (Gibco), 0.05 mM L-ascorbic acid, 20 mM β-glycerophosphate and 100 nM dexamethasone. To induce chondrogenic differentiation, $4x10^5$ cells were cultured in 15 mL conical tubes with 0.5 mL of DMEM supplemented with 10% FBS (Gibco), 50 nM L-ascorbic acid, 6.25µg/mL bovine insulin, and 10 ng/mL transforming growth factor-β (TGF-β) (Peprotech Inc.). Each differentiation medium was replaced every 2-3 days for 21 days. Finally, cells were fixed and stained with Alizarin Red S (osteogenic differentiation), Oil Red O (adipogenic differentiation), and Alcian Blue (chondrogenic differentiation). Reagents were purchased from Sigma-Aldrich.

The multipontecy of the embedded MSCs into PRP-SF bioscaffolds after cryopreservation was also tested (Passage 2-6). MSCs were released from PRP-SF bioscaffolds by shaking the sample with 400µL Urokinase VEDIM 250000 UI/ vial (UCB Pharma S.A) for 3 hours at 37ºC in a 5% incubator. Released cells were seeded and cultured into 6-well culture plates until achievement of 70-80% confluency, following the same aforemetioned differentiation procedures.

Fibroblast colony forming-units (CFU-F) assay was also performed to MSCs (Passage 2). Cells at passage 2 were seeded at a density of 1,000 cells/60-cm² in 6 dishes, and cultured in DMEM supplemented with 10% HS in a humidified incubator at 37°C in the presence of 5% CO2. Dishes were stained after 14 days with 0.5% crystal violet and colonies were counted.

2.3. Cryopreservation

All cryoprotectant solutions were fresh prepared by diluting the cryoprotectant (CPA) agent in maintenance medium. The following CPA solutions were prepared by the combination of dimethyl sulfoxide (DMSO) (ATCC), sucrose (Sigma-Aldrich) and human serum: DMSO 10% (DMSO), Human Serum 10% (HS), DMSO 10% + Human serum 10% (DMSO + HS), Sucrose 0,2M (Sucrose), DMSO 10% + Sucrose 0,2M (DMSO + Sucrose), Human Serum 10% + Sucrose 0,2M (HS + Sucrose) and DMSO 10% + Human Serum 10% + Sucrose 0,2M (DMSO + HS + Sucrose). One bioscaffold/cryovial was cryopreserved with 1 ml of each CPA solutions, storing at least three bioscaffold/CPA in each experiment. Cryovials without CPA or additives (wo CPA) were also stored for study. Cryovials followed the next procedure: 20 minutes on ice at 0-4 Cº, overnight at -80ºC on a CoolCell (Biocision) container and final store into liquid N_2 tanks for at least three weeks before performing any assay. Cryovials were thawed quickly at 37ºC until no ice was observed in the solution. CPA solution was slowly diluted in fresh medium to inhibit osmotic damage. Next, bioscaffolds were rinsed twice with 10 ml of maintenance medium to completely remove the CPA solution.

2.4. Factorial experiment

An orthogonal experimental design was followed to study the influence on cell viability of three additives in the CPA solutions: DMSO (X1), Sucrose (X2) and Human Serum (X3). Two levels (no presence and presence) and three variables (each additive, X1, X2, X3) were evaluated in a fractional factorial experiment. The viable cell number in proliferation response at days 7 and 14, were expressed in absorbance for each experimental combination (Table 1). Three different interactions were studied in the orthogonal experimental design (X1X2, X1X3 and X2X3) and the effect of each variable and each interaction was calculated by the following equation:

$$\text{Effect of Interaction} = \frac{\sum \text{responses with postive sign} - \sum \text{responses with negative sign}}{4}$$

2.5. Viable cell number in proliferation

The viable cell number in proliferation of MSCs within PRP and PRP-SF bioscaffolds at different timepoints after thawing was quantified by means of Cell Counter Kit 8 (CCK-8) (Sigma) assay. Briefly, complete medium was removed from bioscaffolds cultures and 1ml of complete medium with 10% CCK-8 reagent was added incubating them for 4 hours

at 37ºC in a 5% incubator. After incubation, supernatants were collected, 100 µL of each sample transferred into a 96 well plate and read on an Infinite M200 (TECAN Trading AG, Switzerland) microplate reader at 450 nm with reference wavelength at 650 nm. At least five wells were placed and three independent experiments were analysed for each condition.

2.6. Cell viability assays with calcein/ethidum staining

The viability of MSCs within PRP and PRP-SF bioscaffolds at different time points was qualitatively assessed by fluorescence microscopy after staining with LIVE/DEAD® Viability/Cytotoxicity Kit. Bioscaffolds were rinsed three times with DPBS and then, mixed with 0.5µM calcein AM and 0.5µM ethidium homodimer-1 in DPBS on 24-well plates. After incubation at room temperature for 40 minutes in the dark, samples were observed under a Nikon TMS microscope with the following excitation/emission wavelengths: 495/515 nm for calcein AM and 495/635 nm ethidium homodimer. At least three independent experiments were analysed for each condition.

Cell viability was also quantified by flow cytometry in non-cryopreserved (control), wo CPA, DMSO, Sucrose and DMSO +Sucrose cryopreserved groups 7 days after thawing with the LIVE/DEAD® Viability/Cytotoxicity Kit (Life Technologies). Briefly, complete medium was removed and cells were released by shaking with 400µL Urokinase VEDIM 250000 UI/ vial (UCB Pharma S.A) for 3 hours at 37ºC in a 5% incubator. Next, cells were stained with 100 nM calcein AM and 8 µM ethidium homodimer-1 solution for 20 minutes at room temperature, protected from light. Fluorescence was determined immediately with a BD FACS Calibur flow cytometerTM. Unstained samples or samples stained only with 100 nM calcein AM or 8 µM ethidium homodimer-1 were studied as controls. All the measurements were conducted in triplicates, and at least three independent experiments were analysed for each condition.

2.7. Cell early assay apoptosis

Early apoptosis of MSCs in control, wo CPA, DMSO, Sucrose and DMSO + Sucrose cryopreserved samples was quantified 7 days after thawing with the Annexin-V-FITC Apoptosis Detection Kit (Sigma-Aldrich). Briefly, complete medium was removed and cells were released by shaking with 400µL Urokinase VEDIM 250000 UI/ vial (UCB Pharma S.A) for 3 hours at 37ºC in a 5% incubator. Next, cells were rinsed twice with DPBS, resuspended in 10 mM HEPES/NaOH containing 0.14 M NaCl and 2.5 mM $CaCl_2$ (binding buffer, pH 7.5) and stained with annexin V-FITC and propidium iodide for 10 minutes at room temperature protected from light. Fluorescence was determined immediately with a

BD FACS CaliburTM flow cytometer (BD Biosciences). Unstained samples or samples stained only with annexin V-FITC or propidium iodide were analyzed as controls. All the measurements were conducted in triplicates, and at least three independent experiments were analysed for each condition.

2.8. Cell membrane integrity assay

The release of lactic dehydrogenase (LDH) on the culture supernatants was quantified in control, wo CPAs, DMSO, Sucrose and DMSO +Sucrose cryopreserved groups 7 days after thawing as an indicator of membrane integrity using the In Vitro Toxicology LDH based Assay Kit (Sigma-Aldrich). Briefly, 1 ml of complete medium was incubated with PRP-SF bioscaffolds for 24 hours. After incubation, all supernatants were collected to determine the amount of released LDH. In parallel, PRP-SF bioscaffolds were also incubated for 24h hours with 1000 µl of complete medium and lysed to determine the total LDH activity. All supernatants were subjected to enzymatic analysis based on the reduction of NAD by LDH and its further reaction with tetrazolium dye following manufacture´s recommendations. The resulting coloured compound absorbance was read out on the Infinite M200 microplate reader at a wavelength of 490 nm, with absorbance reading at 690 nm as background. All the measurements were conducted in triplicates, and at least three independent experiments were analysed for each condition.

2.9. Statistics

Statistical analysis was performed using SPSS software, version 21.00.1. Data are expressed as means standard deviation. $p < 0.05$ and $p < 0.001$ were considered significant for comparison of groups using ANOVA, Tukey's Post Hoc and Kruskal-Wallis H test.

3. RESULTS AND DISCUSSION

3.1. Isolated knee MSCs characterization

SF is an appropriate source of MSCs because of its direct application on OA. However, SF provides lower cell number than other traditional sources, such as bone marrow [29]. Therefore, a bioscaffold that preserves the environment of the isolated MSCs is very valuable. For experimental purposes, the low number of MSCs in just 450µL of SF is not enough for analysis, and the addition of previously isolated MSCs at higher amount in the range of at least $5x10^4$ cells helps to overcome this drawback. Moreover, the addition of external MSCs to the bioscaffold standardise the cell quantity in each construct. With this purpose in mind, MSCs were isolated from a donor with patellar chondropathy. Cells were cultured

into a culture flask where they adhered to the bottom showing a fibroblast-like morphology (Figure 1A). Isolated MSCs phenotype was next characterized by flow cytometry. Among several markers, it is remarkable that isolated knee MSCs expressed the mesenchymal surface markers CD73, CD105 and CD90, lacking expression of CD34 and CD45, therefore meeting the criteria established by the International Society for Cellular Therapy position for MSCs (Figure 1B) [30] that identify the following qualifying criteria for MSCs: 1) cells must be adhesive to plastic, 2) cells must differentiate into chondrocytes, osteocytes, and adipocytes, and 3) cells must express the surface markers CD73, CD90, CD105 (≥95% expression) with no expression of the hematopoietic markers CD34, CD45, CD14 or CD11b, CD79α or CD19 (≤2%) and absence of HLA Class II molecules. Moreover, knee MSCs expressed the surface markers CD44, CD13 and CD271 (Figure 1B), that correspond to the receptor of hyaluronic acid, the membrane alanyl aminopeptidase and the low-affinity nerve growth factor receptor respectively, that have been described to be expressed in MSCs [31-33].

We also tested if knee MSCs were able to differentiate into the three mesodermal lineages. The detection of calcium deposits with the Alizarin Red, the cytoplasmatic accumulation of vacuoles filled with neutral lipids by Oil Red O, and the positive Alcian blue staining for cartilage matrix confirmed the mesenchymal potential of the isolated knee MSCs (Figure 1C). In addition, the capacity of knee isolated MSCs to form colonies for 21 days was assessed. MSCs showed values of 53 ±5,6 % CFU-F percentage indicating its clonogenicity [34]. Therefore the isolated MSCs were used in the following experiments as an external source of cells to standardize the cell quantity in each studied bioscaffold.

3.2. Cryoprotective comparison of PRP and PRP-SF bioscaffolds

The number, growth potential and replicative capacity of isolated MSCs decrease with the age of the patient [18,19], a reduction that is also reflected in embedded MSCs within bioscaffold obtained from the patients. Therefore, the cryopreservation of bioscaffolds results of high interest since it allows the isolation of young MSCs for their future clinical applications.

PRP-SF bioscaffolds are composed of the mixture of PRP and SF. PRP contains fibrin, a cytocompatible structure that promotes cell proliferation due to the high diversity of binding sites that contains [35], while SF contains HA, a glycosaminoglycan that facilitates the MSCs migration in the extracellular matrix through the interaction between HA and CD44 receptors [36]. Protective effects of HA have been described in the cryopreservation of human fibrotic monolayers [37,38]. Taking into account the additional protective properties of HA, we first aimed to determine if PRP-SF bioscaffolds, formed by PRP and SF mixture,

provide a better environment than bioscaffold formed exclusively of fibrin (PRP bioscaffold), maintaining higher MSCs viabilities after cryopreservation following a conventional slow freezing protocol. Furthermore, we studied if both bioscaffolds required the presence of CPA for their cryopreservation by adding DMSO, the most common CPA in clinic and labs, and compared viabilities with non-cryopreserved bioscaffolds.

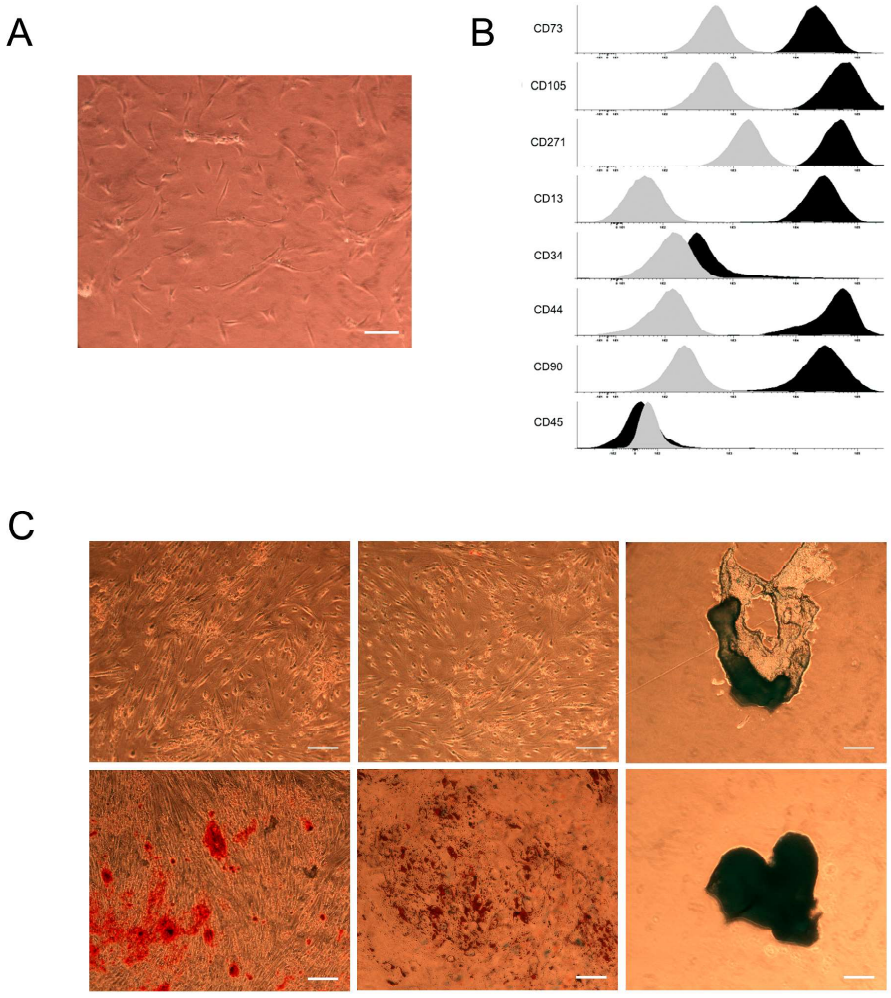

Figure 1. Characterization of human MSCs extracted from Synovial Fluid. A) Micrograph of adhered MSCs. B) Phenotypic characterization by flow cytometry of the following markers: CD73, CD105, CD90, CD34, CD45 ,CD271 ,CD13 and CD44. Grey and black histograms display minus one control and sample respectively. C) Microscopic images of 3 weeks-differentiated MSCs into osteocytes (Osteo), adipocytes (Adipo) and chondrocytes (Chondro). Note: First row: undifferentiated MSCs (contr), second row: differentiated MSCs (diff). Scale bar: 100 µm

Once the MSC derived from the SF were characterized, PRP and PRP-SF bioscaffolds containing MSCs were tested, analyzing their capacity to maintain MSCs viability after cryopreservation and thawing. Both bioscaffolds showed different macroscopic appearance the next day after formation. PRP bisocaffold resulted in a compact solid hydrogel

structure (Figure 2A1) while PRP-SF showed a surrounding matrix formed of non-retracted SF (Figure 2A2), that was reduced overtime (data not shown). Next day after forming bioscaffolds, no differences were quantified between the viable cell number in proliferation of non-cryopreserved PRP and PRP-SF bioscaffolds. Similarly, no differences were detected when both bioscaffolds were cryopreserved with or without DMSO, with similar viable cell number in proliferation than non-cryopreserved bioscaffolds (Figure 2B).

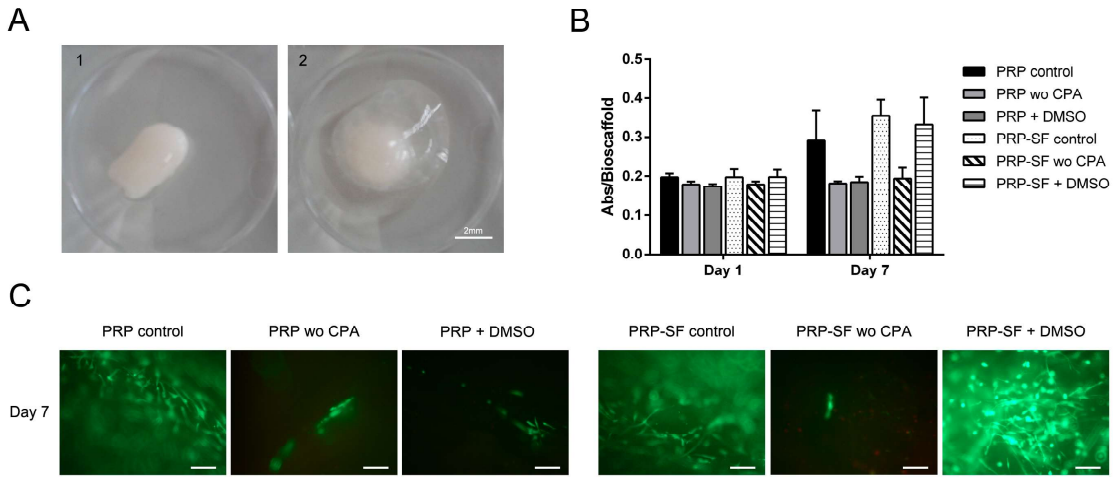

Figure 2. Cryoprotective comparison of PRP and PRP-SF bioscaffolds A) Macroscopic images of 1 day cultured PRP (1) and PRP-SF (2) bioscaffolds. B) Viable cell number in proliferation of embedded MSCs within PRP and PRP-SF bioscaffolds 1 and 7 days after thawing. C) Micrographs of calcein/ethidium stained MSCs within PRP and PRP-SF bioscaffolds 7 days after thawing. Note: PRP control: non-cryopreserved PRP bioscaffold; PRP wo CPA: PRP bioscaffold cryopreserved without CPA and additives; PRP DMSO: PRP bioscaffold cryopreserved DMSO 10%: PRP-SF control: non-cryopreserved PRP-SF bioscaffold; PRP-SF wo CPA: PRP-SF bioscaffold cryopreserved without CPA and additives; PRP-SF DMSO: PRP-SF bioscaffold cryopreserved DMSO 10%. Scale bar: 100 µm

However, 7 days in culture after formation or cryopreservation of bioscaffolds, differences were detected between both bioscaffolds, although not statiscally significant. PRP bioscaffolds did not protect the viable cell number in proliferation of MSCs after cryopreservation either with or without the addition of DMSO (Figure 2B). However, PRP-SF was able to protect the viable cell number in proliferation of embedded MSCs when DMSO was added, showing similar viable cell number between non-cryopreserved embedded MSCs and those cryopreserved with DMSO (Figure 2B), and therefore indicating that PRP-SF results in a more convenient bioscaffold for the cryopreservation of the scarce knee MSCs number. All these results were confirmed by fluorescent microscopy micrographs after calcein/ethidium staining (Figure 2C). However, although we hypothesised that HA could have a cryoprotective effect in the PRP-SF embedded cells, we detected a decrease in

embedded MSCs viable cell number in proliferation when they were cryopreserved without DMSO. In previous studies conducted with human fibrotic monolayers and solutions at high concentration (5%) of low molecular weight HA, the cryoprotective effect of HA was related to its high hydration capacity and its cell internalization CD44 receptor-mediated [37,38]. However, HA in PRP-SF bioscaffolds comes from a natural HA source and has higher molecular weight than the described in those studies. Moreover, HA in these bioscaffolds is not free in solution. Altogether precludes HA to exert its cryoprotective effect, and therefore the addition of an external CPA is required.

The lack of protection by the cryopreserved PRP bioscaffold with DMSO 10% compared to PRP-SF bioscaffold cryopreserved with DMSO 10% at day 7, could be related to cell proliferation after bioscaffold preparation. Even the desirable properties of fibrin to promote cell proliferation, and the reduction of ice creation by DMSO in cryopreservation, it seems that HA, fibrin and DMSO combination enhances cell growth after thawing. In accordance with our results, other authors have described that the proliferation and spreading of osteosarcoma cells within fibrin-HA scaffolds was significantly higher than in single (HA or fibrin) network analogues [15]. Therefore, because PRP bioscaffolds provide lower viable cell number in proliferation in embedded MSCs than PRP-SF after cryopreservation, we performed the following experiments with the MSCs embedded in the PRP-SF bioscaffolds.

3.3. Effects and interactions of CPAs and human serum

The cryopreservation with the conventional slow freezing protocols of tissues or structures with embedded cells is still challenging. The selection and combination of CPAs is one of the most important parameters when determining the conditions for an optimal cryopreservation of such complex structures [39,40]. In function of their nature, CPAs show different cryoprotective mechanisms. Penetrant CPAs, such as DMSO, displace the internal water from the cell, minimizing the intracellular ice crystal formation while, non-penetrant CPAs, such as sucrose, act from the outside of the cells, promoting their dehydration [23]. DMSO shows several disadvantages in the preservation process such as toxicity or loss of multipotency [41], but its replacement is difficult since no other CPA has shown the same results maintaining embedded cell viabilities after cryopreservation [42]. However, the combination of DMSO and sucrose enhance also the post-thawing viability of human MSCs [43,44]. Fetal bovine serum (FBS) has been described as another additive included in several cryopreservation protocols with beneficial effect. FBS stabilizes cell membrane, decreases the extracellular ice formation, minimizes cell dehydration and prevents excessive concentration of solutes during the freezing/thawing process [45]. However, clinicians try to avoid FBS

in the cryopreservation of MSCs to reduce the risk of xeno-derived infection [46,47], for example, with the use of human serum (HS). With all of this in mind, we studied the effect of three additives (DMSO, sucrose and HS) in the cryopreservation of MSCs embedded within PRP-SF.

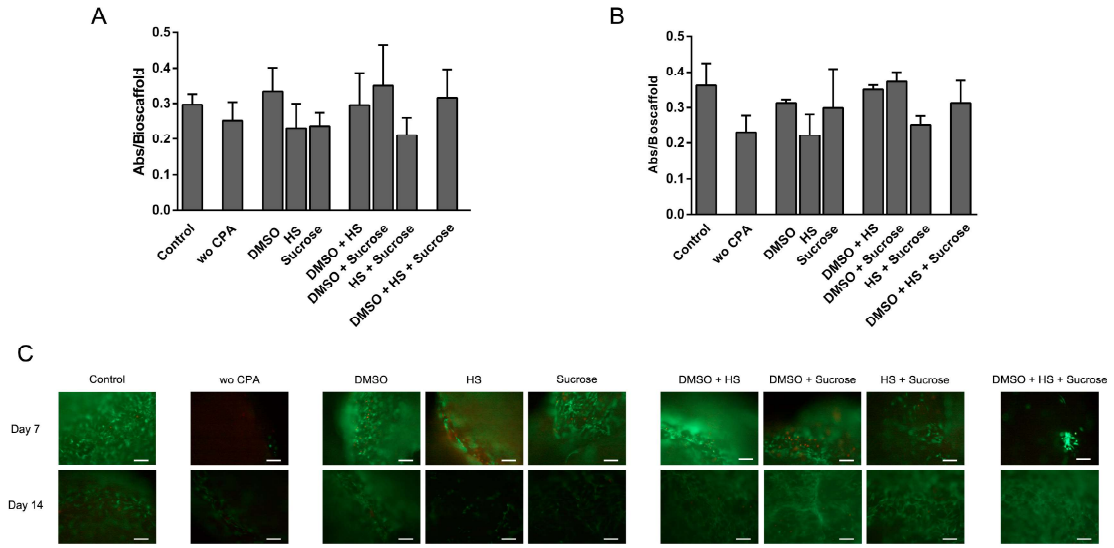

Figure 3. Effects and interactions of CPAs and human serum in the viable cell number in proliferation of cryopreserved embedded MSCs within PRP-SF. Viable cell number in proliferation of embedded MSCs within PRP-SF bioscaffolds 7 (A) and 14 (B) days after thawing. C) Micrographs of calcein/ethidium stained MSCs within PRP-SF bioscaffolds 7 and 14 days after thawing Note: Control: non-cryopreserved bioscaffolds; wo CPA : without CPA and additives; DMSO: DMSO 10%; HS: Human Serum 10%; DMSO + HS: DMSO 10% + Human serum 10%; Sucrose: Sucrose 0,2M; DMSO + Sucrose: DMSO 10% + Sucrose 0,2M; HS +Sucrose: Human Serum 10% + Sucrose 0,2M; DMSO + HS + Sucrose: DMSO 10% + Human Serum 10% + Sucrose 0,2M. Scale bar: 100 µm

We carried out a two levels three variables (DMSO, sucrose and HS) simple factorial experiment to characterize the viable cell number in proliferation of cryopreserved embedded MSCs within PRP-SF bioscaffold 7 and 14 days after thawing (timepoints when differences were expected based on previous results). When comparing bioscaffolds preserved with one additive 7 and 14 days after thawing, those preserved with DMSO 10% showed the highest viable cell number in proliferation (Table 1 and Figure 3A,B). This beneficial outcome was also reflected in other CPA solutions where DMSO was present, with an increasing effect on the viable cell number in proliferation at either 7 days (DMSOe= 0,0894) or 14 days (DMSOe= 0,0854) after thawing (Table 2). Fluorescent micrographs after calcein/ethidium staining confirmed the beneficial effect in the cryopreservation with CPA solutions containing DMSO of embedded MSCs within PRP-SF (Figure 3C). We can conclude that the inclusion

of a penetrant CPAs, such as DMSO, is required for the cryopreservation of MSCs embedded in PRP-SF bioscaffold since DMSO have shown the highest maintenance of embedded MSCs viable cell number in proliferation.

Groups	DMSO (%)	HS (%)	Suc (M)	Viable cell number in proliferation			
				Day 7		Day 14	
				MEAN	SD	MEAN	SD
Control	—	—	—	0,302	0,028	0,337	0,0640
Wo CPA	0	0	0	0,253	0,045	0,232	0,043
DMSO	10	0	0	0,333	0,061	0,313	0,009
HS	0	10	0	0,232	0,061	0,222	0,061
Sucrose	0	0	0,2	0,238	0,033	0,299	0,111
DMSO + HS	10	10	0	0,296	0,079	0,351	0,012
DMSO + Sucrose	10	0	0,2	0,349	0,102	0,372	0,029
HS + Sucrose	0	10	0,2	0,213	0,043	0,253	0,025
DMSO + HS + Sucrose	10	10	0,2	0,316	0,071	0,313	0,062

Table 1: Viable cell number in proliferation at day 7 and 14 after thawing of cryopreserved MSCs embedded within PRP-SF bioscaffolds with different CPA solutions. Note: Control: non-cryopreserved bioscaffolds; wo CPA: without CPA and additives; DMSO: DMSO 10%; HS: Human Serum 10%; DMSO + HS: DMSO 10% + Human serum 10%; Sucrose: Sucrose 0,2M; DMSO + Sucrose: DMSO 10% + Sucrose 0,2M; HS +Sucrose: Human Serum 10% + Sucrose 0,2M; DMSO + HS + Sucrose: DMSO 10% + Human Serum 10% + Sucrose 0,2M

When HS was studied as a single preservative additive, a lower viable cell number in proliferation than embedded MSCs within PRP-SF bioscaffolds cryopreserved without CPAs was displayed (Table 1, Figure 3A,B). Subsequently, the HS effect on viable cell number in proliferation was negative in the factorial experiment, with values of -0,0294 at day 7 and in -0,0197 at day 14 post-thawing (Table 2), indicating that HS has not preservative properties in the cryopreservation of embedded MSCs within PRP-SF. When HS was combined with either a penetrant (DMSO) and/or a non-penetrant CPA (sucrose), only combinations in presence of DMSO displayed an increase in the viable cell number in proliferation at day 7 and 14 after thawing (Table 1, Figure 3A,B), indicating that the presence of HS does not improve the cryopreservative effect of the studied CPA solutions. Fluorescent micrographs after calcein/ethidium staining confirmed these results (Figure 3C). In fact, when interactions were analyzed, only some CPA with DMSO showed positive values after thawing (Table 2). In other studies, human ovarian cortical tissues of 1-1.5 mm cryopreserved with HS and combined 1,5 M propanediol and 0,1 M sucrose showed viabilities of 65% in follicles and 75% in oocytes after thawing [48]. Although HS can provide preservative effects in other complex tissues, and it has shown better expansion and proliferation of human synovial

MSCs than fetal bovine serum [49], it seems that it is not appropriate for the cryopreservation of embedded MSCs within PRP-SF bioscaffolds.

Effects and interactions	Viable cell number in proliferation	
	Day 7	Day 14
DMSO[e]	0,0894	0,0854
HS[e]	−0,0294	−0,0197
Sucrose[e]	0,0003	0,0298
DMSO-HS[i]	−0,062	0,0089
DMSO-Sucrose[i]	0,0178	−0,0192
HS- Sucrose[i]	−0,0002	−0,0332
DMSO-HS-Sucrose[i]	0,0019	−0,0152

Table 2: Effects and interactions on viable cell number in proliferation of MSCs embedded within PRP-SF bioscaffolds at day 7 and 14 after thawing. Notes: [e]= effects; [i]= interations.

Lastly, the addition of sucrose 0,2M showed better viable cell number in proliferation activity than embedded MSCs within PRP-SF bioscaffolds cryopreserved without CPAs, 7 and 14 days after thawing (Table 1, Figure 3A,B). These results were reflected in a positive effect at both time-points: 0,003 at day 7 and in 0,0293 at day 14 (Table 2). However, embedded MSCs within PRP-SF bioscaffolds cryopreserved with sucrose 0,2M did not reach the viable cell number in proliferation quantified in embedded MSCs within PRP-SF bioscaffolds non-cryopreserved or cryopreserved with DMSO (Figure 3A,B). In accordance, less viable MSCs were detected in embedded MSCs within PRP-SF bioscaffolds cryopreserved with sucrose 0,2M after calcein/ethidium staining (Figure 3C), indicating that sucrose has protective effects in cryopreservation, but it is not enough to reach non-cryopreserved conditions. When sucrose 0,2M was combined with DMSO, similar viable cell number in proliferation to non-cryopreserved conditions were detected (Figure 3A-C), but this interaction did not overtake the effects obtained by only DMSO (Table 2). Altogether highlights that the studied non-penetrant CPAs have not significant cryoprotective effects, unless they are combined with a penetrant CPA such as DMSO [43,50].

3.4. Characterization of cryopreserved PRP-SF scaffolds

From the results described above, we conclude that the combination of DMSO and sucrose in the cryopreservation of embedded MSCs within PRP-SF bioscaffolds could

provide similar results in viable cell number in proliferation than DMSO alone. Therefore, we aimed to characterized more exhaustively the effects of cryopreservation with either DMSO or DMSO combined with sucrose to define which CPA could maintain embedded MSCs in better conditions. We compared both CPA solutions with embedded MSCs within PRP-SF bioscaffolds non-cryopreserved, cryopreserved without CPA or cryopreserved with sucrose 7 days after thawing.

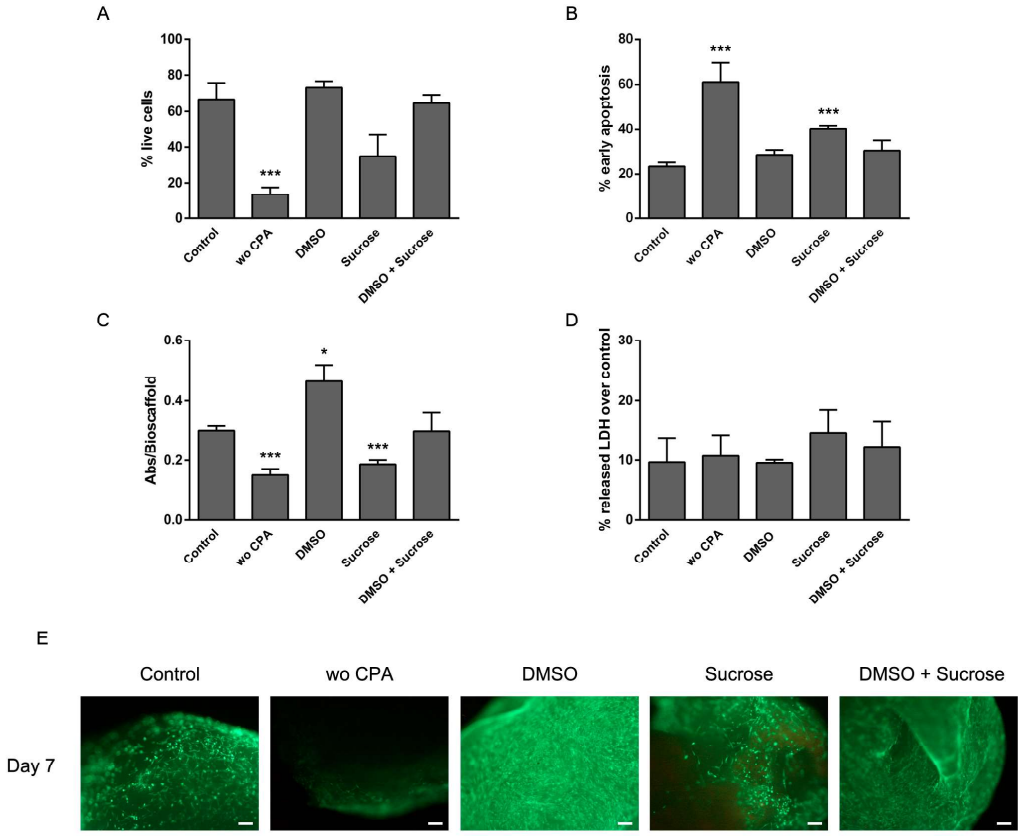

Figure 4. In vitro characterization of cryopreserved embedded MSCs within PRP-SF bioscaffolds 7 days after thawing. Quantification by flow cytometry of A) live cell percentage after calcein/ethidium staining and B) early apoptotic cell percentage after Annexin/PI staining. C) Quantification of viable cell number in proliferation by CCK-8. D) Quantification of membrane integrity by LDH release. E). Micrographs of calcein/ethidium stained samples. Note: Control: non-cryopreserved bioscaffolds; without CPA and additives :wo CPA; DMSO: DMSO 10%; Sucrose: Sucrose 0,2M; DMSO + Sucrose: DMSO 10% + Sucrose 0,2M; Values represent mean ± SD. *: $p < 0.05$, **: $p < 0.01$ and ***: $p < 0.001$ compared to non-cryopreserved group. Scale bar: 100 µm

First, we determined cell viability by flow cytometry after calcein/ethidium staining. Embedded MSCs within PRP-SF bioscaffolds cryopreserved with DMSO at 10% or the combination of DMSO 10% and sucrose 0,2M showed similar alive cells percentage

(70%) than non-cryopreserved embedded MSCs within PRP-SF bioscaffolds (Figure 4A). These viability percentages are in accordance with other studies, such as the cryopreservation of Saos-2 osteosarcoma and HaCaT embedded within poly(vinyl alcohol)-Carrageenan scaffolds [51]. Furthermore, embedded MSCs within PRP-SF bioscaffolds cryopreserved without CPAs or cryopreserved with sucrose showed lower percentage of alive cells than non-cryopreserved bioscaffolds, with significant differences ($p<0,001$) in bioscaffolds cryopreserved without CPAs (Figure 4A).

Next, we quantified the percentage of early apoptosis after Annexin-V staining. In accordance with the quantification of the percentage of alive cells, embedded MSCs within PRP-SF bioscaffolds cryopreserved with DMSO at 10% or the combination of DMSO 10% and sucrose 0,2M showed similar early apoptotic cell percentages than non-cryopreserved embedded MSCs within PRP-SF bioscaffolds (Figure 4B). However, compared to non-cryopreserved bioscaffolds, statistically significant ($p<0,001$) higher percentage of early apoptotic cells were quantified in those samples cryopreserved without CPA or cryopreserved with sucrose, reaching values of 60% and 40% respectively. Other studies have shown the maintenance of cryoinjury in cryopreserved MSCs isolated from Wharton´s Jelly tissue with DMSO after thawing and culturing for several days, with a maximum rate of apoptotic cell percentage at 20% [52]. Although we detected higher apoptotic MSCs percentages in our cryopreserved bioscaffolds, we consider that those percentages represent the addition of two effects: cryoinjury and damaged generated by the bioscaffold breakup process required for their quantification. Nevertheless, we believe that the differences quantified among the different groups are caused by the cryoinjury phenomenon since all the samples followed the same breakup procedure.

We repeated the quantification of viable cell number in proliferation with a higher number of bioscaffolds than in the preliminary studies described above to avoid conclusions slanted by inter-variability among patients. We quantified statistically significant ($p<0,05$) highest viable cell number in proliferation values in bioscaffolds cryopreserved with DMSO 10%. This effect is in accordance with those studies described by other authors, where for example, 1-month storaged MSCs shows higher proliferation rates than non-cryopreserved cells, leading to the theory of a cell selection of “stronger” cells after their storage [28,53]. Moreover, we hypothesized that changes in the PRP-SF bioscaffold network during cryopreservation with only DMSO, that maybe do not occur in the presence of sucrose, could also permit a superior spreading of the embedded cells through the bioscaffold. The combination of DMSO 10% and Sucrose 0,2M showed similar viable cell number in proliferation than non-cryopreserved bioscaffolds, but statistically significant ($p<0,001$) lower viable cell number in proliferation was quantified in samples cryopreserved without

CPA or cryopreserved with sucrose (Figure 4C).

When cell lactate dehydrogenase release was quantified, no differences among all the studied groups were identified (Figure 4D), indicating than even when viable cell number in proliferation and apoptosis are affected, cell membrane integrity was intact. Finally, the detected differences in viable cell number in proliferation and early apoptosis results were confirmed by fluorescence microscopy after calcein/ethidium staining (Figure 4E). Samples cryopreserved with DMSO 10% showed the highest number of alive cells, supporting the proliferation increase of MSCs previously described. Non-cryopreserved and cryopreserved with the combination of DMSO 10% and Sucrose 0,2M samples showed high viabilities, although lower than DMSO cryopreserved samples. Sucrose 0,2M cryopreserved bioscaffolds showed lower number of alive cells and cryopreserved bioscaffolds without CPA only showed few viable cells. Altogether, we can conclude that among the CPA studied the most appropriate for cryopreservation of embedded MSCs within PRP-SF bioscaffolds are DMSO and the combination of DMSO and sucrose, in terms of maintenance of viable cell number in proliferation activity after thawing.

3.5. Differentiation potential of MSCs released from PRP-SF bioscaffolds

The maintenance of the multilineage capacity of the cryopreserved MSCs in the PRP-SF bioscaffolds for their posterior use in cartilage regeneration is a crucial aspect. Although several studies have confirmed that cryopreservation does not affect the differentiation capacity of MSCs [28], the inclusion and cryopreservation of MSCs within PRP-SF could affect its potential to differentiate into osteogenic, adipogenic and chondrogenic lineages after their release from the PRP-SF bioscaffolds. Therefore, after confirming the best CPA solutions for the maintenance of viable cell number in proliferation of embedded MSCs within PRP-SF bioscaffolds, we determine if MSCs were still functional after their release from the cryopreserved PRP-SF bioscaffolds with the selected solutions.

After three weeks with osteogenic differentiation medium, similar calcified matrixes were observed among non-cryopreserved and cryopreserved with DMSO 10% or the combination of DMSO 10% and Sucrose 0,2M (Figure 5). When incubated with adipogenic differentiation medium, the presence of vacuoles were also detected in all the samples with no qualitative differences (Figure 5). Lastly, cell pellets incubated 3 weeks with the chondrogenic differentiation medium showed positive blue staining for cartilage matrixes in all the groups (Figure 5). With all these results it can be concluded that the cryopreservation of embedded MSCs within PRP-SF bioscaffolds does not provoke their loss of multilineage differentiation potential.

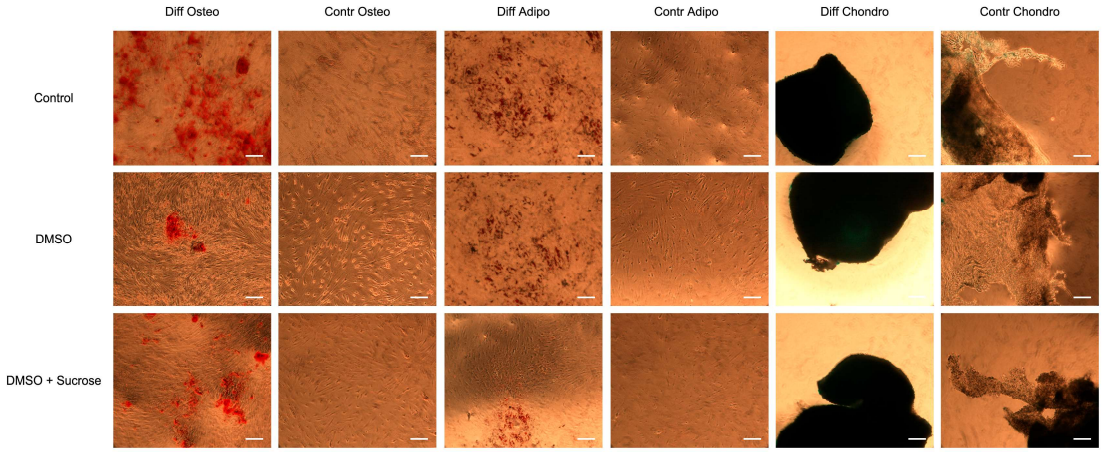

Figure 5: Differentiation potential of cryopreserved MSCs within PRP-SF bioscaffolds. Microscopic images of 3 weeks MSCs released from thawed PRP-SF bioscaffolds and differentiated into osteocytes, adypocites or chondrocytes. Note: Control: non cryopreserved; DMSO: DMSO 10%; Sucrose: Sucrose 0,2M; DMSO + Sucrose: DMSO 10% + Sucrose 0,2M. Osteo: osteogenic differentiation; Contr Osteo: non osteogenic differentiation (control); Adipo: adipogenic differentiation; Contr Osteo: non adipogenic differentiation (control); Chondro: chondrogenic differentiation; Contr Chondro: non chonrogenic differentiation (control). Scale bar: 100 µm

4. CONCLUSIONS

PRP-SF bioscaffold provides an adequate structure and environment for the short time preservation of the scarce MSCs isolated from the synovial fluid of patients. However, healthy young MSCs should be isolated and cryopreserved within PRP-SF bioscaffolds for the future treatment of cartilage defects, since the number, growth potential and replicative capacity of MSCs decrease with age. We have demonstrated that the cryopreservation of embedded MSCs withing PRP-SF with DMSO 10% or the combination of DMSO 10% and sucrose 0,2M provides optimal viable cell number in proliferation after thawing, while maintaining the multilineage potential differentiation properties of MSCs, importantly to chondrogenic lineage, the cell type responsible for cartilage regeneration.

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6

Discussion

6. DISCUSSION

The use of cell therapies for the treatment of diverse diseases has increased enormously in the last decades, promoting the research of the areas that will enable the advance and spread of these therapies. In this respect, cryopreservation, a process that will allow the correct storage, shipment and delivery of these products has been intensively investigated for translating cell therapies from bench to clinics [1]. In the cryopreservation, the cell-based products are stored at deep cryogenic temperatures "holding their biological clock" and facilitating the "on demand" access of patients to the treatments in a clinic far from the manufacturer [2,3]. On this regard, the controlled rate or slow freezing is the most used procedure for preserving cell-based products (e.g., embryos, cells, blood products or stem cells among others) in research [4-6] and clinics [7,8]. During the slow freezing cryopreservation, the cell-based products are subjected to different processes, such as samples preparation to freezing, cooling or thawing among others, and each of these has to be properly performed to provide a quality product that ensures the previous characteristics are kept [9].

In this sense, several research groups are modifying the cryoprotectant (CPA) solution to enhance the cell-based product viability and maintain its structure after its recovery. The combination of permeant (e.g. dimethylsulfoxide (DMSO), glycerol and ethylene glycol) and non-permeant (e.g. sucrose and trehalose) CPAs, with the inclusion of adjuvant molecules (e.g. serum, antioxidants and nucleating agents), is one of the most studied research lines to enhance cell-based product outcome after thawing, and reduce the used DMSO concentration, which was related to adverse effects after the transplantation of cryopreserved cell therapy products [10,11]. For example, trehalose and sucrose have been combined with DMSO in several CPA solutions enhancing cell viability, or even some cell types, such as fibroblasts, have been cryopreserved with only trehalose obtaining good results [12,13]. Moreover, low molecular weight hyaluronan (low MW-HA), a natural and nontoxic anionic non-sulfated glycosaminoglycan, has demonstrated cryoprotective effects in the storage of human dermal fibroblast monolayers [14,15], and in the preservation of mesenchymal stem cells (MSC) in combination with DMSO [16]. Taking these into account, this doctoral thesis is intended to investigate further the CPA election for 3D cell-based products using cell microencapsulation as a model of study.

6.1. DMSO provides the best cryoprotection to 3D cell-based products compared to traditional CPA

With the aim of apporaching the use 3D cell-based product further to clinics, in this

study we determined the optimal current CPA for the cryopreservation of these cell therapy products. In this context, due to our know-how in cell encapsulation technology and its multiple posibilities for the treatment of several diseases, we employed microencapsulated cells as model of study of 3D cell-based products. Compared to traditional pharmacological therapies, microencapsulated cells provide the advantage of being a source of sustained continuous release of "de novo" therapeutic products. Also, the encapsulation technology avoids the need of repeated drugs administration, and protects the inner cells from the immune system, being the microencapsulated cells evaluated for the treatment of a wide range of pathologies [17,18]. However, bearing in mind clinical translation, efficient storage procedures are needed to get therapeutic microencapsulated cells "on demand" [2,3]. Slow freezing cryopreservation has demonstrated to be the best procedure to preserve several microencapsulated cell types, being DMSO the most used CPA for their freezing [9]. Although different cooling protocols have been studied [19-22] and some adjuvants have been added to enhance cryopreservation outcome [20,23], a deep study comparing traditional CPAs to DMSO has not been investigated for the slow freezing cryopreservation of microencapsulated cells [10]. In this study, we have moved a step forward and compared some of the most often used CPAs (DMSO, trehalose and glycerol) in clinical studies for the cryopreservation of D1 mesenchymal stem cells (D1MSC) microencapsulated within 180µm alginate microcapsules, assessing cell function and microcapsules integrity (a parameter unusually determined in alginate microcapsules) after thawing. For generating a suitable cell model for the determination of the best CPA solution on the storage of this cell type after microencapsulation, we transfected D1MSCs successfully to permanently express the therapeutic protein erythropoietin (D1MSC-Epo), generating a suitable cell model for the determination of the best CPA solution on the storage of this cell type after microencapsulation.

First, we intended to asses which CPA were able to maintain microcapsules integrity after thawing, is a critic parameter in cell encapsulation, since the implantation of capsules and devices with wrinkles, acute angles or sharp edges, induce higher foreign body reaction after implantation [24-26]. For that aim, we cryopreserved empty 180µm alginate microcapsules with several CPA combinations of (DMSO, glycerol and trehalose) at different concentrations, and observed the microcapsules integrity by light microscopy, such as circularity and broken microcapsules after thawing.

The empty alginate microcapsules stored with the non-penetrating CPA trehalose at 10% did not provide the appropriate physical recovery (Figure 1 F), presenting similar wrinkles to a study in which human retinal pigment epithelial cells ARPE-19 microcapsules were preserved with trehalose by freeze drying [27]. This phenomenon could be explained by the interaction of trehalose and poly-L-lysine (PLL) that modifies the original secondary

structure of PLL [28]. In this respect, the increasing concentration of trehalose promoted wrinkles formation during the freeze-thaw process, whereas the increasing DMSO concentration protected the alginate microcapsules of this phenomenon (Figure 1 A-I). On the other hand, another penetrating CPA, glycerol, formed wrinkles on microcapsules after thawing, similar to the results reported with alginate poly-L-lysine microcapsules loaded with bifidobacteria [5]. Taking these into account, the CPA solutions that presented high wrinkles formation were excluded from the following studies, with the exception of the trehalose 10%, which was taken as a positive control of impaired microcapsules. Regarding microcapsules integrity after exposition to the extreme conditions of the swelling test, the microcapsules stored with DMSO 10% were the ones that maintained the capsules integrity as non-cryopreserved microcapsules (Figure 1 J). The other cryopreserved groups (DMSO 5%, DMSO 5% + trehalose 5%, glycerol 10% or trehalose 10%,), presented high percentages of broken capsules, although the intact capsules of all cryopreserved groups did not showed significant differences from the initial diameter after the test (Figure 2 K). With this set of experiments it was concluded that the trehalose inclusion in the CPA solution was a negative factor for microcapsules integrity maintenance in slow freezing cryopreservation.

After assessing the influence of CPAs in microcapsules integrity, we determined the effects with the selected CPAs in the microencapsulated cell function in terms of differentiation potential, therapeutic protein secretion, cell viability and metabolic activity after thawing (Figure 2 A-E). Differentiation potential maintenance of MSCs is one parameter assessed after cryopreservation. It has been previously reported that MSCs keep their differentiation potential to osteogenic, adipogenic and chondrogenic lineages after microencapsulation [29], even when they are cryopreserved with DMSO 10% [30]. In this sense, we studied other CPA solutions (DMSO 5%, DMSO 5% + trehalose 5%, glycerol 10% or trehalose 10%,) not observing almost differences on the differentiation capacity of D1MSCs-Epo (Figure 2 A), indicating that the differentiation potential of microencapsulated MSCs is not affected by the CPA solution. However, cell viability and metabolic activity depended on the CPA used (Figure 2 C-D). Trehalose 10% dramatically decreased microencapsulated D1MSCs-Epo viability, maybe due to the formation of wrinkles in the microcapsule surface which could affect the exchange of oxygen, nutrients or cell waste with the medium. Although there are some studies in which fibroblast were successfully cryopreserved with only trehalose as a CPA, compared to this study, the employed cooling rates were much higher, and the cryoprotective effect of trehalose was exerted mainly by the cells prefreezing load with trehalose [31,32]. The combination of trehalose 5% with DMSO 5% was able to increase cell viability and metabolic activity compared to the aforementioned CPA solutions, showing that the presence of DMSO improved the recovery of the microencapsulated D1MSCs-Epo.

It should be taken into consideration that the reduction from 10% to 5% of DMSO resulted in lower viability and metabolic activity on our microencapsulated D1MSCs-Epo, confirmed by the viability reduction in the cryopreservation of microencapsulated primary MSCs in others studies [30].

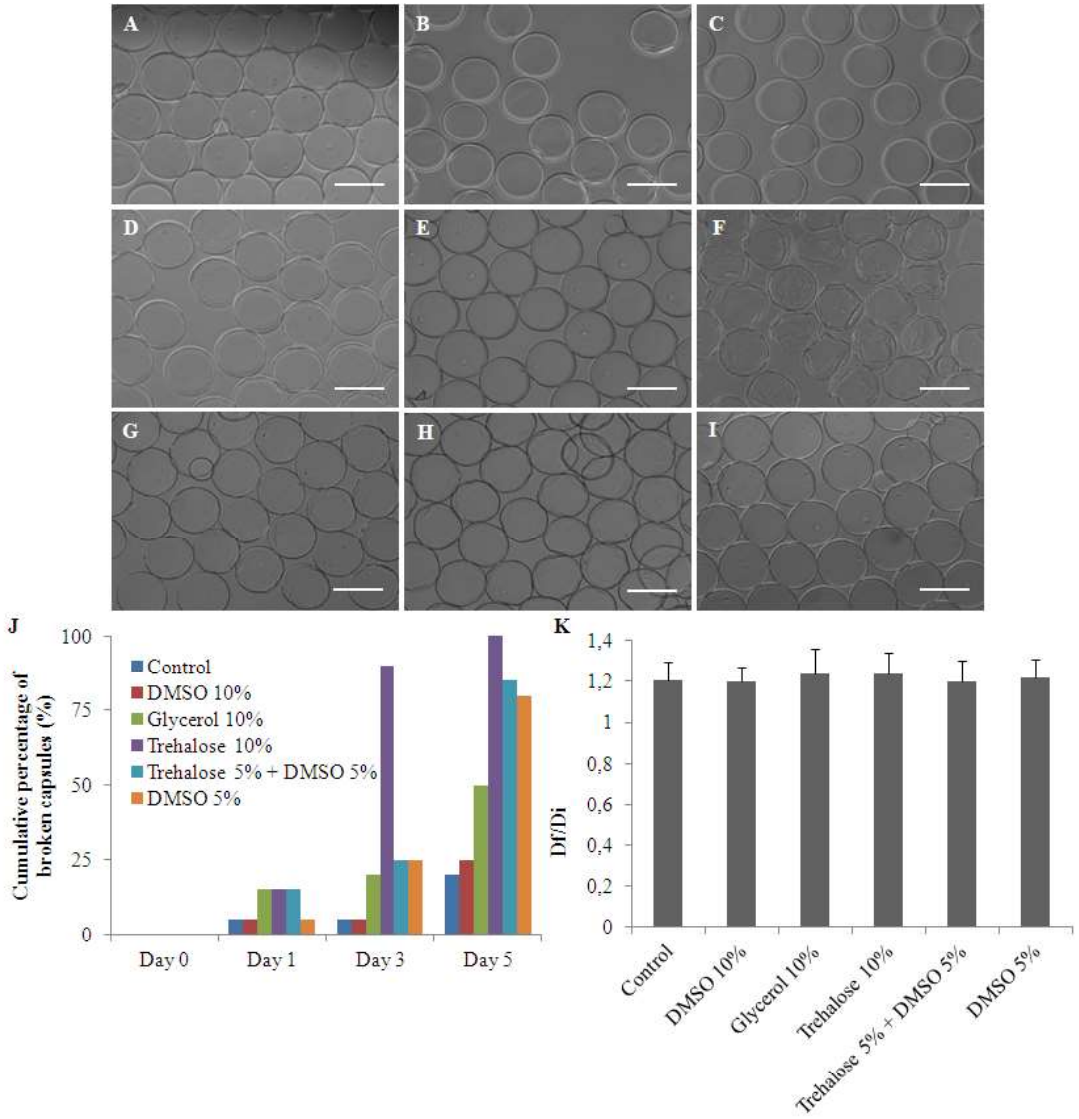

Figure 1: Microcapsules recovery after thawing with several cryoprotectants formulations: Microscopy images after thawing of cryopreserved microcapsules with: A) Non cryopreserved (control), B) DMSO10%, C) DMSO5%, D) Glycerol 10%, E) Trehalose 5% DMSO5% and F) Trehalose 10% G) Trehalose 5% DMSO 2.5%, H) Trehalose 2.5% DMSO2.5% and I) Trehalose 2.5% DMSO5%. Scale bar: 200 um. J) Cumulative percentage of broken microcapsules after 5 days of citrate treatment (swelling assay). K) Ratio (final diameter/initial diameter) from non broken microcapsules after 3 days exposed to sodium citrate.

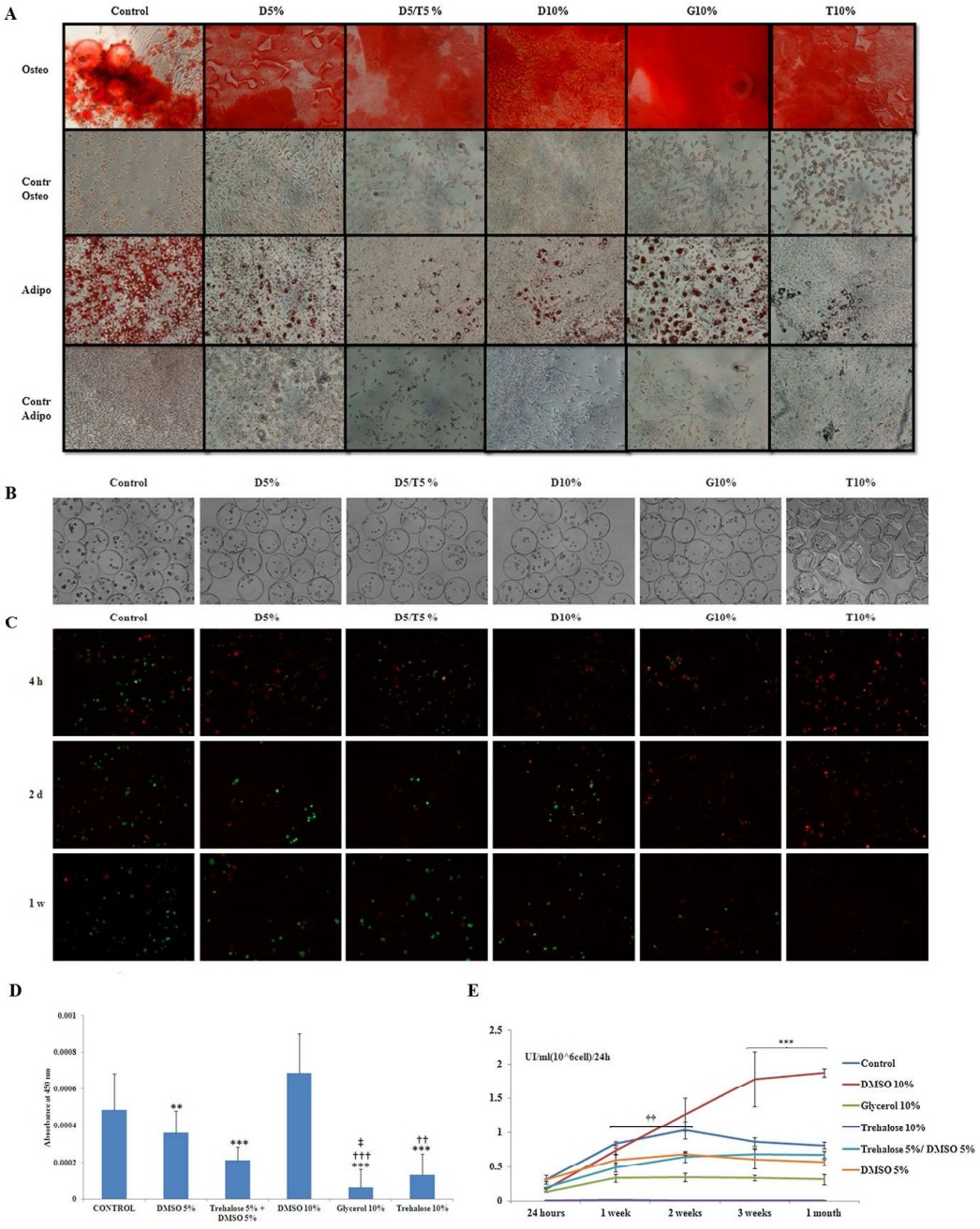

Figure 2: Differentiation potential, viability, metabolic activity and transgene secretion of cryopreserved microencapsulated D1MSCs-Epo with different CPA solutions after thawing. A) Differentiation potential of cryopreserved microencapsulated D1MSCs-Epo after thawing. Microscopic images at 10 x amplification 3 weeks after differentiation. B) Microscopy images of capsules and C) Calcein/ethidium microscopy images at 4 hours (4h), 2 days (2d) and 1 week (1w) after thawing. C) Graphical representation of metabolic activity measured by means of CCK8 assay 1 week after thawing. D) Epo expression and secretion during time of cryopreserved microencapsulated D1MSCs-Epo after thawing. Note: Control: non cryopreserved microcapsules; D5%: DMSO5%; D5/T5%: DMSO 5% + trehalose 5%; D10%: DMSO 10%; G10%: glycerol 10% and T10%: trehalose 10%; Osteo: osteogenic differentiation; Contr Osteo: non osteogenic differentiation (staining control); Adipo: adipogenic differentiation and Contr Adipo: non adipogenic differentiation (staining control). *: compared to DMSO 10% (***: p<0.001, **: p< 0.01 and *: p< 0.05), †: compared to control (†††: p<0.001, ††: p< 0.01)and ‡ compared to DMSO 5% p< 0.05.

Our research group has previously observed a 40% decrease on Epo release when microencapsulated C2C12 myoblasts secreting Epo were cryopreserved with DMSO 10%, and a 80% decrease when were cryopreserved with DMSO 5% [22]. Similarly, two weeks after thawing, we detected a 50% reduction on Epo release from cryopreserved microencapsulated D1MSCs-Epo with DMSO 10% compared to those cryopreserved with DMSO 5% (Figure 2 E). Moreover, cell proliferation seems to enhance over the time after cryopreservation with DMSO 10% compared to non-cryopreserved and, therefore, the release Epo reaches higher rates than non-cryopreserved microcapsules (Figure 2 E). These results are in accordance to studies in which cryopreserved and non-encapsulated MSCs have showed that their proliferation rate with DMSO after 1-month storage was significantly higher than that of unfrozen control cells. Authors proposed a cell selection of "stronger" cells after cryopreservation [33], a hypothesis that can explain the results described on this study. Our in vitro results can be extrapolated to in vivo outcomes on hematocrit percentage when cryopreserved microencapsulated MSCs-Epo were implanted (Figure 3). Microencapsulated D1MSCs-Epo cryopreserved with DMSO 10% were able to increase the hematocrit similarly to non-cryopreserved D1MSCs-Epo during the time studied, while those cryopreserved with DMSO 5% were not (Figure 3), not showing statistical differences on the hematocrit values between mice implanted with non-cryopreserved microcapsules and cryopreserved with DMSO10% (Figure 3).

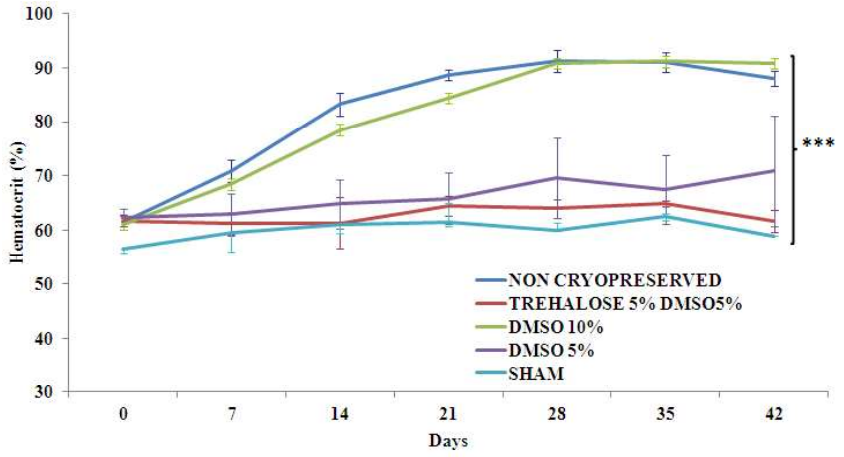

Figure 3: Hematocrit levels of implanted Balb/c mice with cryopreserved microencapsulated MSCs-Epo. Evaluation of non-cyropreserved microcapsule implantation vs cryopreserved microcapsules for 45 days. Sham control: PBS. Values represent mean + SD. Note: ***, $p<0.001$.

With all these results, we concluded that among the assessed CPA combinations, the most suitable CPA for in vitro and in vivo microencapsulated cell function maintenance after

thawing is DMSO 10%. The addition of other CPAs, such as trehalose or glycerol, impairs microencapsulated cell viability and microcapsules integrity after the freeze-thaw process. Altogether, this study remarks the importance of the proper selection of the CPA solution before the use of microencapsulated cells can be translated into the clinic.

6.2. Low molecular weight hyaluronan for the cryopreservation of 3D cell-based products

DMSO is a suitable CPA in the cryopreservation of 3D cell-based products [22,30,34], but its adverse effects after transplantation encouraged us to continue in the research of novel CPAs for the cryopreservation of encapsulated cells. In this respect, hyaluronan (HA) an anionic, nonsulfated glycosaminoglycan with molecular weight (MW) ranging from 10^4 to 10^6 Da, has been postulated as a natural non toxic CPA [14,15]. This macromolecule is integrated by the repetition of a disaccharide unit of an N-acetylglucosamine and a β-glucuronic acid, and interacts with cells through the CD44 surface receptor [35]. HA has been described for being involved in a wide variety of biological procedures (e.g., cell signaling mediation, regulation of cell adhesion and proliferation) [36], and due to its favorable mechanical properties, its use has been extensively promoted in tissue engineering over the last decades [37-39]. Moreover, it has been demonstrated that low MW-HA is able to preserve human dermal fibrotic monolayers [14,15], and improve the viability and cell proliferation of human MSCs after cryopreservation when HA was combined with DMSO in the CPA solution [16]. Based on these evidence, first, we aimed to assess if low MW-HA at 5% displays similar cryoprotective effects than DMSO with D1 mesenchymal stem cells (D1MSCs) or myoblasts (C2C12) microencapsulated within 380µm alginate microcapsules as a model of 3D cell-based products (Figure 4).

In the first set of experiments, microencapsulated C2C12 or D1MSC stored with low MW-HA 5% after 20 minutes equilibration time on ice, did not show statistical metabolic activity and viability differences 1 and 7 days in culture after thawing, independently of the incubation time at 4ºC, when compared with microencapsulated cells cryopreserved with DMSO 10% or the commercial CPA solution called CS10 (Figure 4 A-C). Also, low MW-HA was able to maintain the spherical structure of microcapsules after thawing, displaying similar round smooth surfaces than non-cryopreserved microcapsules (Figure 4 C), which is a critic parameter in cell encapsulation, since the implantation of capsules and devices with wrinkles, acute angles or sharp edges, induce higher foreign body reaction after implantation [24-26]. With these results, we first demonstrated that low MW-HA retains the same metabolic activity than DMSO 10% or CS10 after thawing in both cryopreserved encapsulated cells,

and second, that this effect is achieved with just 20 minutes equilibration time on ice. In this sense, this short period of incubation time indicates that low MW-HA acts outside the cell since biological mechanisms require longer periods than 20 minutes to internalize HA, especially when the cell metabolic activity is slowed down by ice [40]. On this regard, low MW-HA could exert its extracellular cryoprotective effect by acting as a non-permeating CPA, such as sucrose or trehalose, stabilizing biological membranes and proteins by direct interaction of HA with polar residues through hydrogen bonding, and forming a metastable glass upon water cell dehydration [41]. Although non-permeant CPAs, such as low MW-HA, are usually used in combination to permeant CPAs to avoid the intracellular ice formation [42,43], good cell outcome after cryopreservation with only non-permeant CPAs have also been reported [44].

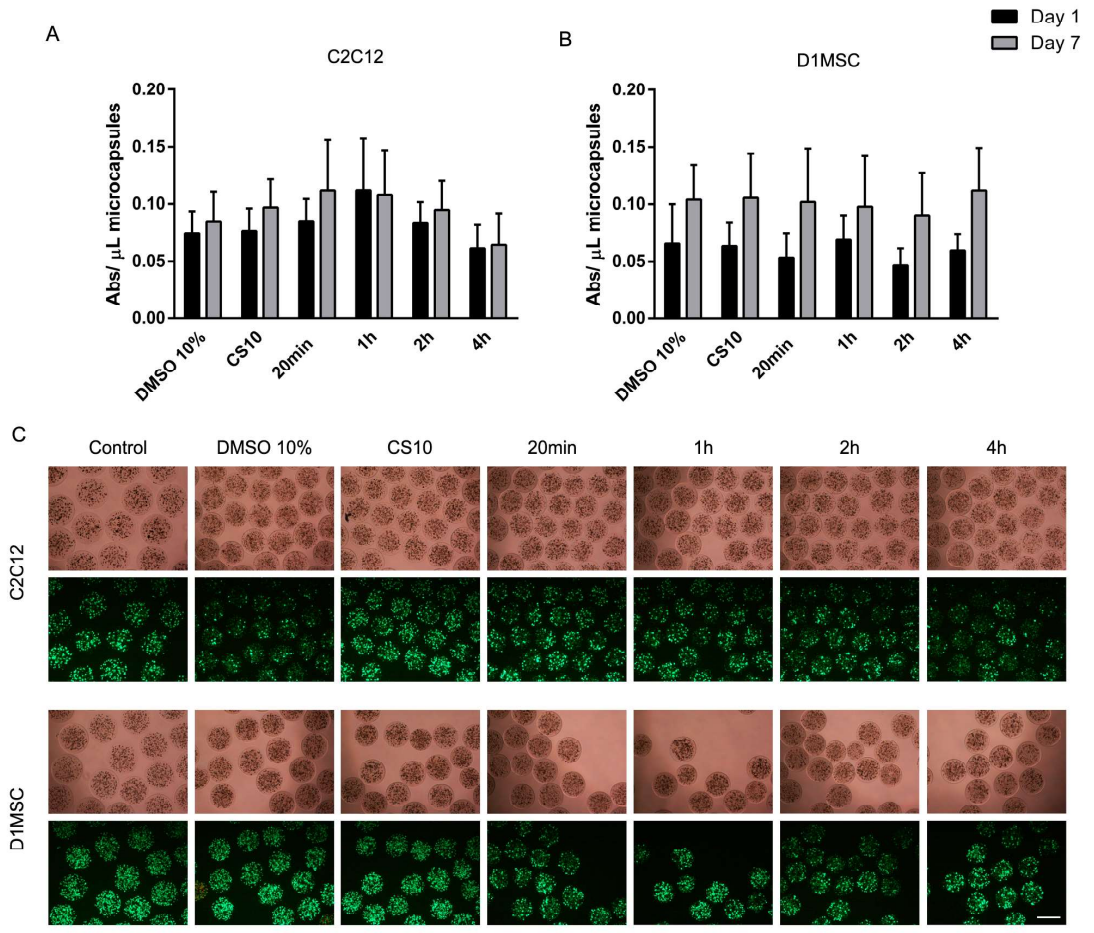

Figure 4. Determination of low MW-HA equilibration time before cryopreservation by comparison with DMSO and CS10. Metabolic activity 1 and 7 days in culture after thawing of microencapsulated C2C12 (A) and D1MSC (B) cells, cryopreserved by pre-incubation at 4ºC for 20 minutes, 1, 2 and 4 hours. C) Micrographs of calcein/ethidium stained cryopreserved microencapsulated C2C12 and D1MSC cells 7 days in culture after thawing. Note: Control: non-cryopreserved bioscaffolds. Values represent mean ± SD. Scale bar: 400µm

Since the preincubation at 37ºC with low MW-HA, followed by cryopreservation with either with DMSO [15] or low MW-HA [14], can improve the viability of human dermal fibroblasts cells after thawing, we studied if this preincubation could improve the outcomes of cryopreserved microencapsulated cells with both CPAs. Thus, we first incubated D1MSCs and C2C12 cells with low MW-HA 0.5% at 37ºC for 45 minutes or 2, 6 and 24 hours, next microencapsulating those cells and freezing with either low MW-HA 5% or DMSO 10% (Figure 5).

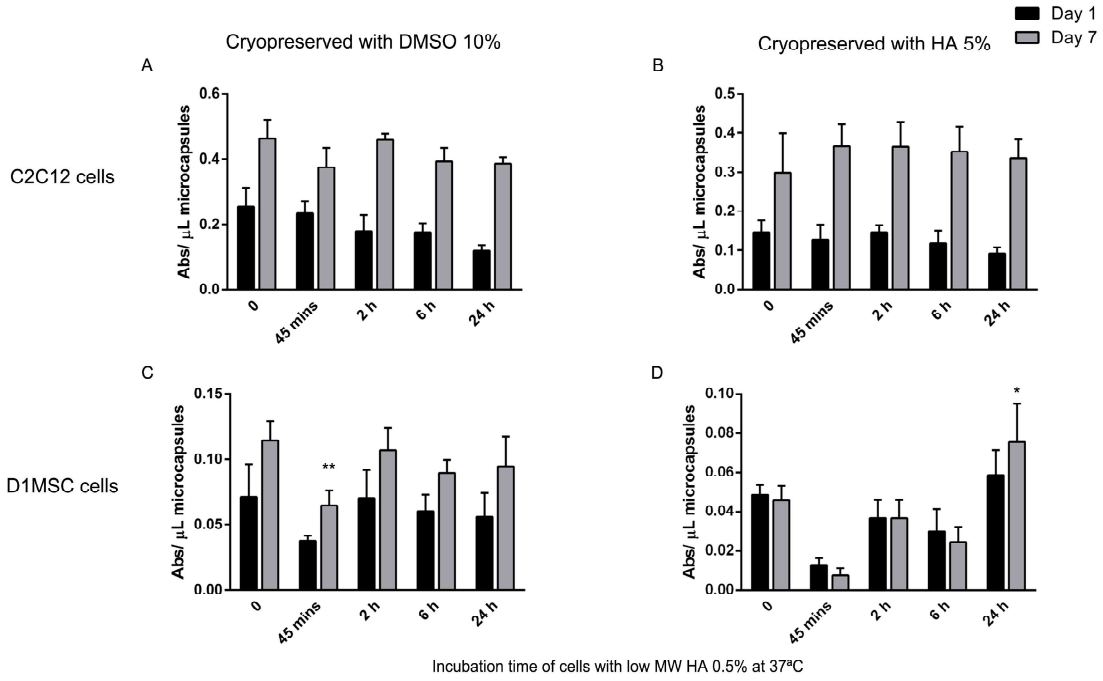

Figure 5. Effect of preincubation with low MW-HA 0,5% on metabolic activity of cryopreserved mictoencapsulated cells after thawing. Metabolic activity 1 and 7 days in culture after thawing of C2C12 cells incubated for 45 minutes, 2, 6 and 24 hours with low MW HA 0,5% at 37ºC, next microencapsulated and cryopreserved with (A) DMSO 10% or (B) low MW-HA 5%. Metabolic activity 1 and 7 days in culture after thawing of D1MSC cells incubated for 45 minutes, 2, 6 and 24 hours with low MW HA 0,5% at 37ºC, next microencapsulated and cryopreserved with (C) DMSO 10% or (D) low MW-HA 5%. Note: Values represent mean ± SD. *: $p < 0.05$ and **: $p < 0.01$ compared to the group with no incubation of low MW HA 0,5%.

Thawed microencapsulated C2C12 maintained the same metabolic activity after 1 and 7 days in culture, independently of the incubation time with the CPA used (Figure 6 A-B). Similarly, microencapsulated D1MSCs following the same procedure with DMSO 10% retained their metabolic activity after thawing (Figure 5 C). This results contrasts with the reported viability enhancement displayed by human dermal fibroblast monolayers [15], or the proliferation improvement in human MSCs [16]. However, the metabolic activity

of microencapsulated D1MSCs previously preincubated with low MW-HA 0.5% for 24 hours, and cryopreserved with low MW-HA 5%, significantly enhanced ($p<0.05$) 7 days after thawing in culture (Figure 6 D), in agreement with the synergistic effect described in human dermal fibroblast monolayers [14]. The discordance with non-permeating CPAs has also been described with trehalose. Preincubation of either suspended or attached fibroblast with trehalose at 37ºC showed different membrane integrity depending on the trehalose concentration in the CPA solution [32]. CPA solutions with low trehalose concentrations (25-50mM) showed a slight enhancement of membrane integrity after thawing the trehalose-preincubated fibroblasts, whereas the cells cryopreserved with CPA solutions with higher trehalose concentrations (100-500mM) diminished the viability after cryopreservation.

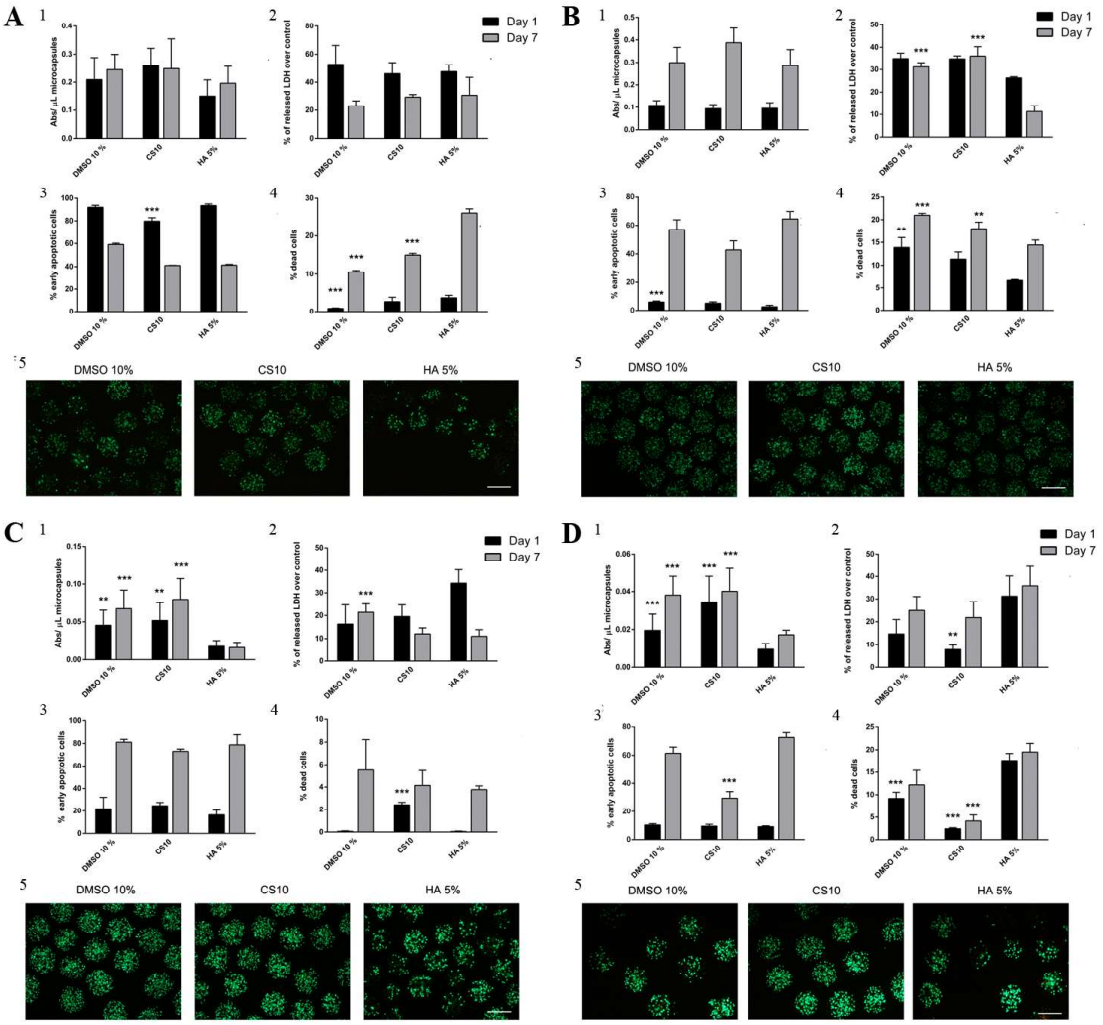

Figure 6. Assesment of cryopreserved several microencapsulated cells with the studied CPAs. Quantification 1 and 7 days after thawing of A) metabolic activity, B) membrane integrity, C) early apoptotic cell percentage, and D) dead cell percentage. E) Micrographs of calcein/ethidium stained cryopreserved microencapsulated cells 7 days in culture after thawing. Note: Values represent mean ± SD. **: $p < 0.01$ and ***: $p < 0.001$ compared to low MW HA 5%. Scale bar: 400µm

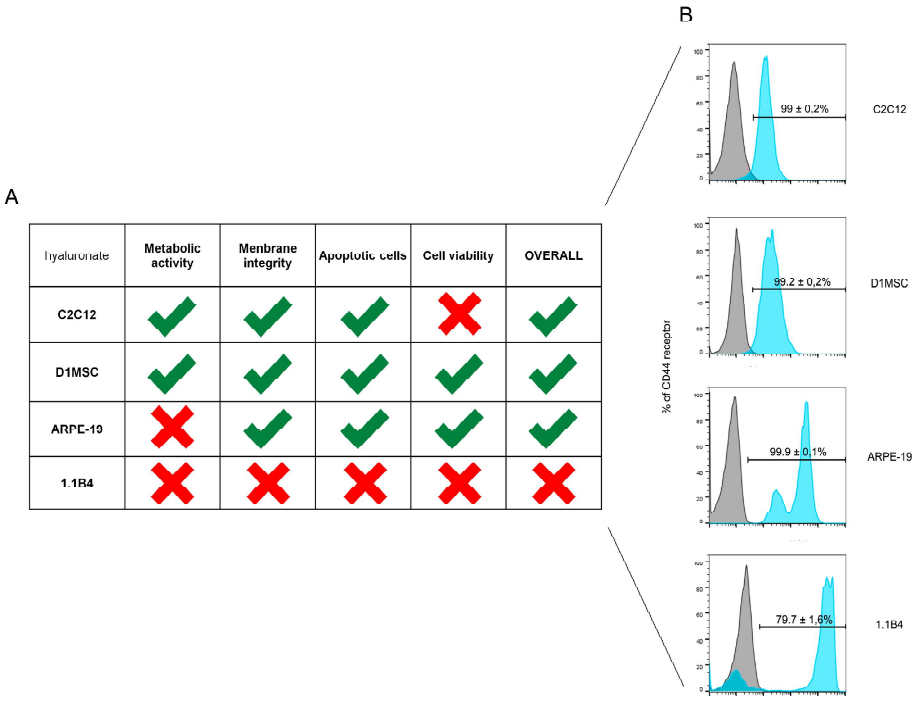

Figure 7: Overall effect of cryopreservation with low MW-HA 5%. A) Chart describing the outcomes from each assay performed in the different cryopreserved microencapsulated cell types. Green tick: similar or better outcome than the DMSO containing CPA solutions; red X: lower outcome than the DMSO containing CPA solutions. B) Flow cytometry quantification of CD44 receptor in the studied cell lines.

Therefore, after the lack of concordance in the results preincubating with low MW-HA, and in order to standardize the cryopreservation protocol of encapsulated cells with the natural non-toxic HA, we further studied the cryoprotective effect of low MW-HA, without preincubating cells with low MW-HA 0,5% at 37ºC, in the following microencapsulated cell types: the murine cell lines, C2C12 and D1MSC, and the human cell lines Arpe-19 (human retinal pigment epithelial cell line) and 1.1B4 (human insulin-secreting cell line). In microencapsulated and cryopreserved C2C12, D1MSC and Arpe-19 cells with low MW-HA 5%, thawed cells presented in overall good results after thawing compared to the other DMSO containing solutions (Figure 6 A-C,6 E). However, although microencapsulated and cryopreserved D1MSC cells presented similar or better outcomes with low MW-HA 5% compared to the other CPA solutions in all the studied assays (Figure 6 B), microencapsulated and cryopreserved C2C12 showed higher dead cells percentages with low MW-HA after 7 days in culture (Figure 6 A-4), and similarly, thawed Arpe-19 with low MW-HA displayed statisticaly lower metabolic activity after 1 ($p<0.01$) and 7 ($p<0.001$) days in culture (Figure 7 C-1). In contrast to these promising results, low MW-HA 5% did not protect microencapsulated 1.1B4 during slow freezing cryopreservation, displaying lower metabolic activity and membrane integrity, and higher early apoptosis and dead cell percentage after

1 and 7 days in culture (Figure 6 D). With the aim of summarizing the cryoprotective effect of low MW-HA 5% in microencapsulated cells, we compiled the quantified results from each assay in the different cryopreserved microencapsulated cell types in one chart (Figure 7). A positive results was considered when low MW-HA provided similar or better outcome than the DMSO containing CPA solutions, and when at least three assays displayed positive results. Overall, we concluded that low MW-HA represents an alternative DMSO free CPA solution for certain microencapsulated cell lines (Figure 7 A).

In order to discern the causes of the different responses by the microencapsulated cell lines, we quantified the single cell expression of the transmembrane CD44 receptor by flow cytometry, since CD44 is the primary receptor that interacts with HA [36,45]. As expected, all cell lines expressed CD44 (Figure 7B) [34,46,47]. However, only C2C12, D1MSC and Arpe-19 cells extensively expressed (>90%) this receptor, in contrast to 1.1B4 cell line that did not homogeneously express CD44 (<70%), correlating with the worst cryoprotective effect of low MW-HA. Complexes formed by the HA-CD44 interaction activate cell survival anti-apoptotic proteins [35,48-50]. In fact, this HA-receptor complex interacts with multiple tyrosine kinase receptors, such as ErbB2, ErbB3, or EGFR, and, consequently, activates intracellular pathways that lead to alterations in cytoskeleton and regulate certain gene expression promoting cell survival. For example, the recruitment of ErbB2 by HA-CD44 enhances the transcription of cyclooxygenase-2 through PI3K-Akt and β-catenin, further strengthening the apoptosis resistance through the increase of prostaglandin E2 expression (Figure 8)[50,51]. The expression of some CD44 isoforms has also been correlated with the apoptosis resistance in human colon cancer cells. For example, cancer cells that do not express CD44 present high apoptosis after the exposure to a cytotoxic cancer drug, whereas cells that express the CD44 receptor upregulate Akt-phosphorylation levels and downregulate the pRb tumor suppressor, presenting lower apoptosis percentages [52]. On this regard, several CPA solutions include anti-apoptotic factors in order to downregulate the overall mechanisms involved in cell apoptosis and necrosis after cryopreservation, known as “cryopreservation-induced onset cell death”, indicating a mechanism to explain the cryoprotection exerted by HA. For example, benzyloxycarbonyl-Val-Ala-DL-Asp-fluoromethylketone, a pan-caspase inhibitor, is able to enhance hepatocyte-specific functions and reduce apoptosis of microencapsulated rat hepatocytes significantly 24 hours after thawing [53]. Moreover, low MW-HA has also demonstrated high antioxidant properties [54,55]. Low MW-HA exhibites significant inhibitory effects on lipid peroxidation and glycation end products formation, and also enhances 2,2-diphenyl-1-picrylhydrazyl and nitrogen oxide radical-scavenging capacity. In this sense, the addition of antioxidants in CPA solutions is also investigated for optimizing cryopreservation procedures [56,57], since antioxidants would block the reactive

oxygen species created during the freeze-thaw processes and would decrease the induced onset cell death. Therefore, it could be postulated that HA can enhance cell recovery after cryopreservation by inhibiting the reactive oxygen species created in the freezing process.

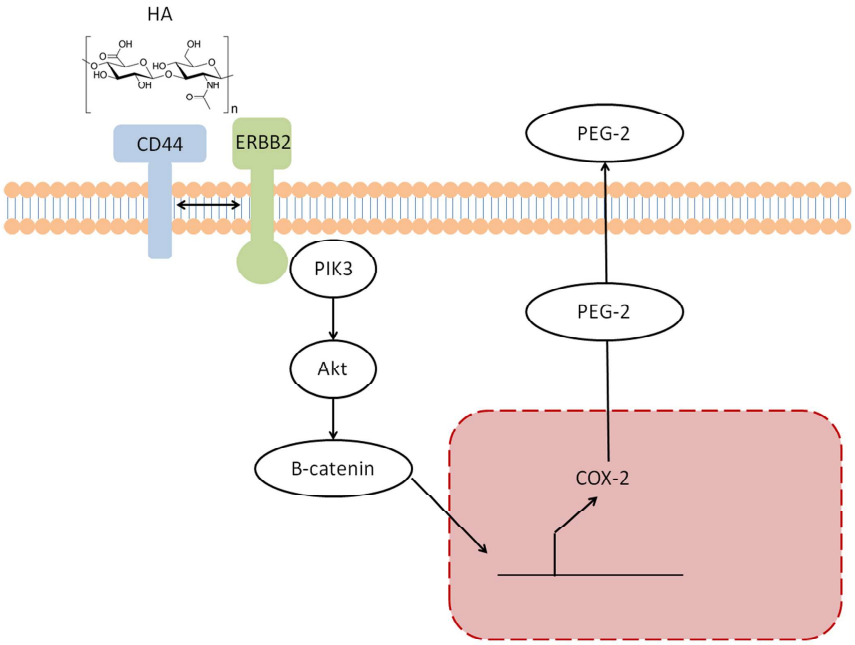

Figure 8: Apoptosis resistance strengthen model mediated by the HA-CD44 and ErbB2 interaction. The recruitment of ErbB2 by HA-CD44 enhances the transcription of cyclooxygenase-2 through PI3K-Akt and β-catenin, further strengthening the apoptosis resistance through the increase of prostaglandin E2 expression.

With this study, it can be concluded that the cryoprotective effect of low MW-HA might not only be performed following the mechanisms of a non-permeant CPA, but also acting as an anti-apoptotic factor interacting with the CD44 transmembrane receptor. This interaction would reduce apoptosis and enhance cell survival after thawing, explaining the beneficial outcomes observed with low MW-HA in microencapsulated C2C12, D1MSCs and Arpe-19 cells that homogeneously express CD44. Moreover, this study demonstrated for the first time the cryoprotective effect of low MW-HA for the slow freezing cryopreservation of several microencapsulated cells, postulating low MW-HA as a potential non-permeant CPA for the cryopreservation of several cells.

6.3. Cryopreservation of human MSCs in an allogeneic bioscaffold based on platelet rich plasma and synovial fluid combining several CPAs

Osteoarthritis (OA) is a highly prevalent degenerative joint disease that involves the cartilage and the surrounding tissue, with the pain as the clinical disease hallmark

[58]. In this sense, the implantation of autologous MSCs has emerged as an alternative to conventional therapies for the treatment of knee OA. Although nowadays MSCs from bone marrow are being used in the knee OA for cartilage repair [59-61], other MSC types such as from synovial fluid (SF) or synovial membrane, have been proposed as alternatives to their higher chondrogenic capacity and cartilage regeneration than bone marrow MSCs [62,63]. Moreover, since the migration of MSCs to the SF is enhanced and SF volume is increased when the articular cartilage, synovial membrane, subchondral bone or the knee joint are affected [64,65], and SF is routinely extracted without harming other tissues when inflammation occurs, this fluid have been postulated as a viable and adequate MSCs source for the treatment of patients suffering OA.

In collaboration with the Arthroscopic Surgery Unit of Dr. Mikel Sanchez, we developed an allogeneic and biomimetic scaffold, composed of SF and blood plasma enriched with platelets, hereafter called Platelet Rich Plasma (PRP). The mixture of PRP and SF permits the formation of an autologous bioscaffold (PRP-SF) due to the synthesis of a fibrin structure after plasma activation, showing high viabilities of embedded MSCs extracted from SF [66]. This bioscaffold can be formed during SF extraction, allowing a short preservation of embedded MSCs without the need of cell attachment and culture, simplifying the labor of the clinician in terms of time and cost, and therefore, representing an alternative to the standard procedure of isolation and preservation of MSCs from SF. However, since the number, the growth potential and the replicative capacity of the MSCs from the patient decrease, while the age of the patient increases [67,68], the long-time preservation of MSCs within PRP-SF bioscaffold would be extremely convenient for the future clinical treatment of OA patients. Thus, after determining the benefitial outcomes of HA in the cryopreservation of microencapsulated cells, we assesed in a clinical 3D cell-based product such as PRP-SF bioscaffold which contains natural HA in its structure, the slow freezing of SF derived MSCs using other conventional CPAs such as DMSO as a first approach, allowing to move this biosystem from the bench to the clinic.

First, we isolated and characterized the MSCs extracted from a patient SF, confirming their mesenchymal stem cell nature. Since SF is a viscous liquid composed of lubricin, hyaluronan (HA), growth factors and cytokines [69], and based in our previous observation on HA cryoprotective properties [70], we intended to analyze if HA could exert cryoprotective effects in the slow freezing of MSCs within the PRP-SF bioscaffolds without the addition of DMSO in the 3D cell-based product. With that aim, we made two different bioscaffolds for the storage of the SF derived MSCs: one composed only of PRP, which contains fibrin, a cytocompatible structure that promotes cell proliferation due to the high diversity of binding sites that contains [71], and the other, the PRP-SF, which combines fibrin

and HA. Furthermore, we studied if both bioscaffolds required the addition of DMSO and compared viabilities with non-cryopreserved bioscaffolds.

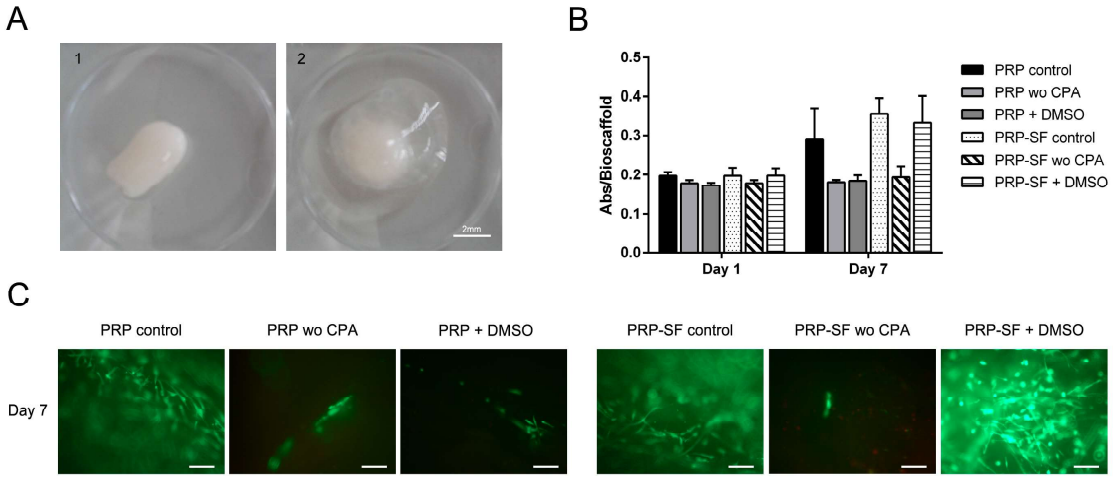

Figure 9. Cryoprotective comparison of PRP and PRP-SF bioscaffolds A) Macroscopic images of 1 day cultured PRP (1) and PRP-SF (2) bioscaffolds. B) Viable cell number in proliferation of embedded MSCs within PRP and PRP-SF bioscaffolds 1 and 7 days after thawing. C) Micrographs of calcein/ethidium stained MSCs within PRP and PRP-SF bioscaffolds 7 days after thawing. Note: PRP control: non-cryopreserved PRP bioscaffold; PRP wo CPA: PRP bioscaffold cryopreserved without CPA and additives; PRP DMSO: PRP bioscaffold cryopreserved DMSO 10%: PRP-SF control: non-cryopreserved PRP-SF bioscaffold; PRP-SF wo CPA: PRP-SF bioscaffold cryopreserved without CPA and additives; PRP-SF DMSO: PRP-SF bioscaffold cryopreserved DMSO 10%. Scale bar: 100 µm

Both bioscaffolds showed different macroscopic appearance the next day after formation. PRP bioscaffold resulted in a compact solid hydrogel structure (Figure 9 A-1) while PRP-SF showed a surrounding matrix formed of non-retracted SF (Figure 9 A-2), that was reduced overtime (data not shown). Next day after thawing, no differences were quantified between the viable cell number in proliferation of non-cryopreserved or cryopreserved PRP and PRP-SF bioscaffolds (Figure 9 B). However, 7 days in culture after formation or cryopreservation of bioscaffolds, non-cryopreserved PRP-SF and PRP-SF cryopreserved with DMSO 10% displayed higher viable cell number in proliferation of MSCs (Figure 9 B), which was confirmed by fluorescent microscopy micrographs after calcein/ethidium staining (Figure 9 C). These results indicated that PRP-SF is a more convenient bioscaffold for the cryopreservation of the MSCs. However, although we hypothesized that HA could be enough to cryopreserve the PRP-SF embedded cells, we detected a decrease in embedded MSCs viable cell number in proliferation when they were cryopreserved without DMSO (Figure 9 B). In previous studies conducted with human fibrotic monolayers and with solutions at high concentration (5%) of low molecular weight HA, the cryoprotective effect of HA

was related to its high hydration capacity and its cell internalization mediated by the CD44 receptor [14,15]. However, HA in PRP-SF bioscaffolds comes from a natural HA source and has higher molecular weight than the described in those studies. Moreover, HA in these bioscaffolds is not free in solution. Altogether precludes HA to exert its cryoprotective effect, and therefore the addition of an external CPA is required. Furthermore, the lack of protection by the cryopreserved PRP bioscaffold with DMSO 10% compared to PRP-SF bioscaffold cryopreserved with DMSO 10% after 7 days in culture, could be related to HA, fibrin and DMSO combination enhancing cell growth after thawing compared to fibrin alone in the PRP bioscaffolds. In accordance with our results, other authors have described that the proliferation and spreading of osteosarcoma cells within fibrin-HA scaffolds was significantly higher than in single (HA or fibrin) network analogs [72].

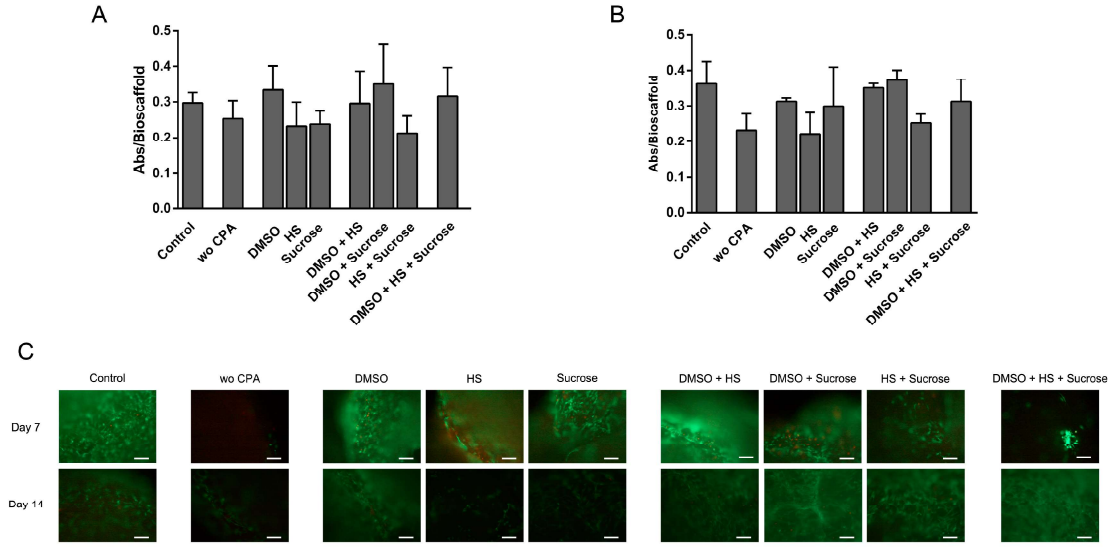

Figure 10. Effects and interactions of CPAs and human serum in the viable cell number in proliferation of cryopreserved embedded MSCs within PRP-SF. Viable cell number in proliferation of embedded MSCs within PRP-SF bioscaffolds 7 (A) and 14 (B) days after thawing. C) Micrographs of calcein/ethidium stained MSCs within PRP-SF bioscaffolds 7 and 14 days after thawing Note: Control: non-cryopreserved bioscaffolds; wo CPA : without CPA and additives; DMSO: DMSO 10%; HS: Human Serum 10%; DMSO + HS: DMSO 10% + Human serum 10%; Sucrose: Sucrose 0,2M; DMSO + Sucrose: DMSO 10% + Sucrose 0,2M; HS +Sucrose: Human Serum 10% + Sucrose 0,2M; DMSO + HS + Sucrose: DMSO 10% + Human Serum 10% + Sucrose 0,2M. Scale bar: 100 µm

Since HA was not sufficient for the cryopreservation of the 3D bioscaffolds, we intended to analyze whether the combination of a permeant CPA (DMSO), a non-permeant CPA (sucrose) and an adjuvant (human serum (HS)) were able to storage appropriately the MSC within the HA containing PRP-SF bioscaffold [73,74]. With that aim, we carried out a two levels three variables (DMSO, sucrose and HS) simple factorial experiment in which

the viable cell number in proliferation of cryopreserved MSC within the PRP-SF bioscaffold were determined 7 and 14 days after thawing. Whithin the PRP-SF bioscaffolds preserved with only one additive, the MSCs preserved with DMSO 10% showed the highest viable cell number in proliferation (Table 1 and Figure 10 A,B). This beneficial outcome was also reflected in other CPA solutions where DMSO was present, with an increasing effect on the viable cell number in proliferation at either 7 days (DMSOe= 0,0894) or 14 days (DMSOe= 0,0854) after thawing (Table 2), indicating that the inclusion of a permeant CPAs, such as DMSO, was required for the cryopreservation of MSCs within the PRP-SF bioscaffold. Although serum displayed beneficial properties in cells cryopreservation [75], HS decreased the viable cell number in proliferation in the MSCs within PRP-SF bioscaffolds (Table 1, Figure 10 A,B), obtaining a negative effect in the factorial experiment, with values of -0,0294 at day 7 and in -0,0197 at day 14 post-thawing (Table 2). Lastly, the addition of sucrose 0,2M enhanced the viable cell number in proliferation compared to those cryopreserved without CPAs, which was reflected in a positive effect at both time-points: 0,003 at day 7 and in 0,0293 at day 14 7 and 14 days after thawing (Table 1-2, Figure 10 A,B). However, this positive values did not reach to the non-cryopreserved or DMSO cryopreserved bioscaffolds, indicating that sucrose has not significant cryoprotective effects, unless they are combined with a permeant CPA such as DMSO [42,76].

				Viable cell number in proliferation			
				Day 7		Day 14	
Groups	DMSO (%)	HS (%)	Suc (M)	MEAN	SD	MEAN	SD
Control	—	—	—	0,302	0,028	0,337	0,0640
Wo CPA	0	0	0	0,253	0,045	0,232	0,043
DMSO	10	0	0	0,333	0,061	0,313	0,009
HS	0	10	0	0,232	0,061	0,222	0,061
Sucrose	0	0	0,2	0,238	0,033	0,299	0,111
DMSO + HS	10	10	0	0,296	0,079	0,351	0,012
DMSO + Sucrose	10	0	0,2	0,349	0,102	0,372	0,029
HS + Sucrose	0	10	0,2	0,213	0,043	0,253	0,025
DMSO + HS + Sucrose	10	10	0,2	0,316	0,071	0,313	0,062

Table 1: Viable cell number in proliferation at day 7 and 14 after thawing of cryopreserved MSCs embedded within PRP-SF bioscaffolds with different CPA solutions. Note: Control: non-cryopreserved bioscaffolds; wo CPA: without CPA and additives; DMSO: DMSO 10%; HS: Human Serum 10%; DMSO + HS: DMSO 10% + Human serum 10%; Sucrose: Sucrose 0,2M; DMSO + Sucrose: DMSO 10% + Sucrose 0,2M; HS +Sucrose: Human Serum 10% + Sucrose 0,2M; DMSO + HS + Sucrose: DMSO 10% + Human Serum 10% + Sucrose 0,2M

From the results described above, we concluded that the combination of DMSO

and sucrose in the cryopreservation of embedded MSCs within PRP-SF bioscaffolds could provide similar results in viable cell number in proliferation than DMSO alone. Therefore, we aimed to characterized more exhaustively the effects of cryopreservation with either DMSO or DMSO combined with sucrose to define which CPA could maintain embedded MSCs within PRP-SF bioscaffolds in better conditions, also comparing both CPA solutions with non-cryopreserved, cryopreserved without CPA or cryopreserved with sucrose 0,2M bioscaffolds 7 days after thawing. Accordingly to previous results, the cryopreserved MSCs within PRP-SF bioscaffold with DMSO 10% or DMSO 10% and sucrose 0,2M were the ones obtaining higher viable cell number in proliferation and live cells percentages, and lower early apoptosis cell percentages with similar outcomes to the non-cryopreserved bioscaffold (Figure 11 A-C). Interestingly, bioscaffolds cryopreserved with DMSO 10% displayed statistically significant ($p<0,05$) higher viable cell number in proliferation values compared to the non-cryopreserved and DMSO 10% and sucrose 0,2M cryopreserved bioscaffolds (Figure 11 C). This effect is in accordance with those studies described by other authors, where for example, 1-month stored MSCs showed higher proliferation rates than non-cryopreserved cells, leading to the theory of a cell selection of "stronger" cells after their preservation [33,34]. We also hypothesized that changes in the PRP-SF bioscaffold network during cryopreservation with only DMSO, that maybe do not occur in the presence of sucrose, could also permit a superior spreading of the embedded cells through the bioscaffold. On the other hand, the bioscaffolds cryopreserved without CPAs or with sucrose 0,2M displayed lower overall cell function compared to the non-cryopreserved one, which was confirmed by fluorescence microscopy after calcein/ethidium staining (Figure 11 A-E).

Effects and interactions	Viable cell number in proliferation	
	Day 7	Day 14
DMSO[e]	0,0894	0,0854
HS[c]	−0,0294	−0,0197
Sucrose[e]	0,0003	0,0298
DMSO-HS[i]	−0,062	0,0089
DMSO-Sucrose[i]	0,0178	−0,0192
HS- Sucrose[i]	−0,0002	−0,0332
DMSO-HS-Sucrose[i]	0,0019	−0,0152

Table 2: Effects and interactions on viable cell number in proliferation of MSCs embedded within PRP-SF bioscaffolds at day 7 and 14 after thawing. Notes: [e]= effects; [i]= interations.

Finally, since the maintenance of the multilineage capacity of MSCs for their posterior use in cartilage regeneration in OA is a crucial aspect, we assessed how the MSCs inclusion within the PRP-SF bioscaffold and their posterior cryopreservation could affect its potential to differentiate into osteogenic, adipogenic and chondrogenic lineages after their release from the best cryopreserved bioscaffolds. After three weeks with osteogenic, adipogenic and chondrogenic differentiation medium, non-cryopreserved, DMSO 10% and DMSO 10% and sucrose 0,2M displayed similar calcified matrixes, presence of vacuoles and positive blue staining for cartilage matrixes (Figure 12). With all these results we concluded that the cryopreservation of embedded MSCs within PRP-SF bioscaffolds does not provoke their loss of multilineage differentiation potential.

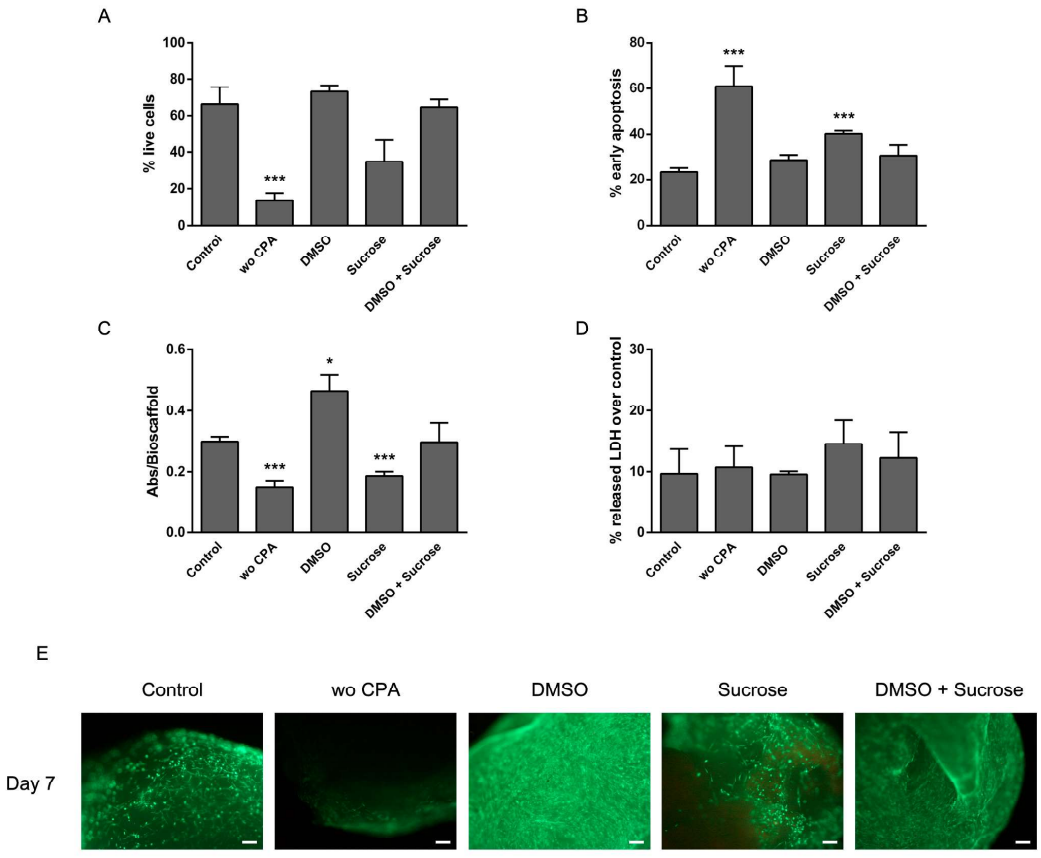

Figure 11. In vitro characterization of cryopreserved embedded MSCs within PRP-SF bioscaffolds 7 days after thawing. Quantification by flow cytometry of A) live cell percentage after calcein/ethidium staining and B) early apoptotic cell percentage after Annexin/PI staining. C) Quantification of viable cell number in proliferation by CCK-8. D) Quantification of membrane integrity by LDH release. E). Micrographs of calcein/ethidium stained samples. Note: Control: non-cryopreserved bioscaffolds; without CPA and additives :wo CPA; DMSO: DMSO 10%; Sucrose: Sucrose 0,2M; DMSO + Sucrose: DMSO 10% + Sucrose 0,2M; Values represent mean ± SD. *: $p < 0.05$, **: $p < 0.01$ and ***: $p < 0.001$ compared to non-cryopreserved group. Scale bar: 100 μm

Overall, this research work demonstrated that healthy young MSCs can be cryopreserved in combination with CPAs within the PRP-SF bioscaffold for the future use of this clinical cell-based product in the treatment of OA, enabling the preservation of thawed MSCs cell function and differentiation capacity, importantly to chondrogenic lineage, the cell type responsible for cartilage regeneration. It can be concluded that the combination of HA, fibrin and DMSO are needed to preserve appropiatelly the MSCs within the studied bioscaffolds, indicating that HA plays a crucial role in the maintenance of the proliferation capacity of the SF derived MSCs.

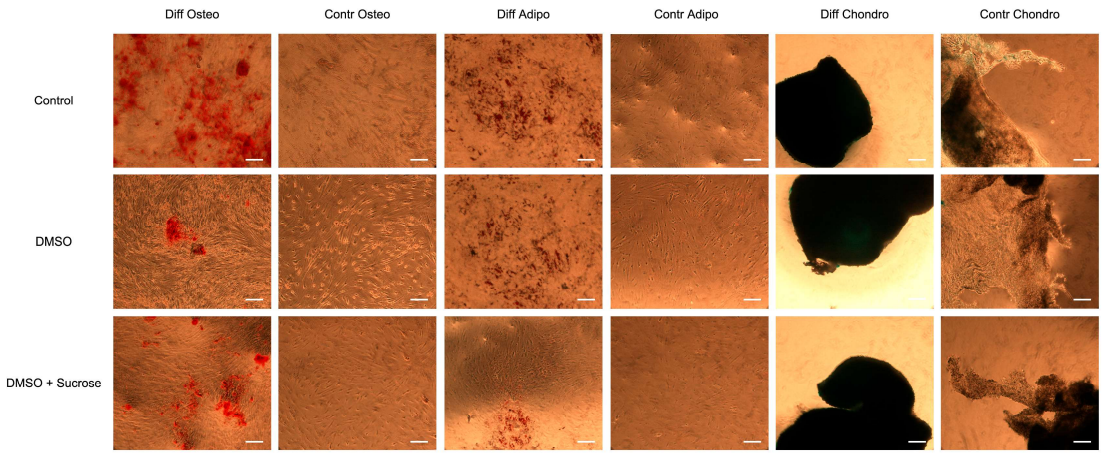

Figure 12: Differentiation potential of cryopreserved MSCs within PRP-SF bioscaffolds. Microscopic images of 3 weeks MSCs released from thawed PRP-SF bioscaffolds and differentiated into osteocytes, adypocites or chondrocytes. Note: Control: non cryopreserved; DMSO: DMSO 10%; Sucrose: Sucrose 0,2M; DMSO + Sucrose: DMSO 10% + Sucrose 0,2M. Osteo: osteogenic differentiation; Contr Osteo: non osteogenic differentiation (control); Adipo: adipogenic differentiation; Contr Osteo: non adipogenic differentiation (control); Chondro: chondrogenic differentiation; Contr Chondro: non chonrogenic differentiation (control). Scale bar: 100 µm

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7

Conclusions

7. CONCLUSIONS

According with the results obtained in the previously described experiments, we can conclude that:

1) The most suitable cryoprotectant agent for the slow freezing cryopreservation of alginate microencapsulated D1MSC maintaining their in vitro and in vivo cell function after thawing is DMSO 10%. The addition of other cryoprotectant agents, such as trehalose, impairs cell viability and microcapsules integrity after the freeze-thaw process.

2) The natural non-toxic low MW-HA exerts cryoprotetive effect in the slow freezing cryopreservation of microencapsulated cells, postulating low MW-HA as a potential non-permeant cryoprotectant agent for the cryopreservation of microencapsulated cells. However, not all microencapsulated cells respond similarly to low MW-HA, making necessary preliminary studies for each micoencapsulated cell type.

3) The cryoprotective effect of low MW-HA might not only be performed following the mechanisms of a non-permeant cryoprotectant agent, but also acting as an anti-apoptotic factor. Further investigations will reveal the mechanisms underlying the cryopreservation by low MW-HA.

4) Healthy young MSCs from synovial fluid can be cryopreserved within the platelet rich plasma and synovial fluid bioscaffold with either DMSO 10% and DMSO 10% and sucrose 0,2M. The combination of hyaluronan, fibrin and DMSO acts synergistically to preserve MSCs isolated synovial fluid within this bioscaffold, maintaining thier proliferation capacity.

5) Alternative non-toxic natural compounds such as low MW-HA, may represent an alternative cryoprotectant agent in the cryopreservation of 3D cell-based products.