Application of sugars and starch in the cryopreservation of cells

Dissertation
zur Erlangung des akademischen Grades *doctor rerum naturalium* (Dr. rer. nat.)

vorgelegt dem Rat der Biologisch-Pharmazeutischen Fakultät der Friedrich-Schiller- Universität Jena

Seit 1558

von Master in Biochemie Yahaira M. Naaldijk

geboren am 02 Juni 1979 in San Juan, Puerto Rico

Gutachter:

1. Prof. Dr. Michael Glei (Friedrich-Schiller-Universität Jena)
2. Prof. Dr. Lars-Oliver Klotz (Friedrich-Schiller-Universität Jena)
3. Prof. Dr. Friedemann Horn (Universität Leipzig)

Day of the oral defense: 21.05.2014

Table of contents

1. List of Abbreviations

ALP	Alkaline Phosphatase
ATMP	Advanced Therapy Medicinal Product
BM	Bone Marrow
CD	Cluster of Differentiation
CM	Conventional Method
CFU	Colony Forming Unit
CPA	Cryoprotectant Agent
cAMP	Cyclic Adenosine Monophosphate
ddH2O	Deionzed Water
DMEM	Dulbecco's Modified Eagle's Medium
DMSO	Dimethyl Sulfoxide
EDTA	Ethylenediaminetetraacetic acid
FBS	Fetal Bovine Serum
FCS	Fetal Calf Serum
FSP	Fibroblast Specific Protein
GLP	Good Laboratory Practice
GM-CSF	Granulocyte Macrophage Colony Stimulating Factor
GMP	Good Manufacturing Practice
H_2O_2	Hydrogen Peroxide
hES	Human Embryonic Stem
HES	Hydroxyethyl Starch
ICE	Intracellular Ice
kDa	Kilodaltons
MHC-II	Major Histocompatibility Complex II
MS	Molar Substitution
MSC	Mesenchymal Stem Cells
MTT	3-[4, 5-dimethyltiazol-2-yl]-2, 5-diphenyltetrazolium bromide
MW	Molecular Weight
PBS	Phosphate Buffered Saline
PEG	Polyethylene glycol
PFA	Paraformaldehyde
PVP	Polyvinylpyrrolidone
QRM	Quality Risk Management
RBC	Red Blood Cells
ROS	Reactive Oxygen Species
SEM	Standard Error of the Mean
SMA-α	Alpha-Smooth Muscle Actin
Tg	Glass-Transition Temperature
TGF-β	Tumor Growth Factor beta
UC	Umbilical Cord

2 Introduction:

In the field of regenerative medicine constant optimization of cryopreservation protocols for cells are on demand to enhance cell viability and to maintain cellular activities. In order to cryopreserve cells, freezing protocols and cryoprotectant agents have been developed but with varying degrees of success. The two main problems encountered by cryopreservation are: cryo-injury and cryoprotectant agent toxicity. Both affect cell recovery as well as cell functionality. Therefore, the studies presented in this thesis focus in the improvement and comparison of different freezing protocols and cryoprotectant agents for the cryopreservation of mesenchymal stem cells, fibroblasts and keratinocytes. In addition, toxicity of the cryoprotectant agent was analyzed and replacement with non-toxic solutions was performed.

2.1 Cryopreservation overview

2.1.1 Cryopreservation

Cryopreservation is the process wherein biomaterials such as cells, tissues and organ, among others can be stored at subzero temperatures without affecting the molecular and biophysical structures. The idea of cryopreservation was reported in 1776 by Spallanzani who exposed human sperm to low temperature and observed that its motility was maintained. It was followed by Mantagazza in 1886 who suggested the idea of creating a human frozen sperm bank. The concept of cryopreservation was first established in 1948 by Polga et al when a glycerol-based solution was accidentally used instead of levulose for the preservation of bull spermatozoa [1]. From that moment on an increased interest in the study of cryopreservation processes and methods emerged.

During the cryopreservation process water and aqueous solutions freeze below their melting point before nucleation of ice takes place, also known as supercooling. At this point, “seeding” can occur if the water or aqueous solution is touched with a metal object or crystal allowing for crystallization to occur after which the solution will return to its freezing point. The melting point is when the water or substance changes from their liquid state to a solid state, and the initial formation of ice is called nucleation. Formation of ice during the preservation process results in release of heat from the solution followed by nucleation (Fig 1). The main challenge that cryopreservation faces is not the prolonged storage of cells at low temperatures but rather the lethality that the cells have to endure by passing twice the intermediate zone of temperature (~-15°C to -60°C), once during cooling and once during warming through. Fowler and Toner described that reaching -138°C without ice formation is possible if freezing occurs very fast or ice crystal formation is inhibited by the addition of cryoprotectants and molecular ice inhibitors [2].

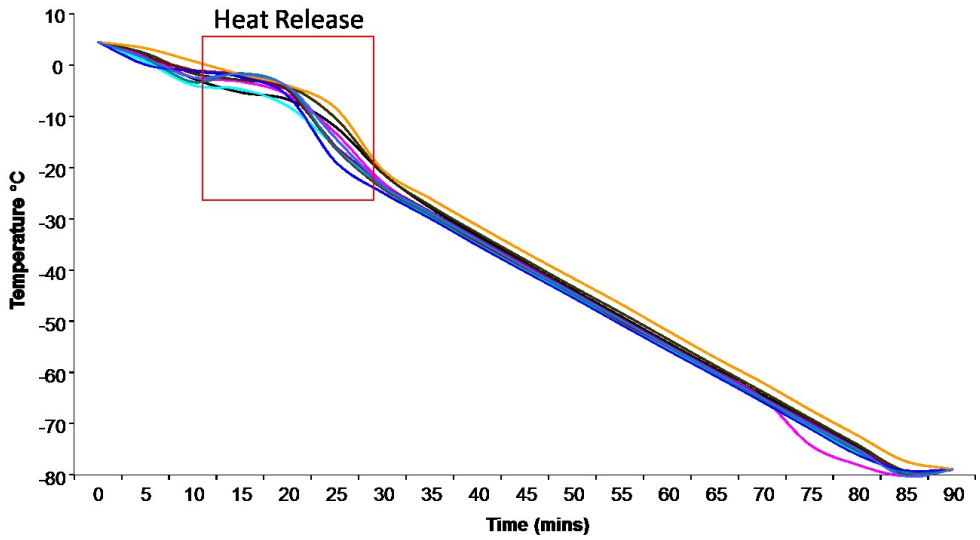


Figure 1.

Representation of heat release during the cooling process utilizing different cryoprotectant agents. Heat release of the samples occurred between -2°C to -15°C for a slow cooling rate.

2.1.2 Cryo-induced damaged

During cryopreservation procedures injuries to the cells, tissues and organs are incurred which are lethal resulting in apoptosis or necrosis in most instances, and which are possibly due to osmotic imbalances, cryoprotectant toxicity, cooling and warming rates, among other things.

In 1972 Mazur reported the “two-factor hypothesis” which stated that the correlation between cooling rates and survival is due to toxic effects of the cryosolution at low freezing rate or by the formation of intracellular ice at high freezing rates (Fig 2) [3].

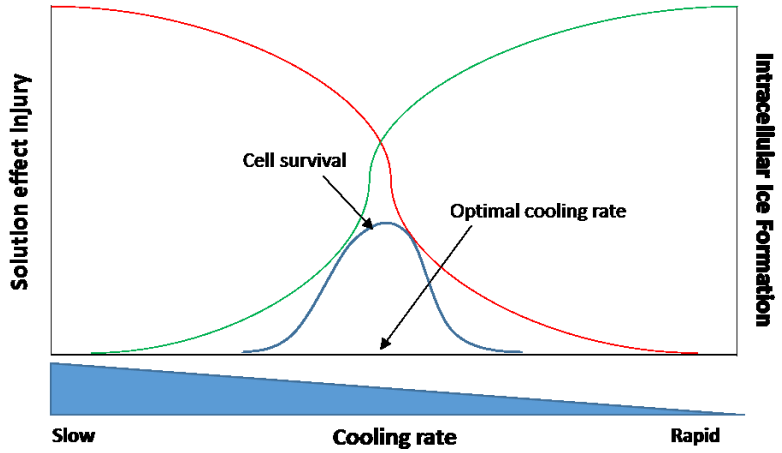


Figure 2. Inversed U-shaped curve for the correlation between cell survival and cooling rate curve correlation in injury mechanism (Representative figure from Mazur [4]).

The main hypothesis behind this is that avoidance of these 2 factors would lead to an optimal cryopreservation. But for an optimal cooling rate to be achieved, cellular survival is dependent on a freezing rate in which water can be retained inside the cell in order to prevent slow-freezing damage but which is slow enough that dehydration can occur without the formation of intracellular ice [2]. A correlation between cooling rate, ICE formation and cell survival can been seen in Figure 3. At higher freezing rates ICE formation increases thereby reducing cellular survival. However, this correlation is cell-dependent.

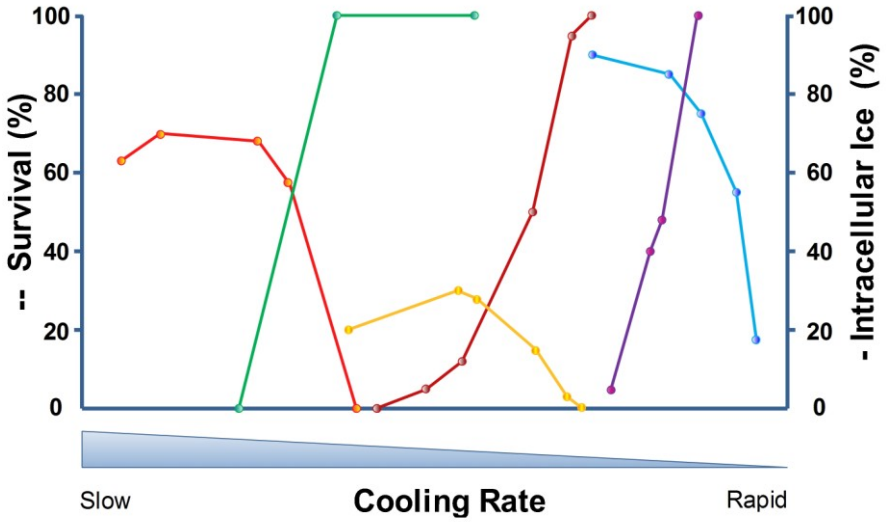


Figure 3.

Comparison of the effect of cooling rates and its correlation with cell survival and intracellular ice formation for different mammalian cell types. Each color represents one cell type (Representative diagram from P. Mazur [3] [5]).

Mazur described that around -5°C the cell and its surroundings stay at an unfrozen state, and that between -5°C and -15°C ice is formed in the surrounding area either spontaneously or due to seeding but that the cell contents remain unfrozen and supercooled, as seen in Fig 4 [5].

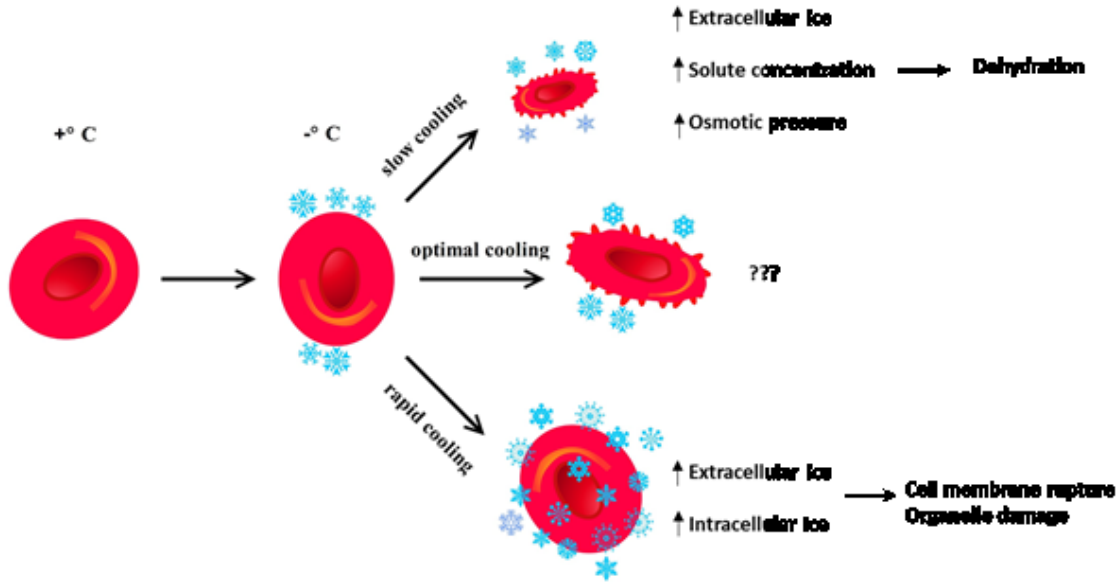


Figure 4. Representation of physical events that occur during the freezing process (hexagomers represent ice crytals). Slow cooling rates resulted in cell dehydration, while rapid cooling rates cause cell damage. Conditions for an optimal cooling rate are unknown but it is believe to be a rapid cooling rate without formation of ice crystals. Representative diagram from P. Mazur [5]).

During slow freezing water is diffused out of cells by a process called exosmosis, resulting in an increase in extracellular ice formation. Meanwhile inside the cell, solute concentrations increase causing dehydration of the cell [6, 7]. During rapid cooling water exosmosis is

inhibited and the possibility of intracellular ice formation increases causing destruction of the cell membranes.
Intracellular ice is the main mediator of cryo-induced damage. Ice formation results in the rupture of cellular membranes leading to cell death, however re-crystallization during the warming process (thawing) is also a cause of cellular damage. There is evidence that intracellular ice can be propagate between cells [8]. The more times that the cells are in close contact with each other, the greater the propagation of ice [9, 10]. Additionally, extracellular ice that is present can facilitate the formation of intracellular ice [5, 11, 12].
Most of the cryopreserved cells are kept at -196°C for long-term storage and at this temperature biological processes are ceased. Improper cryopreservation can lead to a chain of events in which metabolic processes are altered by way of changes in membrane composition due to chilling injury [13-17]). Examples of events following damage to the membranes are: i. activation of apoptosis and calcium dependent phosphatases [6, 18-20], ii. activation and release of lysosomal and lipoprotein hydrolase [21, 22], iii. release of free fatty acids [23], iv. disruption of the cytoskeletal structure [24-27], and v. activation of apoptotic cascades [6]. Very slow freezing can result in toxicity effects due to concentration of solutes within the cell that result in delayed necrosis and apoptosis [6].

2.1.3 Cryopreservation freezing methods

In cryopreservation there exist various methods to cryopreserve cells either by vitrification, computer-controlled rate freezing, or by conventional methods (isopropyl alcohol bath based container Nalgene Mr. Frosty).
Vitrification is defined as a process wherein a liquid solution or substance is converted into a glassy state (vitreous state) due to rapid cooling through the glass transition of the substance [28]. Importantly, this rapid cooling can avoid the formation of ice crystal. There are several ways to accomplish vitrification by using open pulled straw, cryoloop or cryo-electron microscopy in combination with a cryoprotectant agent (CPA). Vitrification is mainly used for the cryopreservation of mammalian embryos, but is mostly used for oocytes [29, 30]. The main limitation of vitrification is that it requires high cooling rates and high CPA concentrations [31, 32].
The conventional method (CM) is commonly used in laboratories as a method for the cryopreservation of cells. It provides a cooling rate of 1°C/min. CM is easy to perform, cost-effective and requires low concentrations of CPA [33]. The conventional method has 3 disadvantages: a. rapid cooling, more than 1°C/min is difficult to reach, b. freezing is limited to -80°C and c. the procedure needs an overnight incubation at -80°C.
The use of controlled rate freezing allows the creation of specific temperature profiles for specific cell types along with specific cryoprotectant agents in order to optimize cell viability and recovery [34, 35]. By controlling the cooling rate, the prevention of intracellular ice formation is accomplished [34]. Freezing temperatures of either the chamber or sample can be controlled to match the time profiles. The benefit of using controlled rate freezing is that it can be applied to most compliance standards (GMP- and GLP-based) since the device and software records each freezing event (Fig 5) and optimizations of freezing protocols are easy to perform.

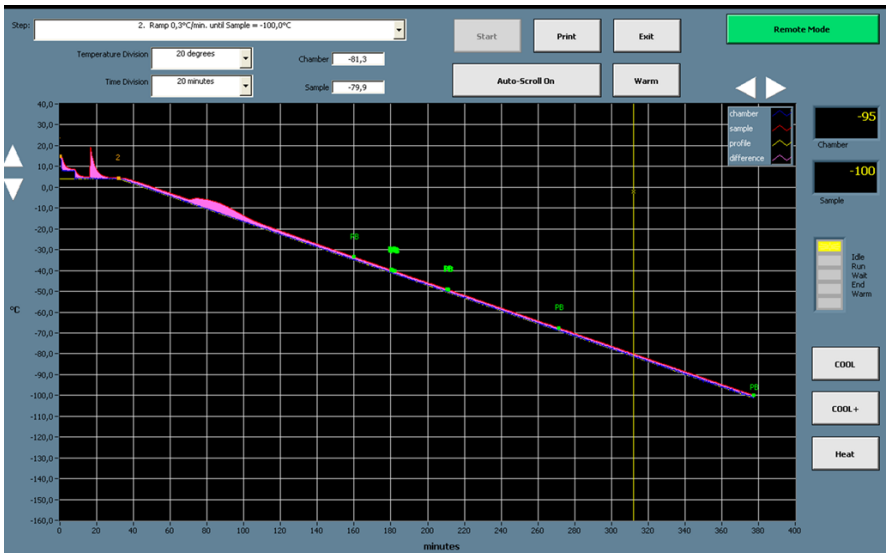


Figure 5. Schematic representation of recorded controlled rate freezing program for GLP/GMP standards where cooling rate of the sample, freezer and program are obtained for every independent freezing process.

The advantage of using controlled rate freezing is the low requirement of cryoprotectant concentrations which are needed to accomplish intracellular freezing thereby reducing the toxicity effects of the cryoprotectant [33]. On the other hand, controlled rate freezing is time consuming and requires expensive equipment.

2.2 Cryoprotectant Agents

In order to protect cells and tissues during the cryopreservation procedure a cryoprotectant agent (CPA) is used. CPA is a substance use to minimize damage during freezing by increasing the solute concentration in the cell. A good CPA should be non-toxic, be able to permeate the cell membrane, have a low molecular weight, and avoid solution effects as well as osmotic shock. There are two types of CPA: permeating (e.g., DMSO, propylene glycol, glycerol) and non-permeating (e.g., sucrose, trehalose, hydroxyethyl starch). Permeating CPAs are able i. to form hydrogen bonds with water molecules thereby preventing ice crystallization, ii. at low concentrations, to decrease the freezing temperature and at high concentrations to inhibit the formation of intracellular ice crystals, iii. to cause electrolyte concentrations to increase at a certain temperature during the freezing process and, iv. to increase the final size of the cells [2]. On the other hand, non-permeating CPAs are often of a high molecular weight, provide extracellular protection and prevent osmotic shock. A disadvantage of using penetrating CPAs is that prolonged exposure can be toxic to the cells due to disruption of intracellular signaling [36] therefore removal after thawing is necessary.

2.2.1 Dimethylsulfoxide (DMSO – C_2H_6OS)

DMSO belongs to the group of small MW penetrating CPAs. It is an amphiphilic molecule with two hydrophobic methyl groups and a hydrophilic sulfoxide group [37, 38]. DMSO cryoprotectant properties were first reported in 1959 for the preservation of red blood cells [39]. DMSO acts as a CPA through a preferential exclusion mechanism by increasing the freeze energy of unfolding and stabilizing cellular proteins [40] and by interacting electrotrastically with phospholipid bilayers thereby stabilizing the plasma membranes [41]. However, DMSO is also known to cause denaturation and destruction of proteins by interacting with their hydrophobic residues [42, 43]. Besides its cryoprotective properties,

DMSO has several other biological functions such as being an inducer of cell fusion, increasing cell permeability and promoting cell differentiation [41, 44-46].

2.2.2 Fetal Bovine Serum (FBS)

FBS is commonly used as a CPA in combination with DMSO in many research laboratories since it supports good cell recovery. FBS contents are unknown but it is clear that its composition provides cryoprotective effects by stabilizing biomembranes and by balancing osmotic pressure [35, 39, 47, 48]. A big disadvantage of using FBS as a CPA results from its composition being unknown therefore animal product contamination with viral or prion diseases, or xenogeneic immune responses may be possible [49].

2.2.3 Sugars

The concept of sugars as CPAs was introduced in the 1900s by Maximov as protective agents were observed to induce a natural freeze tolerance in plants [50]. In nature sugars like glucose, sucrose and trehalose are used by many organism, e.g. frogs [51], bacteria [52], brine shrimps [53], yeast [52] and plants [54] to resist extreme freezing conditions [55, 56]. Accumulation of intracellular and extracellular sugars is required for their survival [55]. The protective effects of sugars were also determined in liposomes, membranes and proteins in freeze-drying cryopreservation [57, 58]. Sugars have been used as CPA since they are non-toxic and act by protecting proteins and cell membranes [58-60]. The advantages of using sugars for cryopreservation is due to their high glass transition temperature allowing for long term storage of cells [61, 62], but complex sugars (e.g. dissacharides - trehalose) are impermeable to mammalian cells membrane due to the presence of hydrophilic residues or the lack of active transport system [63, 64]. Trehalose is the most common sugar used as extracellular CPA in cryopreservation since it can interact more strongly with water molecules [53, 59, 60, 65], and it protects cell membranes [55] and membrane vesicles [66, 67]. Efforts have been performed to introduce trehalose into cells by different mechanisms in order to enhance survival rates\recovery after cryopreservation [68-71].

In addition to trehalose, glucose is another common extracellular CPA often used in combination with intracellular CPA [72-76]. Glucose is a simple monosaccharide, the primary energy source of cells, and is involved in many biochemical pathways and mechanisms of action. In cryopreservation the mechanism of glucose protection is mediated by its binding to proteins and lipids and stabilization of membranes [77, 78]. At higher concentrations, however, glucose causes toxicity by its osmotic effects [79]. In addition, protein glycations are more likely to occur in presence of glucose. Glycation of proteins interfere with enzyme activities, membrane stability and promote the formation of reactive oxygen species [80-84].

2.2.3.1 Sorbitol ($C_6H_{14}O_6$)

Polyalcohols are defined as alcohol that contains more than one hydroxyl group. They show cryoprotective effects for the cryopreservation of oocytes and embryos [85] and human erythrocytes [86]. Sorbitol is a sugar alcohol which can be obtained by reduction of glucose and an organic osmolyte that is known to have protective activities during cryopreservation by preventing osmotic cell damage [86]. There is no real evidence as to whether sorbitol is able to penetrate the cell membrane [87] but it has been proposed to be an intracellular CPA [88]

2.2.3.2 Dextran ($H(C_6H_{10}O_5)xOH$)

Dextran belongs to the high molecular CPAs that were first used in 1947 in clinical settings as a plasma expander [89-92]. It has shown cryoprotective functions for different cell types [93, 94]. Given its high molecular weight (MW) dextran acts by i. increasing the solution viscosity, ii. reduction of the cooling rate for optimal survival during the vitrification process, iii. increasing the predisposition of supercooling and iv. inhibiting ice formation [95, 96].

2.2.4 Hydroxyethyl starch

Hydroxylethyl starch (HES) is a synthetic modified polymer of amylopectin (highly branched polymer of glucose) based on purified starch from corn or potato that has been modified by hydroxyethylation at carbon position C2, C3 or C6 [90-92]. This hydroxyethylation process increases HES solubility and affinity with water. The physical and chemical properties of HES depend: i) in the manufactured MW, ii) degree of molar substitution (MS, total number of hydroxylethyl group present per total glucose subunit), iii) the C2/C6 ratio (the hydroxyethylation at carbon position C2 and C6), and iv) the mean molecular weight. HES molecular weight is classified based on the manufacturing and it ranges from high MW ≥ 450 kDa, medium MW 200-400 kDa, and low MW < 200 kDa [97]. The molar substitution (MS) and C2/C6 determine HES hydrolysis rate therefore the higher the molar substation and the C2/C6 ratio, the slower the breakdown rate of HES.

In vivo, HES is metabolized by alpha amylase or excreted in urine. Alpha-amylase is a calcium metalloenzyme that cleaves complex starch into sugars (maltose and dextrin). The rate of breakdown of HES is dependent on its molecular weight; high and medium MW HES breakdown into a wide range of HES fragments and lower MW HES is excreted in urine. The resulting HES fragments, however, are the ones that determine HES function, activity, and the secretion rate.

The advantages of using HES in the cryopreservation process are non-toxicity, poor antigenicity and the ability to absorb water molecules [97-99]. The mechanism of HES cryoprotection is different from that of commonly used penetrating cryoprotectors such as DMSO and glycerol which are known to depress the freezing point of solution and delay damaging salt concentrations to lower temperatures. In contrast the cryoprotective effect of HES depends on its ability to absorb water molecules and keep them thermally inert in a glassy state without experiencing any phase transition during cooling. Accumulation of HES in extracellular space leads to dehydration of cells during cooling at high sub-freezing temperatures and allows them to be cooled rapidly thereby avoiding intracellular ice crystal formation and osmotic stress, as well as chilling injury which can occur during the process of slow cooling and can lead to serious damage [96, 100, 101]. During warming the high intracellular viscosity suppresses intracellular ice crystal growth and helps overcome the problem that occurs during devitrification – ice crystals growing when the temperature in samples rises slowly to -60°C and above.

2.3 Cell banking of skin cells and mesenchymal stem cells

2.3.1 Skin cell types: fibroblasts and keratinocytes

The skin is the largest organ of the human body and its main role is to maintain the stability of the body's internal milieu and to separate the internal environment from the external. It has various functions such as providing protection from biological invasions, as well as from UV radiation and damage, and is also involved in thermoregulation, the feelings associated with

sensations, the excretion of salt and waste, and the production of vitamin D [102, 103]. The skin is composed of 3 layers: epidermis, dermis and hypodermis.

2.3.1.1 Fibroblasts

Fibroblasts are mesodermal in origin and are the main cells of the dermis. These cells are able to produce extracellular matrix and collagen. They are the most common cell type of the connective tissue in animals and play an essential role in wound healing. Fibroblasts show mesenchymal phenotypes and can be characterized based on marker expression such as vimentin, FSP, SMA-α, or lack of colony-forming capacity and inability to differentiate into other cell types [104].

2.3.1.2 Keratinocytes

Keratinocytes have an ectodermal origin and play an essential role in the skin anatomy (epidermis). They emerge from the epidermal stem cells through a controlled differentiation process in which the differentiated cells are dead thereby forming the skin surface [105-107]. Keratinocytes have physical and immunological functions, and mostly cultured today using optimized keratinocyte media. Under these conditions keratinocyte viability is decreased and a stage of advanced differentiation is observed [108, 109]. Therefore, optimization of cryopreservation protocols are commonly performed using keratinocyte cell lines since no requirement of feeder cells and extended life span make them a great model for the determination of cooling rates and cryoprotectants [110, 111].

2.3.1.3 Skin tissue-engineered fibroblasts and keratinocytes

Skin substitutes are commonly used as tissue replacement for the treatment of wounds and burns. In most cases it does not require tissues and/or cells from the donor and improvement of the healing process can be observed [112]. The end result of tissue-engineered skin is the production of skin by either mainly using cells, only the extracellular matrix materials or a combination of both [113, 114]. In addition, tissue-engineered skin has been used for the testing of drugs and other agents thereby replacing the use of animals [115-117].

Dermal fibroblast and keratinocyte cells are commonly used as skin substitutes for the treatment of skin-related problems such as wounds, ulcers and burns. Both autologous or allogeneic fibroblasts and keratinocytes can be used for transplantation. Allogeneic fibroblasts can be used for pre-conditioning the wound bed prior to implantation [118]. Phillips et al proposed that cultivation of fibroblasts and keratinocytes *in vitro* reduce the likelihood of having any type of rejection because Langerhans cells, antigen-presenting cells, are lost during the cultivation procedure [119].

Optimization of tissue-engineered skin substitute is necessary since long-term storage might result in a decrease in the viability of the cells. Cryopreservation of skin substitutes might have adverse effects after implantation since stress proteins and a stress reaction can occur during the cryopreservation process [120]. Therefore, suboptimal cryopreservation protocols and solutions are on high demand.

2.3.2 Mesenchymal stem cells

Mesenchymal stem cells (MSC) are non-hematopoietic stem cells with multi-lineage potential which have a mesoderm origin. These cells were firstly isolated from the bone marrow by Friedenstein in 1976 [121]. However, recently MSCs have been reported, among others, in adipose tissue, umbilical cord blood and tissue, and placental and peripheral blood. MSCs have the ability to differentiate into different cell types, among others: osteoblast (bone), adipocytes (fat) and chondrocytes (cartilage). MSC characterization is based on the expression of cluster of differentiation (CD) markers (positive: CD90, CD73, CD105;

negative: CD45, CD34, CD11b or CD14, CD79α or CD19), adherence to plastic and *in vitro* differentiation [122]. Also, MSCs lack expression of immunohistocompatibility complex II (MHC-II) and co-stimulatory molecules making them immunosuppressive. Since MSCs can be expanded *ex-vivo* showing similar functionality as *in-vivo* they are a very great source for autologous and allogeneic cell therapies for the treatment of injuries or age –related diseases [123-125].

2.3.2.1 MSCs in regenerative medicine

Since MSCs are multipotent cells and induce non-immune response after transplantation it makes them a very promising tool in the field of regenerative medicine. MSCs have been successfully used in the treatment of spinal cord injury [126] cartilage lesions [127] and bone defects [128]. In addition, clinical trials using MSCs for the treatment of cirrhosis, graft-versus-host disease, left ventricular dysfunction, osteogenesis imperfecta, metachromic leukodystrophy and Hurley syndrome [129, 130] are in process. MSCs are currently used as substitute in autologous bone marrow transplantation [131].

It is known that MSCs have a limited lifespan in culture (population doubling time 15-50) [132] and with increases in passages a decrease in their proliferative capacity and differentiation is observed [133-137]. In addition, a correlation was observed between low cell recoveries of cryopreserved MSCs and poor *in vivo* differentiation [131] indicating the need to improve cryopreservation protocols for MSCs. Therefore, MSCs banking is necessary to control batch variations and quality control to ensure its use in research and clinical application in stem cell-based therapy.

2.4 Cryopreservation of fibroblasts, keratinocytes and mesenchymal stem cells

2.4.1 Fibroblasts cryopreservation

Since fibroblasts are used as dermal substitutes in skin tissue engineering, several attempts have been performed for the optimization of fibroblast cryopreservation by different cooling rates and/or cryoprotectant agents. Table III summarizes relevant data regarding human fibroblast cryopreservation in suspension and adherent comparing freezing protocols and CPAs.

Cell Type	Method	Freezing Protocol	CPA
Primary Fibroblast [111]	Suspension	1°C/min	DMSO
Primary Fibroblast in collagen gel [138]	Suspension	0.5°C/min from 20°C□-70°C-5°C/min from -70°C to-170°C	DMSO
Dermal Fibroblast [139]	Suspension	1°C/min □-80°C □N_2 tank	
Lung Fibroblast [140]	Suspension	3°C/min from RT to 4C 4C for 5 min 1C/min from 4C to -50C 5C/min from -50C to -80C □ N_2 tank	DMSO + serum
I-cell disease fibroblast [141]	Suspension	4°C for 30 min □ -70C freezer (5°C/min) □ N_2 tank	DMSO + serum
Dermal Fibroblast [142]	Suspension	~2.4°C/min in -80°C freezer	D-allose
Dermal Skin Fibroblast Slices [143]	Adherent	1°C/min from -4 □ -60C □ → N2 tank	DMSO

Dermal Fibroblast in PGA Scaffold [143]	Adherent	0.5°C/min ┐ -4°C to -60°C → 1°C/min ┘ N_2 tank	DMSO
Dermal Fibroblast [144]	Adherent	3°C/min from 4 to -80°C → - cooled to 185°C	Hyalouronan

Table I. Overview of cooling rates and cryoprotectants for human fibroblast cryopreservation

In most of the experiments for fibroblast cryopreservation, non-permeable sugars and DMSO (among others) were used in order to enhance cell recovery during the cryopreservation procedure. Incubations with different concentrations of DMSO, temperatures, and time showed that toxicity and viability is dependent on the temperature and exposure time [143]. A study performed by Choi et al showed a correlation between cooling rate, intracellular ice formation and fibroblast viability [145]. In addition, differences between suspension and adherent cryopreservation based on cooling rates were observed. Fibroblasts cryopreservation in suspension was possible using a fast cooling rate and as adherent with a slow cooling rate. This result contradicts what previously was shown by others wherein slow cooling resulted in higher cell viability compared to a medium cooling rate and vitrification. In addition, there is not a complete study in which different freezing protocols were compared to determine whether the same cryopreservation protocols can be applied to primary fibroblasts and fibroblast cell lines.

2.4.2 Keratinocytes cryopreservation

In parallel to fibroblasts, keratinocyte cryopreservation is also frequently optimized due to its role in skin-tissue engineering for dermal replacement. A wide range of studies are available wherein keratinocytes were cryopreserved in suspension and adherent and in absence of DMSO. Table IV compiles data available in cryopreservation of human keratinocytes.

Cell Type	Method	Freezing Protocol	CPA
Primary Keratinocyte [146]	Suspension	-3°C/min	HES
Primary Keratinocyte [147]	Suspension	-3.5°C/min	HES
Freshly isolated and primary cultured keratinocytes [148]	Suspension	-1C/min in -20C freezer x 12 min □ 2 hrs in -80C □ N_2 tank	DMSO
Primary Keratinocyte [149]	Suspension	-1°C/min	DMSO
SVK14 cell line [111]	Suspension	-1°C/min 30°C/min	DMSO + serum
HaCaT [142]	Suspension	~2.4°C/min in -80°C freezer	D-allose
Primary Keratinocyte [69]	Suspension	1°C/min from -6°C to -15°C → hold 20min → N_2 tank	Poration trehalose
Primary Keratinocyte [150]	Adherent	1°C/min: 3°C/min cryomicroscope	HES HES
Primary Keratinocyte [146]	Adherent	3.3°C/min in -85°C freezer	-
HaCaT cell line [110]	Adherent	Styrofoam box in -70°C	DMSO

Table II. Overview of cooling rates and cryoprotectants for human keratinocyte cryopreservation

In addition, keratinocyte cryopreservation encounters a major problem which is that of cell viability. Variation in cell recovery numbers within the experiments were often encountered

and not described or deviations not reported. Therefore, it is crucial to study the cryopreservation process in more detail and to improve it in a reliable way.

2.4.3 Mesenchymal stem cell cryopreservation

MSCs are frequently used in regenerative medicine as stem cell-based therapy. It is known that cryopreservation affects MSC behavior and functionality.
One main problem in MSC cryopreservation is the use of DMSO as a CPA since it has shown adverse effects in hematopoietic stem cell therapy. Various attempts on MSC cryopreservation have been performed to either eliminate or reduce DMSO concentration by combining it with other CPAs [151].
Cryopreservation protocols and CPA used for the cryopreservation of MSCs from different sources were exemplified in Table V.

Origin	Method	Freezing Protocol	CPA
Umbilical cord [152]	Suspension	Controlled rate freezer → -196°C	DMSO + Autologous plasma
Umbilical cord [153]	Suspension	2°C/min from RT to 0°C → 1°C/min to -40°C → 5°C/min -40°C to -80°C	DMSO + sucrose
Bone Marrow [154]	Suspension	4°C for 10 min → Mr. Frosty (1°C/min) to -80°C for 24hr → N_2 tank	DMSO + PEG + albumin
Bone Marrow [155]	Suspension	4°C for 10min → -30°C for 1h → -80°C for 2-3 days → N_2 tank	DMSO + serum + Cell Bank Solution
Bone Marrow [156]	Suspension	Directly in N_2 tank	DMSO + HES
Bone marrow [157]	Suspension	4°C for 1 h → 1°C/min to -40°C → 10°C/min from -40°C to -70°C→ N_2 tank	DMSO + serum
Bone Marrow [158]	Adherent	4°C for 15 min → 1°C/min, 5°C/min or 10°C/min	DMSO + serum
Adipose [159]	Suspension	0,5°C/min from 4 to -20°C → -80°C freezer for 24h → -196°C for 2 weeks	DMSO + serum

Table III. Overview of cooling rates and cryoprotectants in mesenchymal stem cell cryopreservation

As a result of CPA toxicity and the decrease in differentiation potential, optimization of MSC cryopreservation is necessary for its use in a clinical setting and cell banking.

2.5 Aims of the study

Cryopreservation of cells can be accomplished at temperatures below 0°C. The mechanism of cryopreservation is not well understood therefore accomplishment of the perfect cryopreservation method is difficult. Optimization and creation of freezing protocols and cryoprotectants are on demand. Two major problems in the cryopreservation field are the formation of intracellular ice reducing cell viability and recovery. Cryoprotectants are used to

reduce cryo-injury but toxicity is usually encountered. Therefore, combination of an appropriate cryoprotectant (s) and cooling rate(s) can result in a successful cryopreservation protocol.
The first aim of this study is to improve cryopreservation of mesenchymal stem cells by comparing the effect of cooling rates and DMSO substitution with hydroxyethyl starch. It is widely known that DMSO results in cell toxicity and adverse effects have been observed after intravenous injection of cryopreserved red blood cells with DMSO. Since MSC are used as cell-based therapy, cryopreserved rat MSCs under different conditions were analyzed after thawing based on stemness (differentiation potential and phenotyping), cell viability and recovery.
The findings obtained by the cryopreservation of rat MSC lead to the following study wherein combinations of sugars and hydroxyethyl starch were used as cryoprotectants for the cryopreservation of umbilical-cord MSCs. In this study, the use of DMSO as well as serum were completely omitted, serum being an animal product and contamination with virus agents being possible. Differentiation potential, morphology and viability were analyzed after cryopreservation of UC-MSC using sorbitol, dextran and HES combinations as CPAs.

The second aim of the study presented here was to perform a comparison of cooling rates and cryopreservation of cells in suspension versus adherence. As cell types, tissue engineering skin cells, fibroblasts and keratinocytes, were used for the studies. In addition, comparison between primary cells and cell lines performed. These cells are used as dermal replacement in the treatment of skin-related conditions. Cryopreservation protocols are constantly optimize for these cells but the main limitation presented in most of the studies is the lack of a full comparison of different freezing protocols and suspension versus adherent cryopreservation. Therefore, the purpose of this study was to take in consideration all these factors and to compare the obtained results with published data. Cell viability, recovery and cell morphology were analyzed after cryopreservation. In addition comparison of the different freezing protocols was performed as well as cryopreservation of the cells in suspension versus adherence.

3 Overview of the introduced manuscripts

Published manuscripts

1. **Hydroxyethylstarch in cryopreservation – Mechanism, benefits and problems**

Alexandra Stolzing, **Yahaira Naaldijk**, Viktoriya Fedorova and Sebastian Sethe
Transfusion and Apheresis Science, 2012, 46: 137-147

Summary: This review discusses relevant information about hydroxyethyl starch and its cryoprotective benefits in the cryopreservation of cells, mainly stem cells. A detailed comparison of published data on HES and cryopreservation was performed and described on the paper.

Author Contributions:
AS: design of figure 1-3, writing of subsections and provide the idea for the manuscript.YN: writing of subsections and tables. VF: writing of subsections and tables. SS: wiriting of subsections and revision of the manuscripted

2. **Effect of different frezing rates during cryopreservation of rat mesenchymal stem cells using combinations of hydroxyethyl starch and dimethylsulfoxide**

Yahaira Naaldijk, Marek Staude, Viktoriya Fedorova and Alexandra Stolzing
BMC Biotechnology, 2012, 12(49): 1-10

Summary: The purpose of this study was to compare different cooling rate in combination with different HES-based solutions in order to substitute and/or minimize DMSO concentration from the cryosolution without interfering with MSC stemness and viability.

Author Contributions:
YN: designing of the freezing protocols, chrondrocyte differentiation, flow cytometry data for CD44 and CD11b, freezing curves figure, consolidation of the data and provided the 1st draft manuscript
MS: cryopreservation experiments, viability experiments and differentiation of the cells towards osteogeneic and adipogenic differentiation. In addition, flow cytometry data for CD90 and CD45 and morphology pictures
VF: help with flow cytometry stainings for CD44 and CD11b and contribution in writing the paper
AS: planned the experiments, secured the funding and revised the manuscript.

Manuscript submitted

3. **Comparison of different cooling rates for fibroblast and keratinocytes cryopreservation**

Yahaira Naaldijk, Annett Friedrich-Stöckigt, Sebastian Sethe and Alexandra Stolzing

Summary: The manuscript compares different freezing protocols for the cryopreservation of fibroblasts and keratinocytes in suspension as well as in adherent. The paper demonstrated differences in freezing protocols for the cryopreservation of cells, either in suspension or adherent, and cell type dependent.

Author Contributions:
YN: planned and performed the experiments, analyzed the data and wrote the 1st draft of the manuscript
AF-S: provided technical support with the experiments and cell culture.
SS: provided help on data interpretation and revision of the manuscript
AS: planned the experiments, secured the funding and revised the manuscript, and was responsible for the project.

Publication status: Under the second revision by Journal of Tissue Engineering and Regenerative Medicine

4. **Cryopreservation of human umbilical cord-derived mesenchymal stem cells in complex sugar based cryoprotective solutions**

Yahaira Naaldijk, Viktoriya Fedorova and Alexandra Stolzing

Summary: The manuscript accentuate the use of use of sugars and HES for the cryopreservation of human umbilical cord blood MSC using in which differentiation into bone, adipose and chondrocyte were not affected after cryopreservation. This paper provides with a novel cryosolution based on sorbitol, dextran and HES without having the adverse effect and toxicity of DMSO solution.

Author Contributions:
YN: performed experiments, consolidated the data and revised and help in manuscript writing
VF: planned and performed experiments and wrote the 1st draft of the manuscript
AS: planned the experiments, secured the funding, arranged the tissue collections and revised the final version of the manuscript.

Publication status: Submitted to Journal of Biotechnology Letters

4. Manuscript

4.1 Hydroxyethylstarch in cryopreservation- Mechanisms, benefits and problems

Transfusion and Apheresis Science 46 (2012) 137–147

Contents lists available at SciVerse ScienceDirect

Transfusion and Apheresis Science

journal homepage: www.elsevier.com/locate/transci

Review

Hydroxyethylstarch in cryopreservation – Mechanisms, benefits and problems

A. Stolzing [a,*], Y. Naaldijk [a], V. Fedorova [a], S. Sethe [b]

[a] Fraunhofer Institute for Cell Therapy and Immunology, Leipzig, Germany
[b] NESCI, Center for Life, University of Newcastle, UK

ARTICLE INFO

Article history:
Received 5 July 2011
Received in revised form 19 December 2011
Accepted 23 January 2012

Keywords:
Cryopreservation
Hydroxyethyl starch
Stem cells
Dimethysulfoxide

ABSTRACT

As the progress of regenerative medicine places ever greater attention on cryopreservation of (stem) cells, tried and tested cryopreservation solutions deserve a second look. This article discusses the use of hydroxyethyl starch (HES) as a cryoprotectant. Charting carefully the recorded uses of HES as a cryoprotectant, in parallel to its further clinical use, indicates that some HES subtypes are a useful supplement to dimethysulfoxide (DMSO) in cryopreservation. However, we suggest that the most common admixture ratio of HES and DMSO in cryoprotectant solutions has been established by historical happenstance and requires further investigation and optimization.

Contents

* Corresponding author. Address: Fraunhofer Institute for Cell Therapy and Immunology, Perlickstrasse 1, Leipzig 04103, Germany. Tel.: +49 341 35536 3405; fax: +49 341 35536 9921.
E-mail address: Alexandra.Stolzing@izi.fraunhofer.de (A. Stolzing).

1473-0502/$ - see front matter © 2012 Elsevier Ltd. All rights reserved.
doi:10.1016/j.transci.2012.01.007

1. Introduction

Demands for improved cell, tissue and organ storage are increasing as more and more products of regenerative medicine reach the clinic. The safety of cryopreservation of cell material is one of many emerging considerations in regenerative medicine. In addition use of cell-based assays for drug screening and safety testing raises questions as to what cryopreservation methods are preferable.

After briefly charting considerations in cryopreservation and cryoprotection, we will focus on one cryoprotectant factor in particular: We will consider the capacity of hydroxylethyl starch (HES) to act as a cryoprotectant, its use and its reported efficacy from the available data (which, although reaching back 40 years is still rather sparse) and consider the safety implications of using HES- in particular in comparison with the most common cryoprotectant compound dimethyl sulfoxide (DMSO).

2. Cryoprotection basics

Upon cooling with cryoprotectants, extra- and intracellular viscosities abruptly increase whereas thermal energy is decreased and not sufficient to enable chemical reactions. In this case all biological reactions are slowed down to a minimum that makes long term storage of cells, tissues and organs possible. The damages induced by cryopreservation involve many different cell compartments but the exact mechanisms are surprisingly poorly understood. In general, a distinction can be made between the effects of ice formation and other stresses.

2.1. Ice crystal formation

The high concentration of H_2O in tissues or cells is one of the leading determinants of the physical changes during the cooling and warming process. During phase transition of the aqueous solutions, ice crystal formation occurs, and can lead to great damage to tissues and cells [1]. Membrane-associated cell damage can vary greatly between different species, depending on the membrane composition [2]. However, there are doubts if cell membranes rupture [3] due to ice formation.

Ice crystal formation in water solution can occur at any time and any extend at temperatures below 0 °C [1]. The ice is normally solid and has a regular crystalline structure. Intracellular water can remain in a super cooled unfrozen state, even at temperature between −5 °C and −40 °C. [4,5].

Ice crystals need to have a starting point, a nucleus, from where they are able to grow. Examples for nuclei are: ions, vibration and ice crystals themselves. Ice can also form in the extracellular space leading to increasing concentrations of electrolytes in the remaining extracellular solution. The growing extracellular ice forms channels where the extracellular solution and the cells are displaced [6]. In these channels the resulting pressure can cause lethal cell deformation [3]. The evidence that extracellular ice is harmful for tissues was found on cryopreserved smooth muscle tissue. When ice is forming extra-cellular only 21% recovery is observed, compared to 74% for unfrozen samples [7].

Ice growth can be transferred from one cell to another via gap junctions [8]. In addition, transmembrane proteins, "aquaporines", can initiate ice crystal growth from one side to the other side of the cell membrane [3].

Intracellular ice formation can therefore be induced by extracellular ice without damage of the cell membrane. The intracellular super cooled water tends to flow from the intra- to extracellular space due to its higher vapor pressure than ice. Due to the highly concentrated extracellular solution, the intracellular water diffuses to the outside (osmosis), resulting in cell dehydration [9]. The osmotically induced flow of water through the cell-membrane has also been proposed as a cause of damage [10]. As it is known that intracellular ice damages the cells mechanically [4], the outflow of water may in fact be a damage-reducing factor.

2.2. Other freezing-associated stresses

Cryopreservation can induce apoptosis or necrosis [11] which can be reduced by adding anti-apoptotic factors [11] or antioxidants [12]. One of the possible triggers for those types of damage might be an increase in reactive oxygen species (ROS), usually hydrogen peroxide (H_2O_2), superoxide anions, and hydroxyl radicals, production during cryopreservation [13–15]. There are data showing that human sperm DNA fragmentation is associated with an increase in oxidative stress during cryopreservation, rather than the activation of caspases and apoptosis [16]. Excessive and increased generation of ROS followed by peroxidation of membrane phospholipids are proposed as one of the biochemical basis of damaging effect during sperm cryopreservation [17]. Decreased glutathione levels found in frozen cells [18,19] as well as reduced antioxidative defense activities like SOD, catalase and others [20] will be involved in a net increase of ROS during cryopreservation. Cryopreserved retinal pigment epithelial cells showed increased expression of senescence-associated beta-gal activity, increased single-strand DNA breaks in telomeric regions and subsequently accelerated telomeric loss after thawing [21]. Cryopreservation also promotes DNA strand breaks in other regions and induces alterations in damage repair systems [22]. It was shown in human lymphocytes that cryopreservation lead to a decreased ability to repair DNA damage after hydrogen peroxide challenge [23]. One of the key DNA repair enzyme, H2AX histone protein, was found to be phosphorylated and activated in two different cell lines in response to freezing at −20 °C and −80 °C. There is a possibility that H2AX autophosphorylates at freezing temperature to preserve genetic integrity. But it is also possible that freezing cells induces disulfide bond formation through oxidative stress [24].

2.3. Cryoprotection

In order to improve the survival of cryopreseved cells, cryoprotectant agents (CPAs) are used. Major effects of CPAs are determined by their ability to reduce the freezing and thawing point and to lower the optimal cooling rate.

Ice-blockers specifically prevent the formation of ice nuclei in the solution, binding to nuclei, slowing down ice crystal growth during cooling and warming.

CPAs can be divided in two different groups, the low- and the high-molecular weight CPAs.

Low-molecular cryoprotectants like glycerol, ethylene (propylene) glycol [25,26], dimethylsulfoxide [27] are able to penetrate the cellular membrane and permeate inside the cells.

In contrast, high-molecular cryoprotectants can usually not enter cells. They remain in the extracellular space and participate in cell dehydration and minimization of intracellular ice crystal formation or membrane stabilization. Examples of high-molecular CPAs are: dextran, hydroxyethyl starch and polyvinyl-pyrrolidon and polyvinylalcohol [28,29], and a separate class of antifreeze proteins [30]. However it was found that even very high molecular CPAs like can enter cells and remain there for days or weeks [31–34]. Even non-phagocytotic cells can take up material from the extracellular room via pinocytosis [35].

Sugars and their derivatives, serve as natural cryoprotectors in plants and animals. Sugars like glucose, sucrose and trehalose are found in different plants [36], frogs [37] and brine shrimps [38] which are able to tolerate low temperatures. Both sucrose and trehalose are non-penetrating cryoprotectants, but reduce intracellular ice formation by promoting dehydration.

An alternative to simple sugars as cryoprotectants are complex carbohydrates. One such example which has been used in cryopreservation is hydroxylethyl starch (HES).

3. Hydroxylethyl starch (HES)

3.1. HES structure

An alternative to simple sugars as cryoprotectants are complex carbohydrates made e.g. of glucose. One such example which has been used in cryopreservation is hydroxylethyl starch (HES), a synthetic modified polymer based on purified starch of corn or potatoes that has been modified by hydroxyethylation at carbon position C2, C3 or C6 [39–41] (see Fig. 1).

The physical, chemical and clinical properties of HES are depending on: [40] (see Fig. 2).

(i) The manufactured average molecular weight (MW), ranging from high (⩾450 kDa), medium (200–400 kDa), to low MW(<200 kDa).
(ii) The degree of molar substitution (MS) measured by the number of hydroxylethyl groups present per total glucose subunit, categorized into high ('heptastarch' – 0.62–0.75), medium ('pentastarch' – 0.5), and low ('tetrastarch' – 0.4) MS.

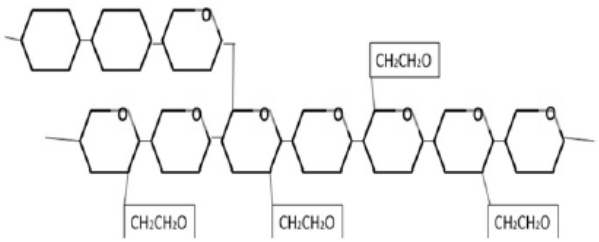


Fig. 1. Structure of HES. The diagram shows the schematic structure of HES. Possible hydroxyethylation positions are on the glucose core at the atom C2 or C5. HES comes with high substitution rates at these sides or low, which affects solubility and degradability of the molecule.

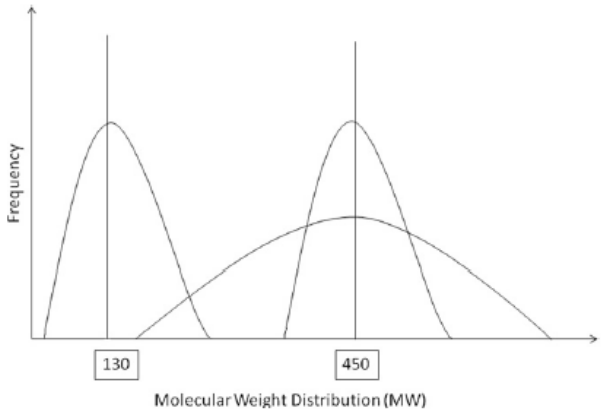


Fig. 2. Molecular weight distribution of HES. On an exemplary illustrative scale the different type of distributions of various HES products. The MW can be identical for two products but the distribution can vary greatly. Some HES do only contain a very narrow range of HES others not.

(iii) The C2/C6 ratio (the hydroxyethylation at carbon position C2 and C6.) classified into high ratio (>8) and low ratio (<8).

The higher the MS and the MW, the slower is the breakdown rate of HES. A higher C2/C6 ratio results in reduced breakdown of HES in vivo [42]. Another important factor (not least for experimental quality control) is not just the mean, but the distribution of the molecular weight of HES. HES can have the same mean weight yet very different weight distributions (see Fig. 2).

It has been observed that the HES solutions available for volume replacement contain low molecular weight impurities (i.e., electrolytes, oligosaccharides) that may differ from one lot to the next from the same manufacturer. Sputtek remarks that "This is one reason why some people have difficulties to reproduce to the work of others (and sometimes their own...)" [43].

3.2. Mechanism of HES cryoprotective effects

Large molecules of HES serve as a non-penetrating cryoprotectant (though some endocytosis might be involved). Thus, the mechanism of HES cryoprotection is different from that of penetrating cryoprotectors like DMSO and glycerol, which are known to depress the freezing point of cryosolutions and delay the point at which the salt concentration becomes damaging to a lower temperature.

In contrast, the cryoprotective effect of HES depends on its ability to absorb water molecules and keep these thermally inert in glassy state without experiencing any phase transition during cooling. It was shown that HES can absorb up to 0.5 g water per 1 g of HES [44]. Due to its physical properties and depending on the concentration and MW, HES influences the viscosity of solutions and decreases the cooling rate required for optimal survival during vitrification, increases propensity for supercooling and kinetically inhibits ice formation [28,45].

The accumulation of HES in the extracellular space leads to changed water flow behavior during the course of cooling and warming: Initially increased extracellular viscosity reduces the rate at which water can be withdrawn from the cells preventing osmotic stress and damage [28,46]. However, as HES attracts and absorbs water, its viscosity is reduced and the rate of dehydration increases, which then allows cells to be cooled rapidly avoiding intracellular ice crystal formation as well as chilling injury [26].

Another factor is the prevalence and proximity of HES to the cell. In spatial distribution, there needs to be sufficient HES present to block water diffusion 'paths' to ice nucleation points in the extracellular space. On the other hand, if there is too much HES present in the immediate vicinity of the cell, water egress is slowed so that too much water can remain trapped in the cell and can form intracellular ice crystals during cooling [28].

20% HES solution which allows successful cryopreservation of human monocytes also has a glass transition temperature close to −20 °C [28].

During warming, the high intracellular viscosity suppresses intracellular ice crystal growing by suppressing molecular motion and helps overcome the problem of ice crystal growth during de-vitrification [47]. The general mechanism of HES cryoprotection in comparison with DMSO is abstractly shown in Fig. 3.

3.3. The history of HES as a cryoprotectant

Synthesized in 1934 [48], the history of HES as a cryoprotectant is closely linked with the use of synthetic colloids in blood transfusion. Polyvinylpyrrolidone (PVP) was the first commercially available synthetic colloid developed during the second world war for blood transfusions [49], interest in the field and the search for PVP alternatives yielded HES as a potential candidate. In a separate strand, HES was used to separate granulocytes from normal human blood in the sedimentation of buffy coats with HES to concentrate leukocytes [50]. This lead naturally to investigating the effect of HES when freezing blood. In 1966 Ballinger et al. pioneered the use of HES as a plasma expander in dogs [51] and proved safety in human application [52]. Even while further safety studies were underway

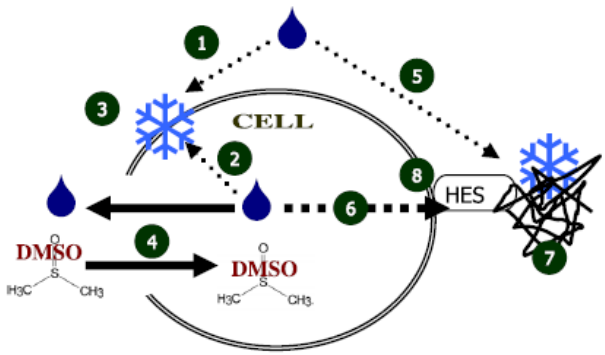


Fig. 3. Upon cryopreservation, extracellular (1) and intracellular (2) water crystallises to ice which can lead to damage including to membranes (3). DMSO opens cell walls and leads to water being removed from the cell (4) while DMSO enters. HES in turn binds extracellular water (5) and establishes a concentration gradient which removes water from the cell (6), and can thereby confine ice formation away from the cell (7). In addition, it is considered to stabilize the cell membrane (8) but normally without entering the cell.

Garzon [53] and Knorpp [54] began investigating HES as a cryoprotectant in erythrocyte cryopreservation [54]. They found 98% cell survival, recommending HES as a viable cryoprotectant, especially in comparison with PVP.

However, by that time a 'competitor' compound was already being promoted: In 1959, DMSO had entered the scene and subsequently attracted significant attention as a 'miracle compound' [55].

Although a 6% HES concentration was used for plasma expansion, Knorpp selected a 15% solution without giving a reason for the chosen concentration. Indeed, when Persidsky [56] later compared HES to DMSO in the cryopreservation of rat bone marrow cells, 15% HES was found to be more effective than 10% or 20%. Ashwood-Smith et al. [57] found 10% and 15% HES equally effective compared to 10% DMSO in survival of cryopreserved Chinese hamster cells.

Thus, by the mid-1970s, the following parameters had been established, albeit on a limited evidence base: (1) HES was useful in cryopreservation of blood and stem cells, not least because it could also be used clinically, (2) it was equally, but not more effective than DMSO and (3) it seemed sensible to use both in conjunction, probably in roughly equal parts making up 10% total.

Building on this, in 1975, Lionetti and colleagues established two paradigms of the field: They suggested that HES on its own was much less effective than HES and DMSO combined [58], and they suggested – at the time without experimental data – that a 6% HES solution might be best: "Ideally the thawed unit could be transfused directly, but a dilution of the thawed unit to reduce HES from 14% to that of the plasma protein level (about 6%) is desirable. This affords the option of additional manipulation of the unit such as a centrifugation and expression of unwanted components (hemoglobin, cell fragments, HES) by extraction of the supernatant fluids." Lionetti et al. then went on to trial a 6%HES/5% DMSO mixture [59] (the '6&5 solution') and found it superior to 4%HES/5%DMSO.

This was corroborated during the 1980s when Stiff [60,61] found 'the 6&5 solution' more effective in bone marrow preservation compared to 10% DMSO. In the fol-

Table 1
Types of cells cryopreserved using HES.

Type of cells	Concentrations of HES used for cryopreservation	Additions	Effect of cryopreservation with HES	Authors
Human pancreatic islets	6% HES MW not specified	5%DMSO + 4% FBS	No comparison to DMSO	[64]
Human granulocytes	4% (w/v) HES MW = 150 kDa DS = 0.75	5% DMSO	4% HES gives 50% cell recovery compared to 4% HES + 5% DMSO	[64]
Canine red blood cells	2.5, 7.5, 12.5, 17.5, 22.5% (w/v) HES MW = 200 kDa	–	12.5% HES has good results on thaw hemolysis, saline stability and osmotic fragility compared to 20% glycerol	[62,100]
Human red blood cells	11.5% (w/w) HES MW = 200 kDa DS = 0.5	–	HES gives high survival rate and high saline stability. Not compared to DMSO	[70,99]
Canine vascular mixed cells	6% HES	5% DMSO	Better viability in HES/DMSO cryopreserved cells compared to 10% DMSO	[101]
Human keratinocytes	Suspension: 2, 4, 6, 8, 10 w/w % HES Monolayers: 4, 6, 8, 10 w/w % HES, MW = 200 kDa; DS = 0.62	+/− 5% NCS	Higher survival rate for suspended and adherent cells in cryosolution with 8–10% HES compared to lower HES concentrations No comparison to DMSO	[102]
Human erythrocytes	12% HES MW = 200 kDa DS = 0.62	–	Erythrocytes show changes in the protein composition after HES cryopreservation. No comparison to DMSO	[103]
Human erythrocytes	6% HES	–	Erythrocytes show high level of damage. No comparison to DMSO	[104]
Human monocytes	0, 10, 20, 30, 40, 50% (w/w) HES MW = 450 kDa	–	70% monocytes survived with 20% HES. 100% monocytes survived with 7% DMSO. HES potentiates the effect of the DMSO	[28]
Human red blood cells	14% HES MW not specified	–	High cell recovery and saline stability of cells No comparison to DMSO	[71]
Human red blood cells	14% HES (w/v) MW = 150 DS = 0.75	–	High cell recovery and saline stability in large volume (405 ml) No comparison to DMSO	[63]
Chinese hamster cells	10, 15, 20% (w/v) HES MW = 450 kDa & 150 kDa	10% (FCS)	Nearly as good as 10% DMSO	[57]

lowing two decades, most research involving HES in comparison with other cryopreservation solutions followed this pattern: comparing 'the 6&5 solution' against 10% in various cell types.

3.4. Efficacy of HES as a cryoprotectant

As established, the assessment of HES as a cryoprotectant can take different forms: (1) 'in principle' demonstrations of some efficacy; (2) in comparison against some other substance, notably DMSO; (3) as an admixture to DMSO. We will briefly discussed it on this section.

A number of different cell types have been cryopreserved with various degrees of success using HES in combination with other cryoprotectors as well as HES alone (Table 1). HES is frequently used as a red blood cell cryoprotectant [58,62] and it has also been successfully used for cryopreservation of granulocytes [63], cultured hamster cells [57] and pancreatic islets [64,65]. Some stem cell types, for example bone marrow and blood stem cells [66,67] (Table 2) and even tissues such as rat liver [68] and human trachea [69] have been cryopreserved using HES (Table 3). Unfortunately in many studies specific HES characteristics and the source of the HES (manufacturer, batch) are not documented.

Only a few "type 2" studies have been conducted: 11.5% HES alone in cryosolution lead to a high survival rate for human red blood cells (RBC) [58,70]. Another study reported cell recovery of 97% and more than 80% saline stability of human red blood cells after cryopreservation with 14% HES [71]. The parameters of thaw hemolysis, saline stability, osmotic fragility and morphological changes after thawing of canine red blood cells cryopreserved with 12.5% HES are noticeably better in comparison with conventional 20% glycerol solution [62]. There are only two 'type 2' studies *comparing* HES to undiluted DMSO: both would suggest that HES alone is slightly less effective than DMSO alone. The first investigated the freezing of Chinese Hamster Ovarien cells and found 22% cell survival for 10% HES in comparison to 39% for 10% DMSO [57]. The other study investigated human monocytes and found them to be surviving better with DMSO then with HES alone [28].

Type (3) is the most common study in this field, usually demonstrating that 'the 6&5 solution' is more effective in

Table 2
Stem cells cryopreserved using HES.

Type of cells	Concentrations of HES used for cryopreservation	Additional substances used	Effect of cryopreservation with HES	Authors
Human peripheral blood progenitors	6% HES	5% DMSO, 4% HSA	Significant higher cell recovery in HES/DMSO samples compared to 10% DMSO	[105]
Human cord blood stem cells	6% HES MW not specified	5% DMSO + 10% DMSO	Greater cell viability and better engraftment in SCID mice for the HES/DMSO samples compared to10% DMSO	[106]
Human peripheral blood stem cells	6% HES pentastarch MW = 150–350	5% DMSO 4 Human serum albumin	Solutions are not comparable as for the 10% DMSO a different freezing protocol was applied	[107]
Canine bone marrow derived hematopoietic stem cells	6% HES MW not specified	5% DMSO + 4% BSA	The HES/DMSO mixture gave better cell survival and CFU frequency compared to 10% DMSO	[108]
Human peripheral blood progenitors	2.5% HES MW not specified	1% HSA 3.5% DMSO	Good engraftment, good cell survival. No comparison to DMSO or other substance	[109]
Human bone marrow & peripheral blood progenitors	6% HES MW not specified	5% DMSO, 4% HSA	Good cell survival and CFU recovery. No comparison to DMSO or other substance	[110]
Human peripheral blood progenitors	6% HES MW not specified	5% DMSO, 4% HSA	Around 80% cell survival. No comparison to DMSO or other substance	[111]
Human cord blood stem cells	4% HES	5% + 2.5% DMSO	From 85% CD34 cell survival with 5% DMSO to 12% with 2.5% DMSO. HES concentration variations in combination with 5% DMSO made no difference	[112]
Human bone marrow stem cells	6% HES MW = 150,7 kDa	5% DMSO + 4% HSA	Greater cell numbers of viable cells and a greater CFU-C recovery for cells in comparison to 10% DMSO	[60]
Human peripheral blood progenitors	5% HES	5% DMSO	Good engraftment after transplantation. Not compared to DMSO	[113]
Human peripheral blood cells	3% HES MW not specified	5% DMSO	Higher viability, granulocyte macrophage colony forming unit (GM-CFU) activity and nucleated cell recovery in comparison to 10% DMSO	[114]
Human bone marrow stem cells	6% HES MW = 150 kDa	5% DMSO + 4% HSA	Not compared to DMSO	[61]
Human bone marrow stem cells	6% HES MW = 150 kDa	5% DMSO + 4% HSA	Higher cell recovery and cell viability compared to cells preserved in 10% DMSO	[66]
Human blood stem cells	6% HES MW not specified	5% DMSO + 4% HSA	Neutrophil and white blood counts recover faster in patients who received HES-cryopreserved cells compared to 10% DMSO cryopreserved cells	[86]

Table 3
Tissues cryopreserved using of HES.

Tissue	Concentrations of HES used for cryopreservation	Additional substances used	Effect of cryopreservation with HES	Authors
Mice osteoblasts and goat MSC derived micro tissues	5% HES	60% serum Ascorbic acid	Micro tissues on carriers had a better cell survival in 10% DMSO	[115]
Canine pancreatic islets	6% HES MW not specified	5% DMSO + 4% (FBS)	No comparison to DMSO	[65]
Rat molars	6% HES	5% DMSO	Molars showed slower regeneration after transplantation compared to non-cryopreserved molars. No comparison to DMSO	[116]
Human trachea	HES (no data on HES concentration; MW not specified)	(a) 20% glycerol; (b) 15% DMSO; (c) 15% DMSO + 4% albumin; (d) none	Histological & mechanical characteristics are well maintained in combination of HES /glycerol	[69]
Rat liver	5% HES (MW not specified)	University of Wisconsin (UW) solution	Presence of HES in the UW solution decreases proteolysis and retains cell volume	[68]

terms of preserving cell viability, recovery rate and in maintaining the colony-forming capacity of stem cells. These studies, ranging from canine and human pancreatic islets, and human keratinocytes but usually focused on 'transplantable cells' such as monocytes, granulocytes, red & peripheral blood cells, human bone marrow cells and 'blood stem cells' are summarized in Tables 1 and 2. Unfortunately in many studies specific HES characteristics and the source of the HES (manufacturer, batch) are not documented.

In case of tissue cryopreservation HES also shows some protection ability: presence of HES limits proteolysis in rat liver during cryopreservation whereas cryopreservation without HES stimulates protein degradation, oxygen consumption, decreases glucose production and promote intracellular volume reduction [68]. Human trachea cryopreserved in presence of HES and 20% glycerol retain all histological and mechanical characteristics [69].

4. Safety

A major disadvantage of cryoprotectants is their toxicity. Low-molecular penetrating CPAs can remove water from intracellular molecules, leading to unwanted interactions between proteins and to protein denaturation [72]. Thus, they have to be very carefully removed after cryopreservation, normally by centrifugation which has its own drawbacks. High-molecular cryoprotectants are usually better tolerated and do not necessarily need to be removed [70].

In pure form, all cryoprotectants are toxic to some degree (see Table 4), although it is difficult to transfer these data to a scenario where residual traces remain after cryopreservation.

4.1. Regulatory specifications

In a regulatory framework regarding the cryopreservation of stem cells, a first consideration is whether cryopreservation alone is a process of more than minimal manipulation. US regulations define "minimal manipulation" as "processing that does not alter the original relevant characteristics of the tissue relating to the tissue's utility for reconstruction, repair, or replacement" for structural tissue (21 CFR 1271.20;) and simply as "processing that does not alter the relevant biological characteristics." (21 CFR 1271.3(f)(2)) for cells. FDA has stated specifically that freezing and cryopreservation constitute minimal manipulation. Similarly, freezing, cryopreservation and vitrification are expressly listed in Annex I to Regulation (EC)

Table 4
Toxicity doses of commonly used cryoprotectants.

Substance	LD50 in rats	Humans	Literature
DMSO	• 17–28 g/kg oral • 5–8 g/kg IV	• 4 g/kg oral and IV toxic in rhesus monkeys	• Noel et al. (1975) • Vogin et al. (1970) • www.gaylordchemical.com
HES	• 50 g/kg oral • 8,3 g/kg IV	• 200 g/day IV found to be safe in humans	• www.bmscanada.ca • www.polymer.de
Glycerol	• 12,6 g/kg oral • 2,1 g/kg IV	• 1,6 g/kg IV toxic in humans	• www.sitcan21.com
Ethylene glycol	• 4,7 g/kg-31 g/kg oral • 17 g/kg IV	• 30 ml IV fatal in humans • 1,4 g/kg oral toxic in humans	• European chemical substances information system • www.inchem.org
Propylene glycol	• 20 g/kg oral • 68 g/kg IV	• 1 g/l in serum toxic in humans • 25 mg/kg oral toxic in humans	• WHO expert committee on food additives (1974) • www.inchem.org

LD50: known doses leading to death in 50% of the tested subjects (rats).

No. 1394/2007, and therefore are not considered as 'substantial manipulations' of cells. Therefore cryopreservation itself does not make a stem cell or blood transfusion into an advanced therapy medicinal product (ATMP). This does not mean however, that the type of cryoprotectant is not on the regulatory radar screen – if a cell is considered 'substantially manipulated' for reasons other than cryopreservation, the whole process of its manufacture and handling will be considered. A system of Quality Risk Management (QRM), according to ICH Q9 Chapter 1 (Annex 20) requires an evaluation of risk to quality based on scientific knowledge and link to the protection of the patient and a level of effort, formality and documentation of the QRM process commensurate with the level of risk. This is where HES MW and MW distribution in production batches could well play a role.

4.2. DMSO toxicity

While findings differ, there is now a substantial body of evidence in support of 'the 6&5' solution as superior to 10% DMSO in preserving cell viability but also in more rapid cell recovery after faster engraftment after stem cell transplantation (see Table 2).

DMSO can decrease membrane thickening and induces temporary water pores when used at low concentrations. Side effects of infusion of DMSO-cryopreserved cells include nausea, emesis, chills, rigors, and cardiovascular events [74–76]. DMSO also shows neurotoxic effects including encephalopathy, when stem cells were infused into cancer patients, as well as gastrointestinal effects [77,78]. DMSO is also directly cell toxic, affecting cell viability, inducing apoptosis and differentiation [79]. DMSO can decrease membrane thickening and induces temporary water pores when used at low concentrations. At higher concentration it induces disintegration of the bilayer structure of the lipid membrane [73].

Given this toxicity, the removal of DMSO before transplantation (or research) is necessary. Different techniques are applied for DMSO removal with centrifugation being the most common. Cells are being centrifuged, the DMSO containing cryomedia removed and the cells washed before they are resuspended in a buffer and transplanted. Centrifugation and washing are labor and time intensive and results in significant cell losses. For human umbilical cord blood (UCB) the loss of cells is around 27–30% of nucleated cells using either a centrifuge [80] or an automated cell washer [81]. Low cell count has an influence on transplant outcome like delayed engraftment and increased morbidity [82,83].

There are no guidelines for the use of DMSO in bone marrow transplantations despite its reported side-effects. The incidence of DMSO-related severe complications is 1 in 70 patients and for general side effects such as nausea, vomiting and abdominal cramp the frequency was reported to be in 50% [84]. In comparison, for HES used as a plasma expander, the reported side-effects were only 0.001–0.007% [85]. Also the used concentration of DMSO or the application of washing is not regulated and no guideline exists. Centers using lower DMSO concentrations or wash the cells before transplantation, report less complications [84]. Similar observations were made in a phase III study on bone marrow transplantation showing that 5% DMSO/6% HES compared to 10% DMSO improved engraftment and reduced hospitalization costs associated with DMSO toxicity [86].

4.3. HES toxicity – effect of MS, MW and degree of substitution

HES is widely used in the clinical setting as a plasma volume substitute due to its colloidal osmotic pressure which increases viscosity of plasma and whole blood [87] and facilitates delivering of oxygen by red blood cells, in hemodilution treatment to enhance the microcirculation and for peripheral arterial stenosis treatment [88].

In vivo, HES is metabolized by glycolytic enzymes, e.g. by α-amylase. The rate of breakdown and excretion of HES is dependent on MS and average MW. Usually enzymatic cleavage with α-amylase provides a wide range of HES fragments and those with lower MW (lower than 40 kDa) are renally excreted with urine [40,89]. It was shown that HES administration leads to increased serum amylase concentration of up to 5 times the initial value. However, this increase does not affect the pancrease or lipase activity and therefore seems to have no pathological relevance [90,91].

In one study, 80% of kidneys transplants where donors were administered HES 200/0.62, developed osmotic nephrosis-like lesions after 6 weeks in comparison to 14% cases without HES administration [92]. A meta-analysis of 34 studies on patients undergoing fluid therapy evaluating kidney function showed that patients receiving HES had a slightly higher rate of kidney complications with sepsis a particular patients concern [93].

As mentioned above, MS, MW and degree of substitution all have an influence on how quickly HES can be cleared in vivo. While HES is not penetrating, it can be taken up by a variety of cells via phagocytosis or pinocytosis and is then excreted more slowly at higher molecular weight [32,34,35,94,95]. This uptake may be one reason why some patients can develop itching symptoms (pruritus) after chronic HES administration [96]. However, the incidence of HES-associated anaphylactoid reaction at an incidence of ~0.006% is considerably lower in comparison to other commonly used plasma substitutes [97]. Infusion of high volumes of high MW (450/07) and medium MW (200/0.62) HES have been shown to result in bleeding complications due to decreased factor VIII/von Willebrandt factor, platelet function defects and incorporation into fibrin clots, probably due to dilution effects. Large HES molecules can cause detrimental effects on rheological parameters of blood. Slow clearance can lead to coagulation disorders in patient but plasma viscosity also depends on the rate of infusion and the HES concentration [41,91,98]. Therefore, low or medium MW HES is recommended for transfusion by some. There is a single study reporting an effect of MW on cryoprotection: in Chinese hamster cells, high MW (450 kDa) was more effective as a cryoprotectant compared to low MW (150 kDa) [66]. Whether this hints at a general trade-off between cryoprotective efficacy and clinical safety remains to be investigated.

4.4. Safety and benefit of HES as a cryoprotectant

What does this mean for the use of HES as a cryoprotectant in a clinical setting? Likely, the potential detriments associated with HES transfusion are less relevant in this context since HES would only be incorporated as a trace amount.

Autologous re-infusion of red blood cells cryopreserved with HES showed no adverse reactions with or without post thaw washing step [70]. In another study, autotransplantation of frozen human RBCs after thawing without any washing steps, more than 85% of the HES was eliminated from plasma within the first day. Viability of the red cells after thawing in terms of saline stability reached 91.9 +/− 0.7% [58,70]. After transfusion with HES (medium MW 200/0.5)-cryopreserved erythrocytes some patients can develop low level of posttransfusional leukocytosis and moderate increase bilirubin, but these effects disappear within 20 h. The concentrations of platelets, electrolytes, urea, protein and creatinine are within their physiological ranges [99].

For the minimization of cell manipulation and subsequently a more easy transfer to production of cells for the clinic, using only HES would be superior. HES has no influence on cells in vitro and has only in very rare cases side effects in vivo.

These results need to be seen in the light of the efficacy studies reported above that would seem to give an implicit indication of reduced toxicity with 'the 6&5 solution' compared to 10% DMSO.

Given these potential detriments of DMSO, the question might arise if it would not be preferable to use HES alone. Given the much lower toxicity of HES, this could then render the 'centrifugation and washing' step obsolete, mitigating cell loss. However, as mentioned above (Section 3.4), the sparse published data may suggest that HES alone is not as effective a cryoprotectant.

5. Summary

Despite the list of successful cryopreservations of cells and tissues using HES, the mechanism of its action is weakly understood. This includes studies on the effect of molecular weight, degree of its molar substitution and purity on its success in cryopreservation. Given the general non-toxic profile of HES and the amounts likely involved in a cryopreserved clinical product, there seems ample scope to employ a wide range of HES with different MW, MS and degree of hydroxyethylation in order to create effective cryoprotective solution without compromising patient safety. Nonetheless, those wishing to use HES in cryopreservation of a clinical product, in particular during the manufacture or handling of an investigational cell therapy are advised to monitor MW variability and distribution within the batch.

Cryopreservation of different cell types using both HES and DMSO (established, for historical reasons in a mixture of 5% DMSO and 6% HES) appears preferable in many scenarios to using DMSO by itself. Since HES is less toxic than DMSO, the possibility of eliminating a 'washing' step in some regenerative medicine therapies may be attractive, but whether HES can fully replace DMSO as a cryoprotectant in this context has yet to be explored.

6. Declaration of interest

FV, NY and AS have in the past been funded by Serumwerke Bernburg AG, a manufacturer of HES.

References

[1] Fowler A, Toner M. Cryo-injury and biopreservation. Ann N Y Acad Sci 2005;1066:119–35.
[2] Muller K, Muller P, Pincemy G, Kurz A, Labbe C. Characterization of sperm plasma membrane properties after cholesterol modification: consequences for cryopreservation of rainbow trout spermatozoa. Biol Reprod 2008;78(3):390–9.
[3] Gage AA, Baust JG. Cryosurgery – a review of recent advances and current issues. Cryo Lett 2002;23(2):69–78.
[4] Mazur P. Cryobiology: the freezing of biological systems. Science 1970;168(934):939–49.
[5] Mishima O, Suzuki Y. Propagation of the polyamorphic transition of ice and the liquid-liquid critical point. Nature 2002;419(6907):599–603.
[6] Diller KR, Aggarwal SJ. Computer automated cell size and shape analysis in cryomicroscopy. J Microsc 1987;146(Pt 2):209–19.
[7] Taylor MJ, Pegg DE. The effect of ice formation on the function of smooth muscle tissue stored at −21 or −60 °C. Cryobiology 1983;20(1):36–40.
[8] Irimia D, Karlsson JO. Kinetics and mechanism of intercellular ice propagation in a micropatterned tissue construct. Biophys J 2002;82(4):1858–68.
[9] Karlsson JO, Toner M. Long-term storage of tissues by cryopreservation: critical issues. Biomaterials 1996;17(3):243–56.
[10] Muldrew K, McGann LE. The osmotic rupture hypothesis of intracellular freezing injury. Biophys J 1994;66(2 Pt 1):532–41.
[11] Baust JM, Van B, Baust JG. Cell viability improves following inhibition of cryopreservation-induced apoptosis. In Vitro Cell Dev Biol Anim 2000;36(4):262–70.
[12] Jeong YJ, Kim MK, Song HJ, Kang EJ, Ock SA, Kumar BM, et al. Effect of alpha-tocopherol supplementation during boar semen cryopreservation on sperm characteristics and expression of apoptosis related genes. Cryobiology 2009;58(2):181–9.
[13] Mazzilli F, Rossi T, Sabatini L, Pulcinelli FM, Rapone S, Dondero F, et al. Human sperm cryopreservation and reactive oxygen species (ROS) production. Acta Eur Fertil 1995;26(4):145–8.
[14] Tselkas K, Saratsis P, Karagianidis A, Samouilidis S. Extracellular presence of reactive oxygen species (ROS) in fresh and frozen-thawed canine semen and their effects on some semen parameters. Dtsch Tierarztl Wochenschr 2000;107(2):69–72.
[15] Sohn IP, Ahn HJ, Park DW, Gye MC, Jo DH, Kim SY, et al. Amelioration of mitochondrial dysfunction and apoptosis of two-cell mouse embryos after freezing and thawing by the high frequency liquid nitrogen infusion. Mol Cell 2002;13(2):272–80.
[16] Thomson LK, Fleming SD, Aitken RJ, De Iuliis GN, Zieschang JA, Clark AM. Cryopreservation-induced human sperm DNA damage is predominantly mediated by oxidative stress rather than apoptosis. Hum Reprod 2009;24(9):2061–70.
[17] Alvarez JG, Storey BT. Evidence for increased lipid peroxidative damage and loss of superoxide dismutase activity as a mode of sublethal cryodamage to human sperm during cryopreservation. J Androl 1992;13(3):232–41.
[18] McAnulty JF, Huang XQ. The efficacy of antioxidants administered during low temperature storage of warm ischemic kidney tissue slices. Cryobiology 1997;34(4):406–15.
[19] Stevenson DJ, Morgan C, McLellan LI, Helen Grant M. Reduced glutathione levels and expression of the enzymes of glutathione synthesis in cryopreserved hepatocyte monolayer cultures. Toxicol In Vitro 2007;21(3):527–32.
[20] Bilodeau JF, Chatterjee S, Sirard MA, Gagnon C. Levels of antioxidant defenses are decreased in bovine spermatozoa after a cycle of freezing and thawing. Mol Reprod Dev 2000;55(3):282–8.
[21] Honda S, Weigel A, Hjelmeland LM, Handa JT. Induction of telomere shortening and replicative senescence by cryopreservation. Biochem Biophys Res Commun 2001;282(2):493–8.

[22] Cabrita E, Robles V, Rebordinos L, Sarasquete C, Herraez MP. Evaluation of DNA damage in rainbow trout (Oncorhynchus mykiss) and gilthead sea bream (Sparus aurata) cryopreserved sperm. Cryobiology 2005;50(2):144–53.
[23] Duthie SJ, Pirie L, Jenkinson AM, Narayanan S. Cryopreserved versus freshly isolated lymphocytes in human biomonitoring: endogenous and induced DNA damage, antioxidant status and repair capability. Mutagenesis 2002;17(3):211–4.
[24] Peng L, Wang S, Yin S, Li C, Li Z, Liu Q. Autophosphorylation of H2AX in a cell-specific frozen dependent way. Cryobiology 2008;57(2):175–7.
[25] Vandevoort CA, Shirley CR, Hill DL, Leibo SP. Effects of cryoprotectants and cryopreservation on germinal vesicle-stage cumulus-oocyte complexes of rhesus monkeys. Fertil Steril 2008;90(3):805–16.
[26] McGann LE. Differing actions of penetrating and nonpenetrating cryoprotective agents. Cryobiology 1978;15(4):382–90.
[27] Lehle K, Hoenicka M, Jacobs VR, Schmid FX, Birnbaum DE. Cryopreservation of human endothelial cells for vascular tissue engineering. Cryobiology 2005;50(2):154–61.
[28] Takahashi T, Hirsh A, Erbe E, Williams RJ. Mechanism of cryoprotection by extracellular polymeric solutes. Biophys J 1988;54(3):509–18.
[29] Bakaltcheva I, Ganong JP, Holtz BL, Peat RA, Reid T. Effects of high-molecular-weight cryoprotectants on platelets and the coagulation system. Cryobiology 2000;40(4):283–93.
[30] Chao H, Davies PL, Carpenter JF. Effects of antifreeze proteins on red blood cell survival during cryopreservation. J Exp Biol 1996;199(Pt 9):2071–6.
[31] Thompson WL, Fukushima T, Rutherford RB, Walton RP. Intravascular persistence, tissue storage, and excretion of hydroxyethyl starch. Surg Gynecol Obstet 1970;131(5):965–72.
[32] Jurecka W, Szepfalusi Z, Parth E, Schimetta W, Gebhart W, Scheiner O, et al. Hydroxyethylstarch deposits in human skin – a model for pruritus? Arch Dermatol Res 1993;285(1–2):13–9.
[33] Dienes HP, Gerharz CD, Wagner R, Weber M, John HD. Accumulation of hydroxyethyl starch (HES) in the liver of patients with renal failure and portal hypertension. J Hepatol 1986;3(2):223–7.
[34] Stander S, Szepfalusi Z, Bohle B, Stander H, Kraft D, Luger TA, et al. Differential storage of hydroxyethyl starch (HES) in the skin: an immunoelectron-microscopical long-term study. Cell Tissue Res 2001;304(2):261–9.
[35] Nohe B, Burchard M, Zanke C, Eichner M, Krump-Konvalinkova V, Kirkpatrick CJ, et al. Endothelial accumulation of hydroxyethyl starch and functional consequences on leukocyte-endothelial interactions. Eur Surg Res 2002;34(5):364–72.
[36] Fernandez O, Bethencourt L, Quero A, Sangwan RS, Clement C. Trehalose and plant stress responses: friend or foe? Trends Plant Sci 2010;15(7):409–17.
[37] Storey KB. Life in a frozen state: adaptive strategies for natural freeze tolerance in amphibians and reptiles. Am J Physiol 1990;258(3 Pt 2):R559–68.
[38] Crowe JH, Crowe LM, Chapman D. Preservation of membranes in anhydrobiotic organisms: the role of trehalose. Science 1984;223(4637):701–3.
[39] Boldt J. Modern rapidly degradable hydroxyethyl starches: current concepts. Anesth Analg 2009;108(5):1574–82.
[40] Kozek-Langenecker SA. Effects of hydroxyethyl starch solutions on hemostasis. Anesthesiology 2005;103(3):654–60.
[41] Treib J, Baron JF, Grauer MT, Strauss RG. An international view of hydroxyethyl starches. Intensive Care Med 1999;25(3):258–68.
[42] Treib J, Haass A, Pindur G. Coagulation disorders caused by hydroxyethyl starch. Thromb Haemost 1997;78(3):974–83.
[43] Sputtek A. HES – A Nightmare? Society for Low Temperature Biology (SLTB) Newsletter February. 2001 Available from: www.sputtek.de/info/HES.pdf.
[44] Korber C, Scheiwe MW. The cryoprotective properties of hydroxyethyl starch investigated by means of differential thermal analysis. Cryobiology 1980;17(1):54–65.
[45] Connor W, Ashwood-Smith MJ. Cryoprotection of mammalian cells in tissue culture with polymers; possible mechanisms. Cryobiology 1973;10(6):488–96.
[46] Thomas MJ, Parry ES, Nash SG, Bell SH. A method for the cryopreservation of red blood cells using hydroxyethyl starch as a cryoprotectant. Transfus Sci 1996;17(3):385–96.
[47] Baust JG, Gage AA, Klossner D, Clarke D, Miller R, Cohen J, et al. Issues critical to the successful application of cryosurgical ablation of the prostate. Technol Cancer Res Treat 2007;6(2):97–109.
[48] Ziese W. Action of amylascs on hydroxyethyl starch. Z Physiol Chem 1934;229:213.
[49] Wiedersheim M. Synthetic macromolecular compounds and their use in medical therapy. S Afr J Lab Clin Med 1957;3(2):118–30.
[50] Roy AJ, Franklin A, Simmons WB, Djerassi I. A method for separation of granulocytes from normal human blood using hydroxyethyl starch. Prep Biochem 1971;1(3):197–203.
[51] Ballinger 2nd WF, Solanke TF, Thompson WL. The effect of hydroxyethyl starch upon survival of dogs subjected to hemorrhagic shock. Surg Gynecol Obstet 1966;122(1):33–6.
[52] Gollub S, Schechter DC, Hirose T, Bailey CP. Use of hydroxyethyl starch solution in extensive surgical operations. Surg Gynecol Obstet 1969;128(4):725–8.
[53] Garzon AA, Cheng C, Lerner B, Lichtenstein S, Karlson KE. Hydroxyethyl starch (HES) and bleeding. An experimental investigation of its effect on hemostasis. J Trauma 1967;7(5):757–66.
[54] Knorpp CT, Merchant WR, Gikas PW, Spencer HH, Thompson NW. Hydroxyethyl starch: extracellular cryophylactic agent for erythrocytes. Science 1967;157(794):1312–3.
[55] Lovelock JE, Bishop MW. Prevention of freezing damage to living cells by dimethyl sulphoxide. Nature 1959;183(4672):1394–5.
[56] Persidsky MD, Ellett MH. Hydroxyethyl starch as a cryopreservative for nucleated mammalian cells. Cryobiology 1971;8(6):586–8.
[57] Ashwood-Smith MJ, Warby C, Connor KW, Becker G. Low-temperature preservation of mammalian cells in tissue culture with polyvinylpyrrolidone (PVP), dextrans, and hydroxyethyl starch (HES). Cryobiology 1972;9(5):441–9.
[58] Lionetti FJ, Hunt SM. Cryopreservation of human red cells in liquid nitrogen with hydroxyethyl starch. Cryobiology 1975;12(2):110–8.
[59] Lionetti FJ, Hunt SM, Mattaliano RJ, Valeri CR. In vitro studies of cryopreserved baboon granulocytes. Transfusion 1978;18(6):685–92.
[60] Stiff PJ, Murgo AJ, Zaroulis CG, DeRisi MF, Clarkson BD. Unfractionated human marrow cell cryopreservation using dimethylsulfoxide and hydroxyethyl starch. Cryobiology 1983;20(1):17–24.
[61] Stiff PJ, Koester AR, Weidner MK, Dvorak K, Fisher RI. Autologous bone marrow transplantation using unfractionated cells cryopreserved in dimethylsulfoxide and hydroxyethyl starch without controlled-rate freezing. Blood 1987;70(4):974–8.
[62] Kim H, Tanaka S, Une S, Nakaichi M, Sumida S, Taura Y. A comparative study of the effects of glycerol and hydroxyethyl starch in canine red blood cell cryopreservation. J Vet Med Sci 2004;66(12):1543–7.
[63] Lionetti FJ, Hunt SM, Gore JM, Curby WA. Cryopreservation of human granulocytes. Cryobiology 1975;12(3):181–91.
[64] Kenmochi T, Asano T, Maruyama M, Saigo K, Akutsu N, Iwashita C, et al. Cryopreservation of human pancreatic islets from non-heart-beating donors using hydroxyethyl starch and dimethyl sulfoxide as cryoprotectants. Cell Transplant 2008;17(1–2):61–7.
[65] Maruyama M, Kenmochi T, Sakamoto K, Arita S, Iwashita C, Kashiwabara H. Simplified method for cryopreservation of islets using hydroxyethyl starch and dimethyl sulfoxide as cryoprotectants. Transplant Proc 2004;36(4):1133–4.
[66] Luo K, Wu G, Wang Q, Sun Y, Liu H. Effect of dimethylsulfoxide and hydroxyethyl starch in the preservation of fractionated human marrow cells. Cryobiology 1994;31(4):349–54.
[67] Sudo K, Asoh S, Ohsawa I, Ozaki D, Yamagata K, Ito H, et al. The anti-cell death FNK protein protects cells from death induced by freezing and thawing. Biochem Biophys Res Commun 2005;330(3):850–6.
[68] Neveux N, De Bandt JP, Charrueau C, Savier E, Chaumeil JC, Hannoun L, et al. Deletion of hydroxyethylstarch from University of Wisconsin solution induces cell shrinkage and proteolysis during and after cold storage of rat liver. Hepatology 1997;25(3):678–82.
[69] Mabrut JY, Adham M, Bourgeot JP, Eljaafari A, DelaRoche E, Ducerf C, et al. Mechanical and histological characteristics of human trachea before and after cryopreservation: an opportunity for tracheal tissue banking. Transplant Proc 2001;33(1–2):609–11.
[70] Sputtek A, Horn EP, Schulte Am Esch J, Kuhnl P. Cryopreservation of red blood cells using hydroxyethyl starch (HES) – from laboratory investigations to clinical application. Anasthesiol Intensivmed Notfallmed Schmerzther 2001;36(Suppl 2):S162–4.
[71] Allen ED, Weatherbee L, Permoad PA. Post-thaw suspension of red cells cryopreserved with hydroxyethyl starch. Cryobiology 1978;15(4):375–81.
[72] Arakawa T, Bhat R, Timasheff SN. Why preferential hydration does not always stabilize the native structure of globular proteins. Biochemistry 1990;29(7):1924–31.

[73] Gurtovenko AA, Anwar J. Modulating the structure and properties of cell membranes: the molecular mechanism of action of dimethyl sulfoxide. J Phys Chem B 2007;111(35):10453–60.
[74] Zambelli A, Poggi G, Da Prada G, Pedrazzoli P, Cuomo A, Miotti D, et al. Clinical toxicity of cryopreserved circulating progenitor cells infusion. Anticancer Res 1998;18(6B):4705–8.
[75] Ferrucci PF, Martinoni A, Cocorocchio E, Civelli M, Cinieri S, Cardinale D, et al. Evaluation of acute toxicities associated with autologous peripheral blood progenitor cell reinfusion in patients undergoing high-dose chemotherapy. Bone Marrow Transplant 2000;25(2):173–7.
[76] Stroncek DF, Strand RD, Hofkes CL, McCullough J. Comparison of the effectiveness of potential marrow donors recruited from apheresis donors or whole blood donors and through appeals to the general public. Transfusion 1991;31(2):138–41.
[77] Rowley SD, Feng Z, Yadock D, Holmberg L, Macleod B, Heimfeld S. Post-thaw removal of DMSO does not completely abrogate infusional toxicity or the need for pre-infusion histamine blockade. Cytotherapy 1999;1(6):439–46.
[78] Chen-Plotkin AS, Vossel KA, Samuels MA, Chen MH. Encephalopathy, stroke, and myocardial infarction with DMSO use in stem cell transplantation. Neurology 2007;68(11):859–61.
[79] Scheinkonig C, Kappicht S, Kolb HJ, Schleuning M. Adoption of long-term cultures to evaluate the cryoprotective potential of trehalose for freezing hematopoietic stem cells. Bone Marrow Transplant 2004;34(6):531–6.
[80] Antonenas V, Shaw PJ, Bradstock KF. Infusion of unwashed umbilical cord blood stem cells after thawing for allogeneic transplantation. Bone Marrow Transplant 2004;34(8):739.
[81] Perotti CG, Del Fante C, Viarengo G, Papa P, Rocchi L, Bergamaschi P, et al. A new automated cell washer device for thawed cord blood units. Transfusion 2004;44(6):900–6.
[82] Migliaccio AR, Adamson JW, Stevens CE, Dobrila NL, Carrier CM, Rubinstein P. Cell dose and speed of engraftment in placental/umbilical cord blood transplantation: graft progenitor cell content is a better predictor than nucleated cell quantity. Blood 2000;96(8):2717–22.
[83] Gluckman E, Rocha V, Chevret S. Results of unrelated umbilical cord blood hematopoietic stem cell transplantation. Rev Clin Exp Hematol 2001;5(2):87–99.
[84] Windrum P, Morris TC, Drake MB, Niederwieser D, Ruutu T. Variation in dimethyl sulfoxide use in stem cell transplantation: a survey of EBMT centres. Bone Marrow Transplant 2005;36(7):601–3.
[85] Brecher ME, Owen HG, Bandarenko N. Alternatives to albumin: starch replacement for plasma exchange. J Clin Apher 1997;12(3):146–53.
[86] Rowley SD, Feng Z, Chen L, Holmberg L, Heimfeld S, MacLeod B, et al. A randomized phase III clinical trial of autologous blood stem cell transplantation comparing cryopreservation using dimethylsulfoxide vs dimethylsulfoxide with hydroxyethylstarch. Bone Marrow Transplant 2003;31(11):1043–51.
[87] Neff TA, Fischler L, Mark M, Stocker R, Reinhart WH. The influence of two different hydroxyethyl starch solutions (6% HES 130/0.4 and 200/0.5) on blood viscosity. Anesth Analg 2005;100(6):1773–80.
[88] Westphal M, James MF, Kozek-Langenecker S, Stocker R, Guidet B, Van Aken H. Hydroxyethyl starches: different products – different effects. Anesthesiology 2009;111(1):187–202.
[89] Hulse JD, Yacobi A. Hetastarch: an overview of the colloid and its metabolism. Drug Intell Clin Pharm 1983;17(5):334–41.
[90] Kohler H, Zschiedrich H, Clasen R, Linfante A, Gamm H. The effects of 500 ml 10% hydroxyethyl starch 200/0.5 and 10% dextran 40 on blood volume, colloid osmotic pressure and renal function in human volunteers (author's transl). Der Anaesthesist 1982;31(2):61–7.
[91] Treib J, Haass A, Pindur G, Grauer MT, Jung F, Wenzel E, et al. Increased haemorrhagic risk after repeated infusion of highly substituted medium molecular weight hydroxyethyl starch. Arzneimittel-Forschung 1997;47(1):18–22.
[92] Legendre C, Thervet E, Page B, Percheron A, Noel LH, Kreis H. Hydroxyethylstarch and osmotic-nephrosis-like lesions in kidney transplantation. Lancet 1993;342(8865):248–9.
[93] Dart AB, Mutter TC, Ruth CA, Taback SP. Hydroxyethyl starch (HES) versus other fluid therapies: effects on kidney function. Cochrane Database Syst Rev 2010(1). Article no. CD007594.
[94] Sirtl C, Laubenthal H, Zumtobel V, Kraft D, Jurecka W. Tissue deposits of hydroxyethyl starch (HES): dose-dependent and time-related. Br J Anaesth 1999;82(4):510–5.
[95] Szepfalusi Z, Parth E, Jurecka W, Luger TA, Kraft D. Human monocytes and keratinocytes in culture ingest hydroxyethylstarch. Arch Dermatol Res 1993;285(3):144–50.
[96] Parker NE, Porter JB, Williams HJ, Leftley N. Pruritus after administration of hetastarch. Br Med J (Clin Res Ed) 1982;284(6313):385–6.
[97] Laxenaire MC, Charpentier C, Feldman L. Anaphylactoid reactions to colloid plasma substitutes: incidence, risk factors, mechanisms. A French multicenter prospective study. Ann Fr Anesth Reanim 1994;13(3):301–10.
[98] Boldt J, Zickmann B, Rapin J, Hammermann H, Dapper F, Hempelmann G. Influence of volume replacement with different HES-solutions on microcirculatory blood flow in cardiac surgery. Acta Anaesthesiol Scand 1994;38(5):432–8.
[99] Sputtek A, Singbartl G, Langer R, Schleinzer W, Henrich HA, Kuhnl P. Cryopreservation of erythrocytes using hydroxyethyl starch: in vivo results of an autologous retransfusion model in humans. Beitr Infusionsther Transfusionsmed 1994;32:44–7.
[100] Kim H, Itamoto K, Une S, Nakaichi M, Taura Y, Sumida S. Application of phosphoenolpyruvate into canine red blood cell cryopreservation with hydroxyethyl starch. Cryo Lett 2005;26(1):1–6.
[101] Hibino N, Shin'oka T, Matsumura G, Watanabe M, Toyama S, Imai Y. Cryopreservation of vascular mixed cell for tissue engineering in cardiovascular surgery. Kyobu Geka 2001;54(6):479–84.
[102] Pasch J, Schiefer A, Heschel I, Dimoudis N, Rau G. Variation of the HES concentration for the cryopreservation of keratinocytes in suspensions and in monolayers. Cryobiology 2000;41(2):89–96.
[103] Singbartl K, Langer R, Henrich HA. Altered membrane skeleton of hydroxyethylstarch-cryopreserved human erythrocytes. Cryobiology 1998;36(2):115–23.
[104] Mantzavinos D, Bailey AI, Rampling MW. Flash freezing of erythrocyte suspensions. Biorheology 1997;34(1):73–83.
[105] Kang EM, Areman EM, David-Ocampo V, Fitzhugh C, Link ME, Read EJ, et al. Mobilization, collection, and processing of peripheral blood stem cells in individuals with sickle cell trait. Blood 2002;99(3):850–5.
[106] Hayakawa J, Joyal EG, Gildner JF, Washington KN, Phang OA, Uchida N, et al. 5% Dimethyl sulfoxide (DMSO) and pentastarch improves cryopreservation of cord blood cells over 10% DMSO. Transfusion 2010;50(10):2158–66.
[107] McCullough J, Haley R, Clay M, Hubel A, Lindgren B, Moroff G. Long-term storage of peripheral blood stem cells frozen and stored with a conventional liquid nitrogen technique compared with cells frozen and stored in a mechanical freezer. Transfusion 2010;50(4):808–19.
[108] Ide K, Matsuura S, Fujino Y, Ohno K, Tsujimoto H. Investigation of various methods for the cryopreservation of canine bone marrow-derived CD34(+) cells. J Vet Med Sci 2008;70(11):1211–7.
[109] Halle P, Tournilhac O, Knopinska-Posluszny W, Kanold J, Gembara P, Boiret N, et al. Uncontrolled-rate freezing and storage at −80 °C, with only 3.5-percent DMSO in cryoprotective solution for 109 autologous peripheral blood progenitor cell transplantations. Transfusion 2001;41(5):667–73.
[110] Ayello J, Semidei-Pomales M, Preti R, Hesdorffer C, Reiss RF. Effects of long-term storage at −90 °C of bone marrow and PBPC on cell recovery, viability, and clonogenic potential. J Hematother 1998;7(4):385–90.
[111] Katayama Y, Yano T, Bessho A, Deguchi S, Sunami K, Mahmut N, et al. The effects of a simplified method for cryopreservation and thawing procedures on peripheral blood stem cells. Bone Marrow Transplant 1997;19(3):283–7.
[112] Donaldson C, Armitage WJ, DenningKendall PA, Nicol AJ, Bradley BA, Hows JM. Optimal cryopreservation of human umbilical cord blood. Bone Marrow Transplant 1996;18(4):725–31.
[113] Takaue Y, Abe T, Kawano Y, Suzue T, Saito S, Hirao A, et al. Comparative-analysis of engraftment after cryopreservation of peripheral-blood stem-cell autografts by controlled-rate versus uncontrolled-rate methods. Bone Marrow Transplant 1994;13(6):801–4.
[114] Clapisson G, Salinas C, Malacher P, Michallet M, Philip I, Philip T. Cryopreservation with hydroxyethylstarch (HES) + dimethylsulfoxide (DMSO) gives better results than DMSO alone. Bull Cancer 2004;91(4):E97–E102.
[115] Lippens E, Cornelissen M. Slow cooling cryopreservation of cell-microcarrier constructs. Cells Tissues Organs 2010;192(3):177–86.
[116] Kawasaki N, Hamamoto Y, Nakajima T, Irie K, Ozawa H. Periodontal regeneration of transplanted rat molars after cryopreservation. Arch Oral Biol 2004;49(1):59–69.

1.

4.2 Effect of different frezing rates during cryopreservation of rat mesenchymal stem cells using combinations of hydroxyethyl starch and dimethylsulfoxide

Naaldijk *et al. BMC Biotechnology* 2012, **12**:49
http://www.biomedcentral.com/1472-6750/12/49

BMC Biotechnology

RESEARCH ARTICLE Open Access

Effect of different freezing rates during cryopreservation of rat mesenchymal stem cells using combinations of hydroxyethyl starch and dimethylsulfoxide

Yahaira Naaldijk, Marek Staude, Viktoriya Fedorova and Alexandra Stolzing*

Abstract

Background: Mesenchymal stem cells (MSCs) are increasingly used as therapeutic agents as well as research tools in regenerative medicine. Development of technologies which allow storing and banking of MSC with minimal loss of cell viability, differentiation capacity, and function is required for clinical and research applications. Cryopreservation is the most effective way to preserve cells long term, but it involves potentially cytotoxic compounds and processing steps. Here, we investigate the effect of decreasing dimethyl sulfoxide (DMSO) concentrations in cryosolution by substituting with hydroxyethyl starch (HES) of different molecular weights using different freezing rates. Post-thaw viability, phenotype and osteogenic differentiation capacity of MSCs were analysed.

Results: The study confirms that, for rat MSC, cryopreservation effects need to be assessed some time after, rather than immediately after thawing. MSCs cryopreserved with HES maintain their characteristic cell surface marker expression as well as the osteogenic, adipogenic and chondrogenic differentiation potential. HES alone does not provide sufficient cryoprotection for rat MSCs, but provides good cryoprotection in combination with DMSO, permitting the DMSO content to be reduced to 5%. There are indications that such a combination would seem useful not just for the clinical disadvantages of DMSO but also based on a tendency for reduced osteogenic differentiation capacity of rat MSC cryopreserved with high DMSO concentration. HES molecular weight appears to play only a minor role in its capacity to act as a cryopreservation solution for MSC. The use of a 'straight freeze' protocol is no less effective in maintaining post-thaw viability of MSC compared to controlled rate freezing methods.

Conclusion: A 5% DMSO / 5% HES solution cryopreservation solution using a 'straight freeze' approach can be recommended for rat MSC.

Keywords: Mesenchymal stem cells, Cryopreservation, Controlled rate freezing, Hydroxyethyl starch

Background

Mesenchymal stem cells (MSC) provide a useful tool for regenerative medicine due to their differentiation capacity, immunosuppressive properties, secretome profile and migratory ability [1,2].

MSC represent a valuable source for research and clinical applications due to their ability to produce a range of different cell types including osteoblasts, adipocytes, chondrocytes and myoblasts [3-6]. Effective cryopreservation MSCs offers an opportunity to advance the potential use and implementation of these cells into clinical applications.

Cryopreservation itself can affect differentiation capacity of stem cells [7,8]. The loss of a variety of pluripotency markers has been associated with cryopreservation [9,10] but the precise reasons for these changes remain to be explored.

Many studies on the cryopreservation of MSCs were carried out using slow-rate cooling methods [11,12]

* Correspondence: Alexandra.Stolzing@izi.fraunhofer.de
Fraunhofer Institute for Cell Therapy and Immunology, Perlickstrasse 1, Leipzig 04103, Germany

Naaldijk et al. BMC Biotechnology 2012, 12:49
http://www.biomedcentral.com/1472-6750/12/49

which is often considered a superior method of preservation [13,14]. However, limited evidence exists whether the freezing rate in fact affects stem cell growth and differentiation potential. Both 'slow' [15-19] and 'fast' [20,21] freezing protocols have reported 'success' as far as maintaining similar phenotypes, cell surface markers and growth rates in comparison with unfrozen MSC.

Several groups have investigated MSC (from different sources) cryopreservation using 10% DMSO and slow freezing protocols. In these studies, the cryopreserved MSC maintained similar phenotypes, cell surface markers and growth rates in comparison with fresh cells [19,22,23]. In addition fast freezing protocols (vitrification) have been investigated with MSC, showing normal proliferation, phenotype and differentiation [20,21].

To facilitate freezing, a cryoprotectant is usually added. An ideal cryoprotection solution should be nontoxic for cells and patients, nonantigenic, chemically inert, provide high survival rate after thawing and allow transplantation without washing. The most commonly used cryoprotector, DMSO, shows cytotoxicity [7,8]. Clinically, DMSO can cause leukoencephalopathy [24], epileptic seizures [25] or elevated lactate dehydrogenase levels [26] after transplantation of DMSO-preserved human bone marrow cells. DMSO is thought to interact with the metabolism and membrane of cells, resulting in cell damage [27]. Nonetheless, DMSO is widely seen as indispensible at least as a component of a cryoprotectants solution.

Additions to DMSO include methycellulose [28], PVP [29], trehalose [30] or others and these components are not investigated here.

Another substitution compound for DMSO in cryoprotection is Hydroxylethyl Starch (HES) which is used in the clinical setting as a plasma volume expander [31-35]. A number of different cell types have been cryopreserved using HES [36-38] with red blood cells being routinely cryopreserved in cryoprotective solutions containing HES [33,39,40]. Bone marrow cells from human and other animal species have also been cryopreserved in HES-containing solutions [41-43].

Physical and chemical properties such as solubility, molecular stability as well as rate of hydrolysis and metabolism depend on molecular weight (MW) and degree of substitution of HES molecules. HESs with lower MW have higher solubility and slower breakdown rates [41,43]. A variety of different HESs with different MW are currently clinically approved and commercially available.

In this study we attempt an initial comparison of two independent factors: freezing rates and cryopreservation solutions. We analysed their effect on viability, growth characteristics and differentiation potential of rat MSCs after cryopreservation.

Methods

Isolation of mesenchymal stem cells

The rat (Sprague Dawley, 2–3 month old, male) was killed by controlled inhalation of CO_2. The hind legs were removed, the soft tissue removed and the separated bones (tibia, femur) were stored in PBS. The bones were centrifuged at 1000 rpm for 5 min and bone marrow was resuspended in 1 ml DMEM.

Cell culture

Cells obtained from each animal were distributed into two T75 flasks and incubated at 37°C. The first medium change was done after 5 days and afterwards every 2 to 3 days. For the experiments we used MSCs from passage 1 to passage 3.

Rat MSCs were cultured in 1x Dulbecco's modified Eagle's medium (DMEM, 1 g/L D-Glucose, Invitrogen) containing 10% fetal bovine serum (FBS, Hyclone) and 1% penicillin/streptomycin (pen/strep, Invitrogen).

Cryopreservation

MSCs were frozen when they reached 80% confluency. Prior to freezing, cell number was determined by trypan blue staining using a Neubauer hemocytometer. 10^5 cells were added to each cryogenic vial (2 ml Nalgene). MSC were centrifuged for 5 min (1000 rpm at room temperature) to pellet the cells, media was removed and CPA was slowly (10 sec) added with a pipette and the cells were carefully re-suspended. CPA were containing 500 µl of prepared cryoprotectant consisting of hydroxyethyl starches of different mean molecular weights (MW = 109, 209, 309, 409, 509, 609 kDa - Serumwerk, Bernburg) and/or DMSO (Sigma-Aldrich)). Cryogenic vials were kept on ice until samples were frozen applying the different freezing protocols (Table 1) using a rate controlled freezing system (Thermo Scientific) where indicated Model 7452 Series). Protocols used on this study were modified or newly designed based on published protocols [35,44-46]. The chamber of the freezing system was pre-cooled to 4°C before each experiment. The samples were stored at −134°C in the vapor phase segment of a liquid nitrogen tank for at least 24 h. Samples were thawed at 37°C in a water bath and 10^4 cells were seeded per well in triplicates into pre-warmed culture medium. Fresh unfrozen MSC were seeded as a control.

MTT assay

The MTT assay was performed 3 days after thawing. Each well was filled with 500 µl of media containing of MTT-reagent, consisting of 5 mg/ml MTT (Carl Roth) in PBS. After incubation for 4 h at 37°C, medium was

Naaldijk et al. BMC Biotechnology 2012, 12:49
http://www.biomedcentral.com/1472-6750/12/49

Table 1 Freezing protocols used

Protocol	Description	Duration [min]	References
1	0,3°C/min to −100°C	347	
	Store in vapour phase at −134°C		[47]*
2	1°C/min to −80°C	84	[46-50]
	Store in vapour phase at −134°C		
3	1°C/min to - 30°C	44	
	5°C/min to −80°C		[44,51]*
	xStore in vapour phase at −134°C		
4	1°C/min to −20°C	34	
	5°C/min to −40°C		
	10°C/min to −80°C		Designed
	20°C/min to −100°C		
	Store in vapour phase at −134°C		
5	1°C/min to −6°C	26	
	25°C/min to −50°C		Designed
	10°C/min to −90°C		
	Store in vapour phase at −134°C		
6	Directly into the vapour phase	0	
7	99°C/min to −100°C	2	
	Store in vapour phase at −134°C		[35,52]*

* Modified.

removed and 500 μl stop-solution (10% SDS (Merck) and 50% dimethylformamide, (VWR International)) was added. The cells were incubated overnight at 37°C and absorbance was measured using a microplate reader (TECAN) at 550 nm and 630 nm as reference wavelength.

Osteogenic differentiation

The day after thawing medium was changed to osteoinductive medium (low Glucose DMEM; 10% FBS; 1% pen/strep; 10 nm dexamethasone, (Sigma-Aldrich); 50 μg/ml ascorbic acid 2-phosphate, (Sigma-Aldrich)). Differentiation media was changed every 2 days for a period of 14 days. For qualitative analysis of osteogenic differentiation, cells were fixed in 70% ethanol for 15 min and washed once with ddH2O.

After washing, cells were stained with ALP buffer pH 8.5 (0.2 M Tris, 1 mg/ml fast red, Sigma and 50 μg/ml naphtol phosphate AS-BI, Sigma) for 1 hr.

Adipogenic differentiation

Adipogenic medium (10% FBS; 1% pen/strep, 10% insulin-transferrin-selenium supplement, (Sigma-Aldrich) 10^{-8} M dexamethasone (Sigma-Aldrich); 0.5 mM isobutylmethylxanthine, Sigma-Aldrich; 100 μM indomethacin, Sigma-Aldrich) was added the day after thawing. The media was changed every 2 days. After 14 days cell phenotype was analyzed by Oil Red O (Sigma-Aldrich) staining.

Chondrogenic differentiation

Chondrogenic media (10% FBS; 1% pen/strep 1% insulin-transferrin-selenium supplement (Sigma-Aldrich), 10-7 M dexamethasone (Sigma-Aldrich), 150 μM ascorbic-2-phosphate (Sigma-Aldrich), 20 μM linoic acid (Sigma-Aldrich) and 0.1 ng/ml TGF-β (Oncogenic Sciences) was added after thawing. After 2 weeks, cells were stained with Alcian Blue (Sigma-Aldrich).

Quantitative alkaline phosphatase (ALP) assay

After 14 days of differentiation, the 24 well-plates were washed and fixed with ice-cold 70% ethanol for 20 min. Ethanol was removed, the plates washed and incubated with 1 ml of p-nitrophenylphosphate (1 mg/ml, Calbiochem) in TRIS (pH 8.0). Cells were incubated for 1 h at RT and absorbance measured at 405 nm using a microplate reader (TECAN). Subsequently, the 24 well-plates were rinsed with ddH_2O and washed with 10 mm borate buffer (pH 8.5, Sigma-Aldrich). 500 μl methylene blue (1 mg/ml, Sigma-Aldrich) in 10 mm borate buffer was added to each well. After 30 min incubation at RT the plates were washed with 10 mm borate buffer and 500 μl 1% hydrochloric acid (VWR International) was used for dye elution. Plates were incubated for 30 min at RT and absorbance was measured at 650 nm using a microplate reader (TECAN).

Phenotyping of mesenchymal stem cells

Cells were incubated with CD90 (1:50, Abcam), CD45 (1:100, AbD Serotec), CD11b (1:100, Abcam), and CD44 (1:50, Millipore) for 1 h at 4°C, washed and incubated with Cy2 (1:750, Jackson ImmunoResearch) for 45 min at 4°C. Cells were washed again and analyzed using the Cytomics FC500 flow cytometer and CXP Analysis 2.1 software (Beckman Coulter).

Cell morphology

Morphology of the cells was analyzed 3 days after thawing by light microscopy. Pictures were taken at 10x magnification.

Statistics

All experiments were repeated at least three times. Statistical analysis was performed using ANOVA followed by Turkey test, with $p < 0.05$ considered an indicator of robustness (although not of absolute statistical significance as the number of experiments was too low).

Results

Rat MSCs were cryopreserved in vials using seven different freezing protocols (Table 1). Different concentrations of DMSO and HES alone or in mixture were tested.

Naaldijk *et al. BMC Biotechnology* 2012, **12**:49
http://www.biomedcentral.com/1472-6750/12/49

Verification of consistent nucleation

Some studies (in the 80's) [53,54] have stressed the importance of capturing a consistent ice nucleation point. While in our experiments (in keeping with many machine-based freezing practices in tissue engineering) nucleation was not initiated 'manually', we can show that nucleation occurs consistently as indicated by a characteristic [55] 'heat release' spike (Additional file 1 Figure S4).

Post-thaw phenotyping

CD90, CD44, CD45 and CD11b expression were measured in samples cryopreserved using protocol 1 and 7, in 90% DMEM and three different cryosolutions: 10% DMSO, 10% HES 450 and 5% DMSO/5% HES 450. CDs were measured directly after thawing and 3 days later and compared to non-cryopreserved cells (Figure 1). As expected, both MSCs after cryopreservation and non-cryopreserved MSCs have low expression of hematopoietic stem cell markers CD45 and CD11b and high expression of mesenchymal-associated marker CD90 and CD44 [21,56,57]. No differences in CD expression were observed between day 0 and 3 in both protocols. Usage of HES 450 and DMEM results in low cell number after 3 days therefore no CD phenotyping was performed.

Post-thaw viability

Directly after thawing, we recorded approximately 85% cell viability with no observed difference between the different protocols and cryoprotectant-solutions (Figure 2A-B). However, viability of cells directly after thawing cannot represent reliable criteria for estimation of cryopreservation efficacy. After cultivation for 3 days, a considerable decrease in viability for some solutions was observed (Figure 2C-D). This post-thaw decrease in cell viability is known to be related to apoptotic and necrotic processes which occur within first 24 hours and are not evident immediately after thawing [16,58,59]. On day 3 after thawing, DMSO concentrations under 4% are associated with reduced MSC viability (Figure 2D). The solution of 8% DMSO / 2% HES 450 shows on average of all protocols the highest viability compared to all

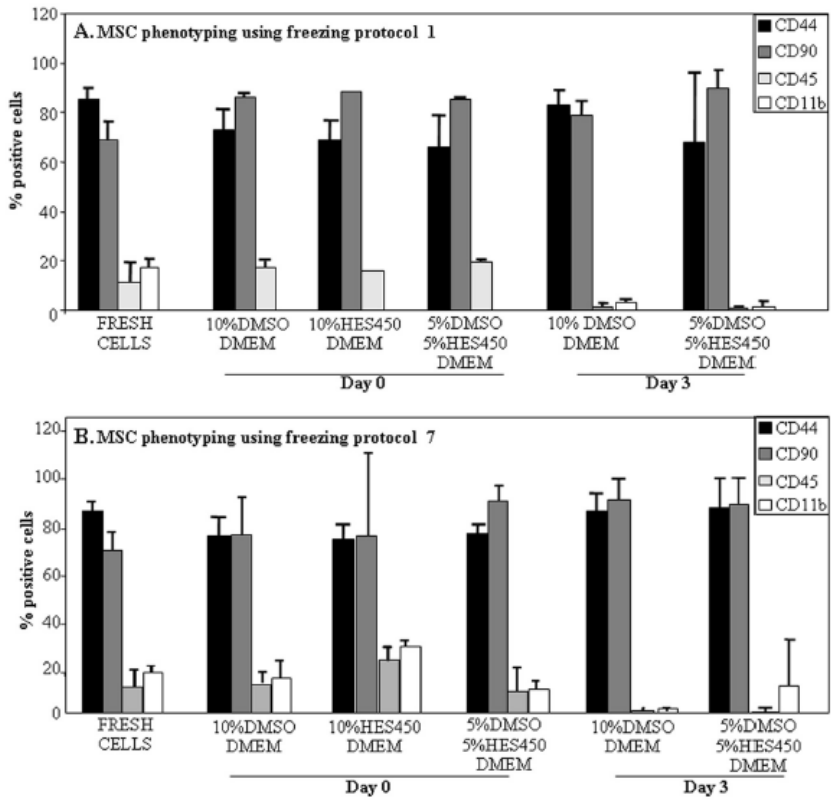


Figure 1 MSC phenotyping. Level of CD-expression in MSCs cryopreserved in different solutions directly after thawing (day 0) and after 3 days with protocol 1 (**A**) and protocol 7 (**B**).

Naaldijk *et al. BMC Biotechnology* 2012, **12**:49
http://www.biomedcentral.com/1472-6750/12/49

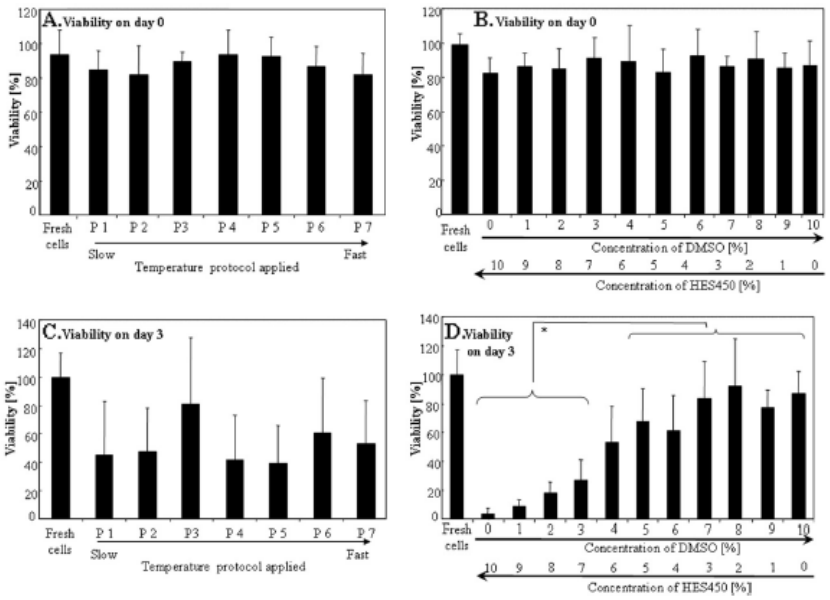


Figure 2 The effect of cryopreservation on MSC viability immediately after thawing and after 3 days at various HES/DMSO combinations and freezing rates. Viability of MSCs after cryopreservation with either different freezing rates (**A**, **C**) or different concentrations of DMSO and HES 450 (**B**,**D**) measured directly after thawing (**A**, **B**) and after 3 days by MTT (**C**, **D**).

solutions with 5% and less DMSO. Reliance on HES alone (10% HES 450) is associated with low cell viability. MSC viability was maintained at 14 days (Figure 3B) showing that proliferation activity is not affected by prolonged culture (Additional file 2 Figure S2).

HES with the same molar substitution (0.7) but different molecular weight distributions were investigated in order to determine the relation between size of HES and its cryoprotective capacity. We used the cooling protocols 1, 5, and 6 (Table 1) which were chosen as the protocols with the most different parameters. No difference in cell viability at day 0 was observed between the solutions and protocols (data not shown). HES solutions in DMEM and FCS showed less cell viability at day 3 post-thawed compared to DMSO controls (Figure 4A).

As a trend, higher HES molecular weight seems to sustain cell viability (with notable exception for protocol 5) but only in FCS not in DMEM (Figure 4A and B).

Post-thaw differentiation capacity

Thawed MSC retain their capacity to differentiate toward osteoblasts, adipocytes and chondrocytes. Qualitative assessment of the osteogenic, adipogenic and chondrogenic differentiation of MSC shows no difference in effect between the different cryosolutions (Additional file 3 Figure S1), but we only pursued osteogenic capacity in greater detail.

ALP activity is lower at 'high' (>5%) levels of DMSO compared to solutions with a higher HES 450 content (Figure 3, C and Additional file 4 Figure S3).

No differences could be observed between HES of different molecular weights and there was no effect attributable to concurrent use of either serum or DMEM (Figure 4c).

Post-thaw phenotyping

CD90, CD44, CD45 and CD11b expression were measured in samples cryopreserved using protocol 3 and 6, in three different cryosolutions: 10% DMSO + medium, 10% HES 450 + medium and 5% DMSO/5% HES 450 + medium. CDs were measured directly after thawing and 3 days later and compared to non-cryopreserved MSC (Figure 1).

As expected, both MSCs after cryopreservation and non-cryopreserved MSCs have low expression of hematopoietic stem cell markers CD11b and CD45 and high expression of mesenchymal-associated marker CD90 and CD44 [56,57]. No differences in CD expression were observed between day 0 and 3 in both protocols. Cryopreservation did lead to a further reduction of hematopoetic makers CD11b and CD45. Usage of HES 450 and DMEM results in such low cell number after 3 days that no CD phenotyping was possible.

Naaldijk *et al. BMC Biotechnology* 2012, **12**:49
http://www.biomedcentral.com/1472-6750/12/49

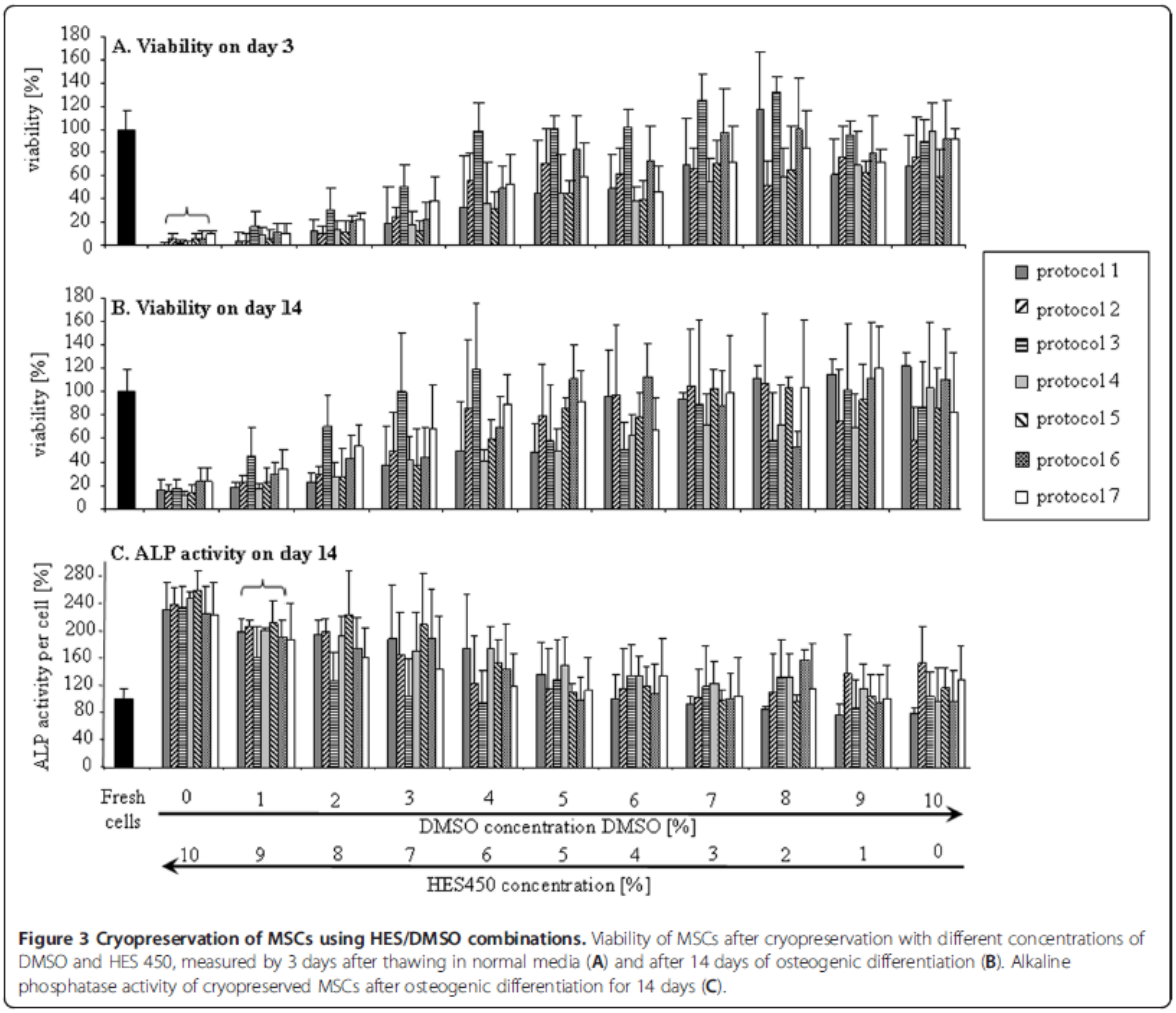


Figure 3 Cryopreservation of MSCs using HES/DMSO combinations. Viability of MSCs after cryopreservation with different concentrations of DMSO and HES 450, measured by 3 days after thawing in normal media (**A**) and after 14 days of osteogenic differentiation (**B**). Alkaline phosphatase activity of cryopreserved MSCs after osteogenic differentiation for 14 days (**C**).

Discussion

It is not uncommon to analyze cell viability directly after thawing [47,48,60-62], however our results clearly show that this is an inaccurate measure of cryopreservation effects in rat MSC, since the different effects of various cryosolutions tested were only observable 3 days after thawing and beyond. Cryopreservation associated cell death is known to occur from 6 h after thawing and beyond [63-66]. We investigated different cooling protocols and found a constant cooling rate of 1°C per min until −30°C followed by 5°C per min until −80°C and protocols with a fast cooling rate (protocols 1 & 6) are equally suitable considering cell viability and recovery in the cryopreservation of rat MSCs. However, the choice of protocol seems to have a rather marginal effect on post-thaw viability, with little difference to a 'straight freeze' approach (protocols 6) that saves considerable time and effort. Survival rates were in the range of previous reports and MSC phenotypes were not affected by cryopreservation. It could be argued that protocols 1–3 are essential 'the same' with regards to the pre-cooling rate of hypothetical importance, i.e., the one that governs the biophysical response of the cells being cooled. Indeed, we observe no difference between these protocols. However, since the different protocols are in practical use we have maintained their differentiated profile for reference.

DMSO reduction using alternative cryoprotectants

As discussed above and previously [43], the use of DMSO has several disadvantages. However, based on these results in rat MSC, a total substitution of DMSO with HES is not advisable if cell viability is a key indicator. Upon reducing DMSO concentration below 4% an observed decrease in cell viability can be measured, probably because of ice crystal growth in the relatively

Naaldijk et al. BMC Biotechnology 2012, 12:49
http://www.biomedcentral.com/1472-6750/12/49

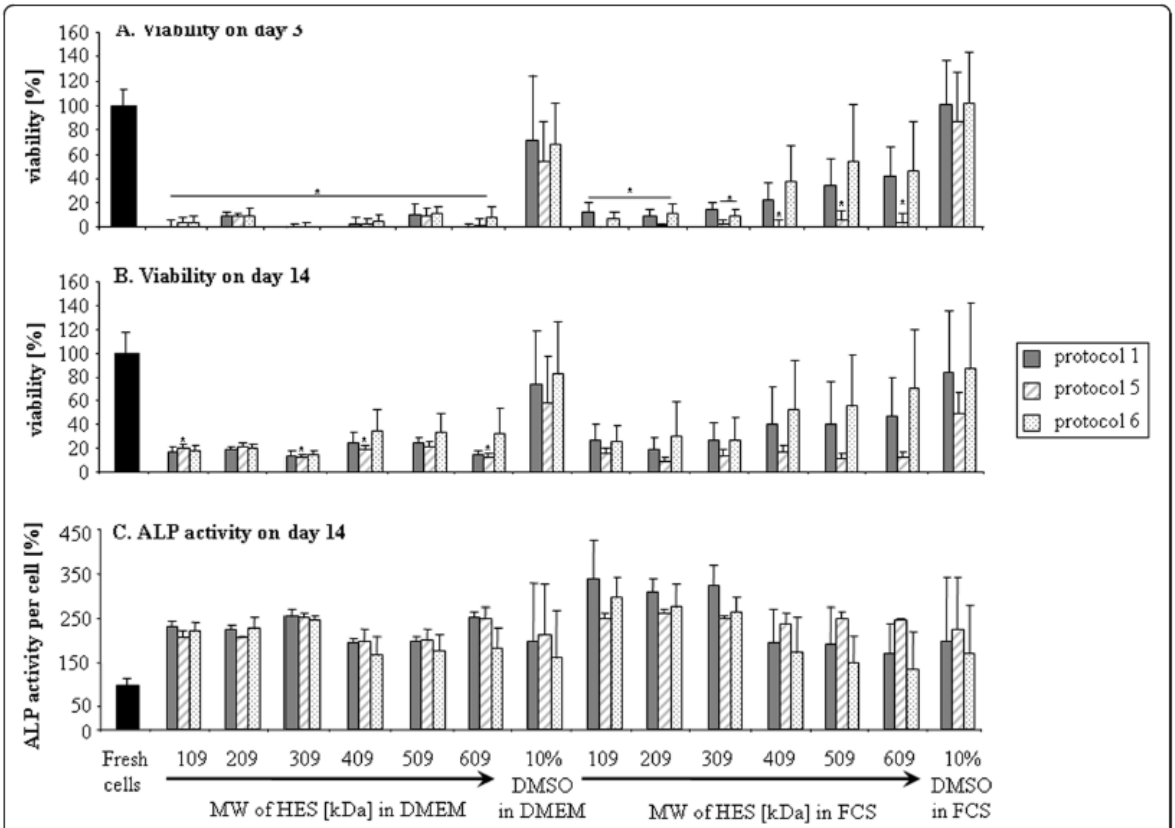


Figure 4 Effect of HES with different molecular weight distributions on MSCs cryopreservation. Viability of MSCs after cryopreservation with 10% HES of different molecular weight distributions (109–609) in combination with DMEM or FCS, measured 3 days after thawing (**A**) and by methylene blue staining after osteogenic differentiation for 14 days (**B**). Alkaline phosphatase activity of cryopreserved MSCs after osteogenic differentiation for 14 days (**C**).

large cell body of the MSC and reduced osmotic exchange of water against DMSO during the relatively short incubation time of the cells in DMSO.

However, we could observe a slight reduction of osteogenic capacity related to higher DMSO concentrations. It is known that oxidative stress induces differentiation [67], which also occurs during freezing [68]. A badly designed freezing protocol might induce differentiation impacting on the quality of the stem cells.

If these or other drawbacks of DMSO are a factor, our results show that a partial replacement of DMSO with HES is certainly possible. Studies using a 6% HES + 5% DMSO solution usually show superior cryopreservation in comparison to 10% DMSO [43]. Regarding the 'optimum' between MSC survival and osteogenic differentiation one can conclude that while lowest DMSO concentration are slightly better for differentiation for practical tissue engineering purposes a DMSO concentration of 5% or slightly higher should be preferable.

Molecular weight of HES

In previous cryopreservation studies different MW of HES ranging from 150 to 450 kDa were used but not compared, making it difficult to know if there are differences between the variable HES solutions [33,69]

For the first time we compared the effects of HES ranging from 109 to 609 kDa with a similar hydroxyl substitution rate on viability and osteogenic differentiation. The cryopreservation with HES of different molecular weights had no effect on survival and differentiation of MSCs.

Conclusion

1. The study confirms that, for rat MSC, cryopreservation effects need to be assessed some time after, rather than immediately after thawing.
2. MSCs cryopreserved with HES maintain their characteristic cell surface marker expression as well

as the osteogenic, adipogenic and chondrogenic differentiation potential.

3. There are no major changes in the expression of surface proteins identifying MSC, proliferation capacity and osteogenic differentiation in MSC frozen with 5% DMSO/ 5% HES.
4. HES alone does not provide sufficient cryoprotection for rat MSCs, but provides good cryoprotection in combination with DMSO, permitting the DMSO content to be reduced to 5%. There are indications that such a combination would seem useful not just for the clinical disadvantages of DMSO but also based on a tendency for reduced osteogenic differentiation capacity in rat MSC cryopreserved with high DMSO concentration.
5. HES molecular weight appears to play only a minor role in its capacity to act as a cryopreservation solution for MSC.
6. The use of a 'straight freeze' protocol is no less effective in maintaining post-thaw viability of MSC compared to controlled rate freezing methods.

As a simplified summary, a 5% DMSO / 5% HES solution cryopreservation solution using a 'straight freeze' approach can be recommended as 'optimal' for 'normal' rat MSC cryopreservation.

Additional file

Additional file 1: Figure S4. Freezing curves of the machine cryopreservation protocols. The curves for the machine based freezing rates were recorded by the machine and summarized here for the protocols 1–6. The curves show just small variations during the heat release phase between 0 and −10°C.

Additional file 2: Figure S2. Cellular Morphology of cryopreserved rat MSC. Morphology of cryopreserved rat MSC after 14 days in culture.

Additional file 3: Figure S1. Differentiation capacity of rat MSCs after cryopreservation. Qualitative ALP-staining of MSCs after 14 days osteogenic differentiation. Magnification 20X (**A**). Oil red O staining of MSCs after 14 days in adipogenic differentiation medium. Magnification 40X (**B**). Chondrogenic staining of differentiated MSCs cells after 14 days. Magnification 20X (**C**).

Additional file 4: Figure S3. Morphology of cryopreserved rat MSC during osteogenesis. Cellular morphology of cryopreserved rat MSC after 14 days in osteogenic differentiation medium.

Competing interests
This work was supported in part by Serumwerke Bernburg a manufacturer of HES.

Authors' contributions
MS carried out the bulk of the experiments, VF contributed to some phenotyping experiments and sections of the manuscript. YN conducted the phenotyping and chondrocyte experiments, consolidated data and provided the first draft of the manuscript. AS planned the experiments, secured the funding and revised the manuscript. All authors read and approved the final manuscript.

Acknowledgements
Thanks goes to Dr. Sebastian Sethe for advice on data interpretation and manuscript revision.

Received: 10 April 2012 Accepted: 31 July 2012
Published: 13 August 2012

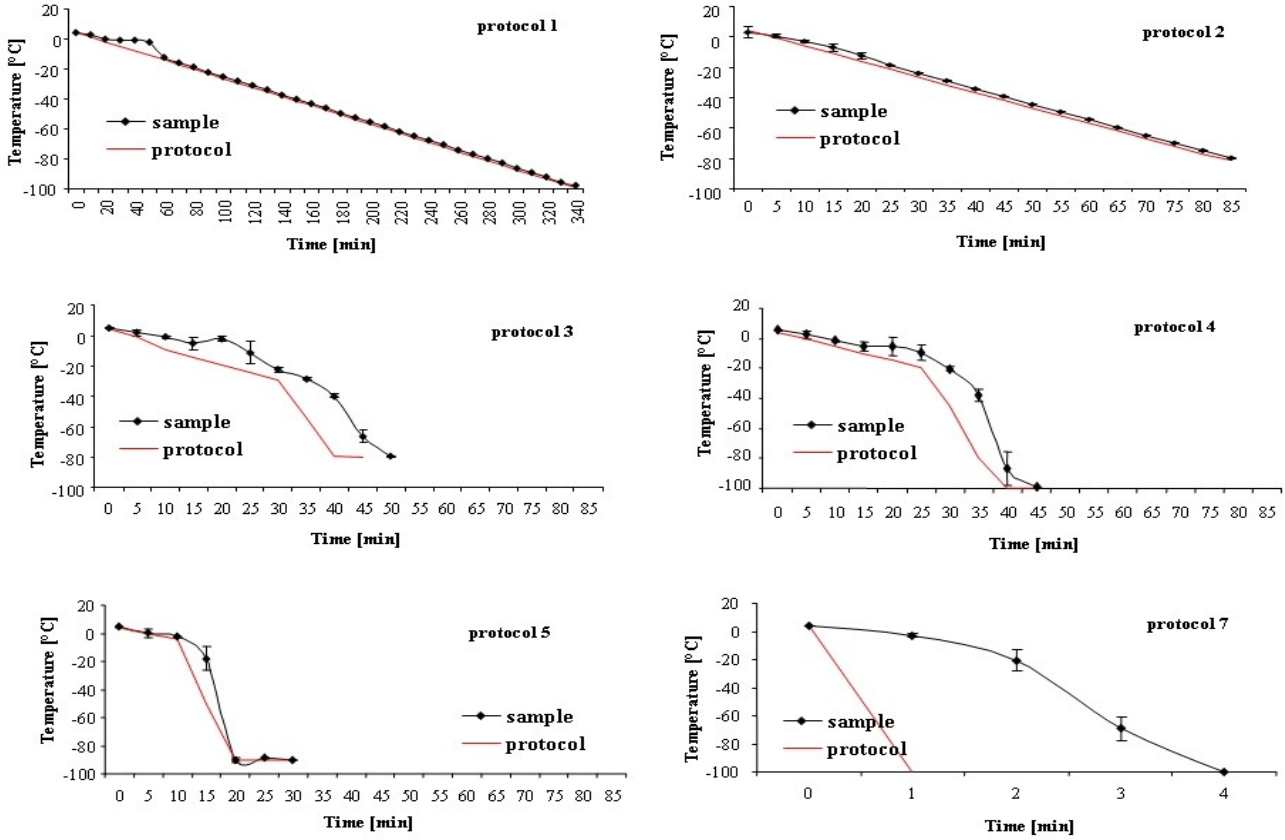
References

1. Abdallah BM, Kassem M: **Human mesenchymal stem cells: from basic biology to clinical applications.** *Gene Ther* 2008, **15**:109–116.
2. Chamberlain G, Fox J, Ashton B, Middleton J: **Concise review: mesenchymal stem cells: their phenotype, differentiation capacity, immunological features, and potential for homing.** *Stem Cells* 2007, **25**:2739–2749.
3. Reyes M, Verfaillie CM: **Characterization of multipotent adult progenitor cells, a subpopulation of mesenchymal stem cells.** *Ann N Y Acad Sci* 2001, **938**:231–233. discussion 233–235.
4. Woodbury D, Schwarz EJ, Prockop DJ, Black IB: **Adult rat and human bone marrow stromal cells differentiate into neurons.** *J Neurosci Res* 2000, **61**:364–370.
5. Pittenger MF, Mackay AM, Beck SC, Jaiswal RK, Douglas R, Mosca JD, Moorman MA, Simonetti DW, Craig S, Marshak DR: **Multilineage potential of adult human mesenchymal stem cells.** *Science* 1999, **284**:143–147.
6. Wakitani S, Saito T, Caplan AI: **Myogenic cells derived from rat bone marrow mesenchymal stem cells exposed to 5-azacytidine.** *Muscle Nerve* 1995, **18**:1417–1426.
7. Qi W, Ding D, Salvi RJ: **Cytotoxic effects of dimethyl sulphoxide (DMSO) on cochlear organotypic cultures.** *Hear Res* 2008, **236**:52–60.
8. Wang X, Hua TC, Sun DW, Liu B, Yang G, Cao Y: **Cryopreservation of tissue-engineered dermal replacement in Me2SO: Toxicity study and effects of concentration and cooling rates on cell viability.** *Cryobiology* 2007, **55**:60–65.
9. Katkov II, Kim MS, Bajpai R, Altman YS, Mercola M, Loring JF, Terskikh AV, Snyder EY, Levine F: **Cryopreservation by slow cooling with DMSO diminished production of Oct-4 pluripotency marker in human embryonic stem cells.** *Cryobiology* 2006, **53**:194–205.
10. Reubinoff BE, Pera MF, Vajta G, Trounson AO: **Effective cryopreservation of human embryonic stem cells by the open pulled straw vitrification method.** *Hum Reprod* 2001, **16**:2187–2194.
11. Haack-Sorensen M, Bindslev L, Mortensen S, Friis T, Kastrup J: **The influence of freezing and storage on the characteristics and functions of human mesenchymal stromal cells isolated for clinical use.** *Cytotherapy* 2007, **9**:328–337.
12. Lee RH, Kim B, Choi I, Kim H, Choi HS, Suh K, Bae YC, Jung JS: **Characterization and expression analysis of mesenchymal stem cells from human bone marrow and adipose tissue.** *Cell Physiol Biochem* 2004, **14**:311–324.
13. Hunt CJ: **Cryopreservation of Human Stem Cells for Clinical Application: A Review.** *Transfus Med Hemother* 2011, **38**:107–123.
14. Djuwantono T, Wirakusumah FF, Achmad TH, Sandra F, Halim D, Faried A: **A comparison of cryopreservation methods: Slow-cooling vs. rapid-cooling based on cell viability, oxidative stress, apoptosis, and CD34+ enumeration of human umbilical cord blood mononucleated cells.** *BMC Res Notes* 2011, **4**:371.
15. Bruder SP, Jaiswal N, Haynesworth SE: **Growth kinetics, self-renewal, and the osteogenic potential of purified human mesenchymal stem cells during extensive subcultivation and following cryopreservation.** *J Cell Biochem* 1997, **64**:278–294.
16. Kotobuki N, Hirose M, Machida H, Katou Y, Muraki K, Takakura Y, Ohgushi H: **Viability and osteogenic potential of cryopreserved human bone marrow-derived mesenchymal cells.** *Tissue Eng* 2005, **11**:663–673.
17. Perry BC, Zhou D, Wu X, Yang FC, Byers MA, Chu TM, Hockema JJ, Woods EJ, Goebel WS: **Collection, cryopreservation, and characterization of human dental pulp-derived mesenchymal stem cells for banking and clinical use.** *Tissue Eng Part C Methods* 2008, **14**:149–156.
18. Rust PA, Costas C, Cannon SR, Briggs TW, Blunn GW: **Characterisation of cryopreserved cells freshly isolated from human bone marrow.** *Cryo Letters* 2006, **27**:17–28.
19. Liu G, Zhou H, Li Y, Li G, Cui L, Liu W, Cao Y: **Evaluation of the viability and osteogenic differentiation of cryopreserved human adipose-derived stem cells.** *Cryobiology* 2008, **57**:18–24.

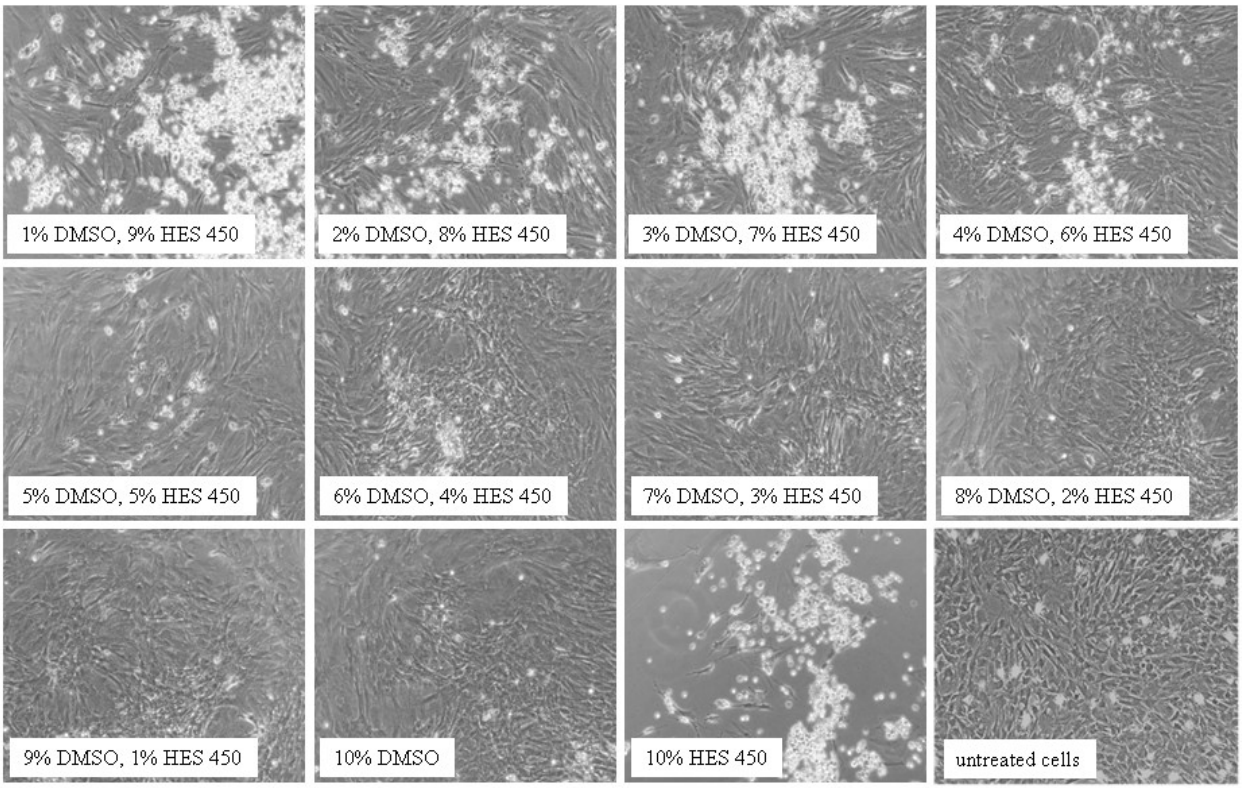
20. Bhakta G, Lee KH, Magalhaes R, Wen F, Gouk SS, Hutmacher DW, Kuleshova LL: **Cryopreservation of alginate-fibrin beads involving bone marrow derived mesenchymal stromal cells by vitrification.** *Biomaterials* 2009, **30**:336–343.
21. Moon JH, Kwak SS, Park G, Jung HY, Yoon BS, Park J, Ryu KS, Choi SC, Maeng I, Kim B, *et al*: **Isolation and characterization of multipotent human keloid-derived mesenchymal-like stem cells.** *Stem Cells Dev* 2008, **17**:713–724.
22. Goh BC, Thirumala S, Kilroy G, Devireddy RV, Gimble JM: **Cryopreservation characteristics of adipose-derived stem cells: maintenance of differentiation potential and viability.** *J Tissue Eng Regen Med* 2007, **1**(4):322–324.
23. Lee MW, Choi J, Yang MS, Moon YJ, Park JS, Kim HC, Kim YJ: **Mesenchymal stem cells from cryopreserved human umbilical cord blood.** *Biochem Biophys Res Commun* 2004, **320**:273–278.
24. Higman MA, Port JD, Beauchamp NJ Jr, Chen AR: **Reversible leukoencephalopathy associated with re-infusion of DMSO preserved stem cells.** *Bone Marrow Transplant* 2000, **26**:797–800.
25. Hequet O, Dumontet C, El Jaafari-Corbin A, Salles G, Espinouse D, Arnaud P, Thieblemont C, Bouafia F, Coiffier B: **Epileptic seizures after autologous peripheral blood progenitor infusion in a patient treated with high-dose chemotherapy for myeloma.** *Bone Marrow Transplant* 2002, **29**:544.
26. Gomez S, Nagler A, Naparstek E, Slavin S: **Transient elevation of serum lactic dehydrogenase following autologous bone marrow transplantation.** *Bone Marrow Transplant* 1991, **7**:487–488.
27. Penninckx F, Cheng N, Kerremans R, Van Damme B, De Loecker W: **The effects of different concentrations of glycerol and dimethylsulfoxide on the metabolic activities of kidney slices.** *Cryobiology* 1983, **20**:51–60.
28. Thirumala S, Gimble JM, Devireddy RV: **Evaluation of methylcellulose and dimethyl sulfoxide as the cryoprotectants in a serum-free freezing media for cryopreservation of adipose-derived adult stem cells.** *Stem Cells Dev* 2010, **19**:513–522.
29. Thirumala S, Gimble JM, Devireddy RV: **Cryopreservation of stromal vascular fraction of adipose tissue in a serum-free freezing medium.** *J Tissue Eng Regen Med* 2010, **4**:224–232.
30. Liu Y, Xu X, Ma XH, Liu J, Cui ZF: **Effect of various freezing solutions on cryopreservation of mesenchymal stem cells from different animal species.** *Cryo Letters* 2011, **32**:425–435.
31. Allen ED, Weatherbee L, Spencer HH, Lindenauer SM, Permoad PA: **Large unit red cell cryopreservation with hydroxyethyl starch.** *Cryobiology* 1976, **13**:500–506.
32. Pasch J, Schiefer A, Heschel I, Dimoudis N, Rau G: **Variation of the HES concentration for the cryopreservation of keratinocytes in suspensions and in monolayers.** *Cryobiology* 2000, **41**:89–96.
33. Sudo K, Asoh S, Ohsawa I, Ozaki D, Yamagata K, Ito H, Ohta S: **The anti-cell death FNK protein protects cells from death induced by freezing and thawing.** *Biochem Biophys Res Commun* 2005, **330**:850–856.
34. Celluzzi CM, Welbon C: **A simple cryopreservation method for dendritic cells and cells used in their derivation and functional assessment.** *Transfusion* 2003, **43**:488–494.
35. Ashwood-Smith MJ, Warby C, Connor KW, Becker G: **Low-temperature preservation of mammalian cells in tissue culture with polyvinylpyrrolidone (PVP), dextrans, and hydroxyethyl starch (HES).** *Cryobiology* 1972, **9**:441–449.
36. Sputtek A, Jetter S, Hummel K, Kuhnl P: **Cryopreservation of peripheral blood progenitor cells: characteristics of suitable techniques.** *Beitr Infusionsther Transfusionsmed* 1997, **34**:79–83.
37. Horn EP, Sputtek A, Standl T, Rudolf B, Kuhnl P, Schulte am Esch J: **Transfusion of autologous, hydroxyethyl starch-cryopreserved red blood cells.** *Anesth Analg* 1997, **85**:739–745.
38. Lionetti FJ, Hunt SM: **Cryopreservation of human red cells in liquid nitrogen with hydroxyethyl starch.** *Cryobiology* 1975, **12**:110–118.
39. Persidsky MD, Ellett MH: **Hydroxyethyl starch as a cryopreservative for nucleated mammalian cells.** *Cryobiology* 1971, **8**:586–588.
40. Stiff PJ, Koester AR, Weidner MK, Dvorak K, Fisher RI: **Autologous bone marrow transplantation using unfractionated cells cryopreserved in dimethylsulfoxide and hydroxyethyl starch without controlled-rate freezing.** *Blood* 1987, **70**:974–978.
41. Boldt J: **Modern rapidly degradable hydroxyethyl starches: current concepts.** *Anesth Analg* 2009, **108**:1574–1582.
42. Westphal M, Lamszus K: **Other experimental therapies for glioma.** *Recent Results Cancer Res* 2009, **171**:155–164.
43. Stolzing A, Naaldijk Y, Fedorova V, Sethe S: **Hydroxyethylstarch in cryopreservation - Mechanisms, benefits and problems.** *Transfus Apher Sci* 2012, **46**:137–147.
44. Rodrigues JP, Paraguassu-Braga FH, Carvalho L, Abdelhay E, Bouzas LF, Porto LC: **Evaluation of trehalose and sucrose as cryoprotectants for hematopoietic stem cells of umbilical cord blood.** *Cryobiology* 2008, **56**:144–151.
45. Rowley SD, Feng Z, Chen L, Holmberg L, Heimfeld S, MacLeod B, Bensinger WI: **A randomized phase III clinical trial of autologous blood stem cell transplantation comparing cryopreservation using dimethylsulfoxide vs dimethylsulfoxide with hydroxyethylstarch.** *Bone Marrow Transplant* 2003, **31**:1043–1051.
46. Pasch J, Schiefer A, Heschel I, Rau G: **Cryopreservation of keratinocytes in a monolayer.** *Cryobiology* 1999, **39**:158–168.
47. Teasdale B, Sieber VK, Riches DJ, Nanchahal J: **Cryopreservation of cultured dermal fibroblast impregnated collagen gels.** *Burns* 1993, **19**:406–410.
48. Kearney JN: **Cryopreservation of cultured skin cells.** *Burns* 1991, **17**:380–383.
49. Ji L, de Pablo JJ, Palecek SP: **Cryopreservation of adherent human embryonic stem cells.** *Biotechnol Bioeng* 2004, **88**:299–312.
50. Stiff PJ, Murgo AJ, Zaroulis CG, DeRisi MF, Clarkson BD: **Unfractionated human marrow cell cryopreservation using dimethylsulfoxide and hydroxyethyl starch.** *Cryobiology* 1983, **20**:17–24.
51. Rowley SD, Anderson GL: **Effect of DMSO exposure without cryopreservation on hematopoietic progenitor cells.** *Bone Marrow Transplant* 1993, **11**:389–393.
52. Leibo SP, Mazur P: **The role of cooling rates in low-temperature preservation.** *Cryobiology* 1971, **8**:447–452.
53. Franks F, Mathias SF, Galfre P, Webster SD, Brown D: **Ice nucleation and freezing in undercooled cells.** *Cryobiology* 1983, **20**:298–309.
54. Mazur P: **Freezing of living cells: mechanisms and implications.** *Am J Physiol* 1984, **247**:C125–C142.
55. Kandra D, Devireddy RV: **Numerical Simulation of Local Temperature Distortions During Ice Nucleation of Cells in Suspension.** *Int J Heat Mass Transf* 2008, **51**:5655–5661.
56. Martinello T, Bronzini I, Maccatrozzo L, Mollo A, Sampaolesi M, Mascarello F, Decaminada M, Patruno M: **Canine adipose-derived-mesenchymal stem cells do not lose stem features after a long-term cryopreservation.** *Res Vet Sci* 2011, **91**:18–24.
57. Martinello T, Bronzini I, Maccatrozzo L, Iacopetti I, Sampaolesi M, Mascarello F, Patruno M: **Cryopreservation does not affect the stem characteristics of multipotent cells isolated from equine peripheral blood.** *Tissue Eng Part C Methods* 2010, **16**:771–781.
58. Heng BC, Ye CP, Liu H, Toh WS, Rufaihah AJ, Yang Z, Bay BH, Ge Z, Ouyang HW, Lee EH, *et al*: **Loss of viability during freeze-thaw of intact and adherent human embryonic stem cells with conventional slow-cooling protocols is predominantly due to apoptosis rather than cellular necrosis.** *J Biomed Sci* 2006, **13**:433–445.
59. Heng BC: **Effect of Rho-associated kinase (ROCK) inhibitor Y-27632 on the post-thaw viability of cryopreserved human bone marrow-derived mesenchymal stem cells.** *Tissue Cell* 2009, **41**:376–380.
60. Carvalho KA, Cury CC, Oliveira L, Cattaned RI, Malvezzi M, Francisco JC, Pachalok A, Olandoski M, Faria-Neto JR, Guarita-Souza LC: **Evaluation of bone marrow mesenchymal stem cell standard cryopreservation procedure efficiency.** *Transplant Proc* 2008, **40**:839–841.
61. Ock SA, Rho GJ: **Effect of dimethyl sulfoxide (DMSO) on cryopreservation of porcine mesenchymal stem cells (pMSCs).** *Cell Transplant* 2011, **20**:1231–1239.
62. Li H, Yan F, Lei L, Li Y, Xiao Y: **Application of autologous cryopreserved bone marrow mesenchymal stem cells for periodontal regeneration in dogs.** *Cells Tissues Organs* 2009, **190**:94–101.
63. Borderie VM, Lopez M, Lombet A, Carvajal-Gonzalez S, Cywiner C, Laroche L: **Cryopreservation and culture of human corneal keratocytes.** *Invest Ophthalmol Vis Sci* 1998, **39**:1511–1519.
64. Baust JM, Van B, Baust JG: **Cell viability improves following inhibition of cryopreservation-induced apoptosis.** *In Vitro Cell Dev Biol Anim* 2000, **36**:262–270.
65. Baust JM: **Molecular Mechanism of Cellular Demise Associated with Cryopreservation Failure.** *Cell Preservation Technology* 2002, **1**:17–31.

66. Baust JMV MJ, Snyder KK, Van Buskirk RG, Baust JG: **Activation of Mitochondrial-Associated Apoptosis Contributes to Cryopreservation Failure.** *Cell Preservation Technology* 2007, **5**:155–164.
67. Hronik-Tupaj M, Rice WL, Cronin-Golomb M, Kaplan DL, Georgakoudi I: **Osteoblastic differentiation and stress response of human mesenchymal stem cells exposed to alternating current electric fields.** *Biomed Eng Online* 2011, **10**:9.
68. Han DW, Kim HH, Lee MH, Baek HS, Lee KY, Hyon SH, Park JC: **Protection of osteoblastic cells from freeze/thaw cycle-induced oxidative stress by green tea polyphenol.** *Biotechnol Lett* 2005, **27**:655–660.
69. Luo K, Wu G, Wang Q, Sun Y, Liu H: **Effect of dimethylsulfoxide and hydroxyethyl starch in the preservation of fractionated human marrow cells.** *Cryobiology* 1994, **31**:349–354.

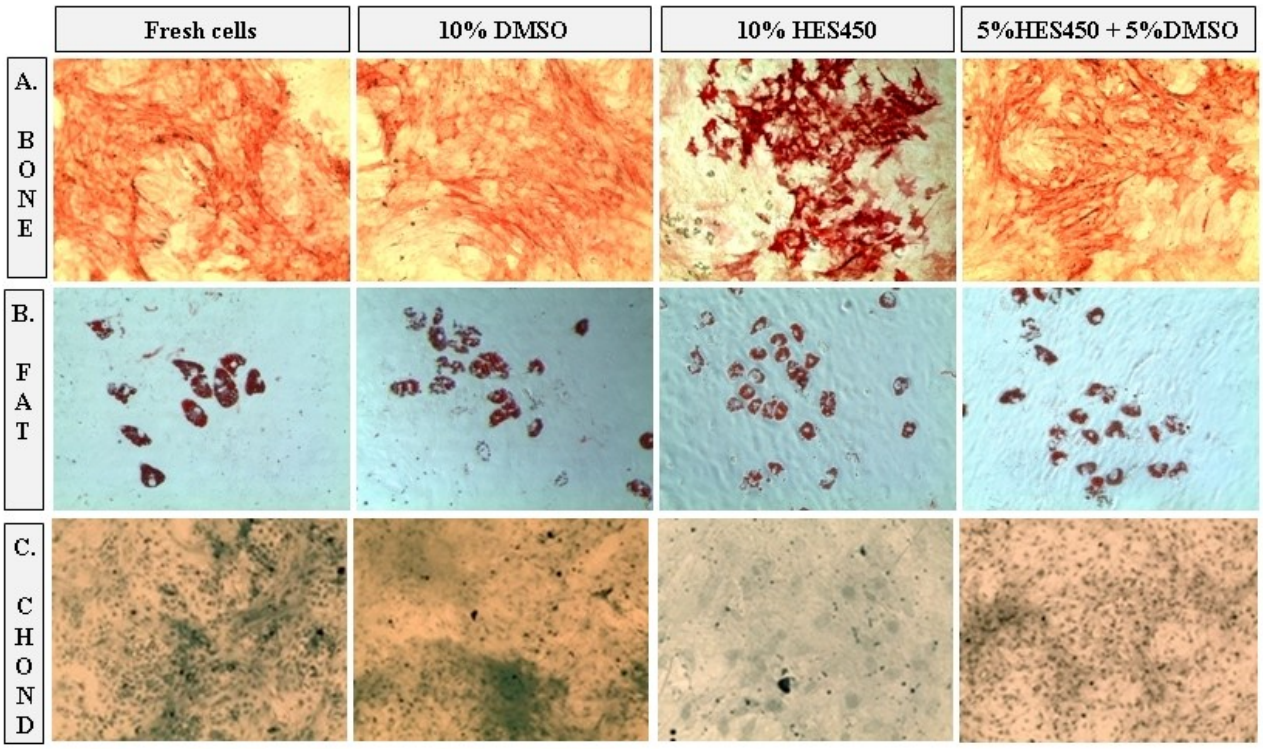
doi:10.1186/1472-6750-12-49
Cite this article as: Naaldijk *et al.*: Effect of different freezing rates during cryopreservation of rat mesenchymal stem cells using combinations of hydroxyethyl starch and dimethylsulfoxide. *BMC Biotechnology* 2012 **12**:49.



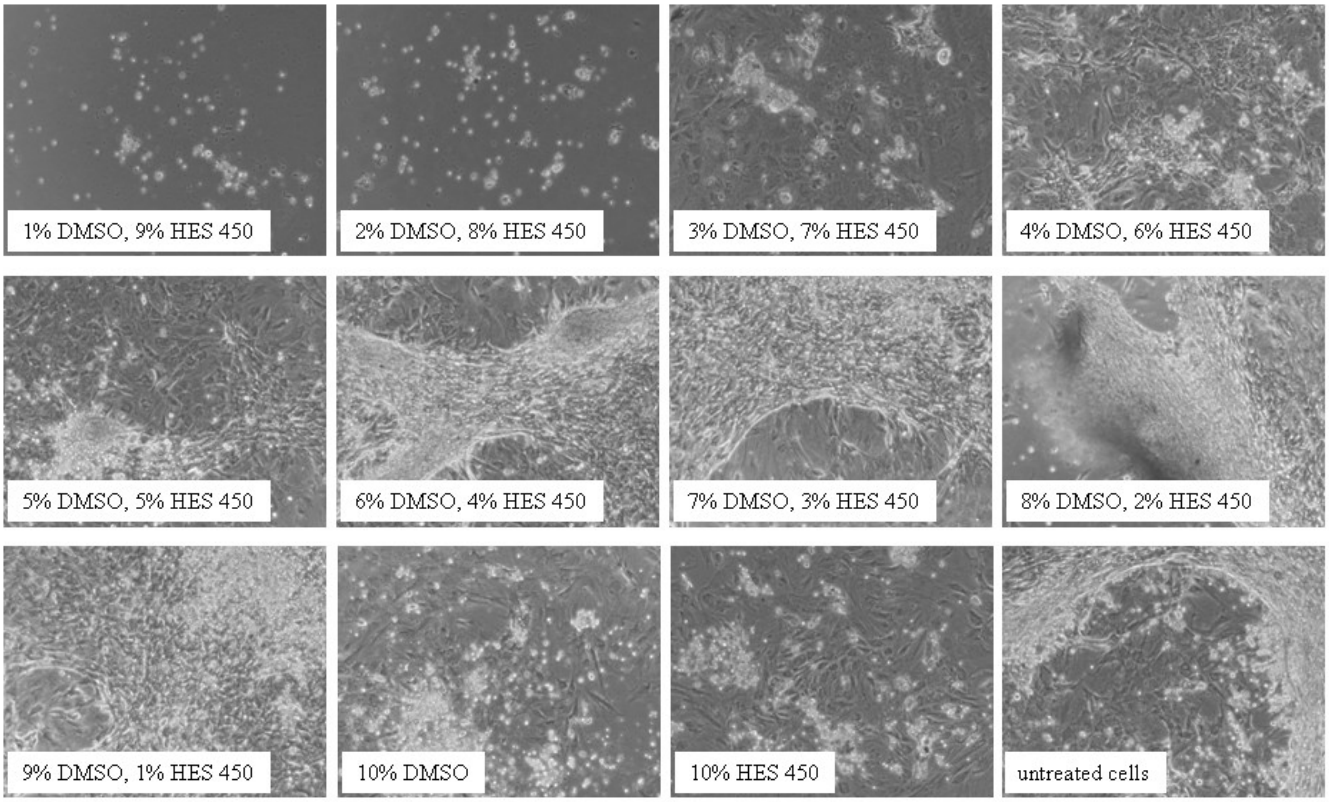
Supplementary Figure 1



Supplementary Figure 2



Supplemenraty Figure 3



Supplementary Figure 4

4.3 Comparison of different cooling rates for fibroblasts and keratinocytes cryopreservation

JOURNAL OF TISSUE ENGINEERING AND REGENERATIVE MEDICINE
J Tissue Eng Regen Med (2013)
Published online in Wiley Online Library (wileyonlinelibrary.com) **DOI:** 10.1002/term.1815

RESEARCH ARTICLE

Comparison of different cooling rates for fibroblast and keratinocyte cryopreservation

Yahaira Naaldijk[1,2], Annett Friedrich-Stöckigt[1], Sebastian Sethe[1] and Alexandra Stolzing[1]*
[1]*Fraunhofer Institute for Cell Therapy and Immunology, Leipzig, Germany*
[2]*Translation Centre for Regenerative Medicine, University of Leipzig, Germany*

Abstract

Easy, cost-effective and reliable cryopreservation protocols are crucial for the successful and effective application of tissue engineering. Several different protocols are in use, but no comprehensive comparisons across different machine-based and manual methods have been made. Here, we compare the effects of different cooling rates on the post-thaw survival and proliferative capacity of two basic cell lines for skin tissue engineering fibroblasts and keratinocytes, cultured and frozen in suspension or as a monolayer. We demonstrate that effectiveness of cryopreservation cannot be reliably determined immediately after thawing: the results at this stage were not indicative of cell growth in culture 3 days post-thaw. Cryopreservation of fibroblasts in an adherent state greatly diminishes their subsequent growth potential. This was not observed when freezing in suspension. In keratinocytes, however, adherent freezing is as effective as freezing in suspension, which could lead to significant cost and labour savings in a tissue-engineering environment. The 'optimal' cryopreservation protocol depends on cell type and intended use. Where time, ease and cost are dominant factors, the direct freezing into a nitrogen tank (straight freeze) approach remains a viable method. The most effective solution across the board, as measured by viability 3 days post-thaw, was the commonly used, freezing container method. Where machine-controlled cryopreservation is deemed important for tissue-engineering Good Manufacturing Practice, we present results using a portfolio of different cooling rates, identifying the 'optimal' protocol depending on cell type and culture method. Copyright © 2013 John Wiley & Sons, Ltd.

Received 18 January 2013; Revised 18 July 2013; Accepted 24 July 2013

Keywords fibroblast; keratinocytes; cryopreservation; controlled-rate freezing

1. Introduction

In vitro-engineered skin is used for the testing of chemicals and cosmetics and in drug development (Griffith and Naughton, 2002). With the growing demand in skin, the development of successful and reliable preservation protocols for the cell source would be beneficial. The use of cryopreserved cells for tissue engineering skin has many advantages, including faster adaptation on demand, pretesting of cells and the production of large standardized batches.

Human allogeneic keratinocytes and fibroblasts have been used to treat burn wounds and chronic skin ulcers, with some success (Leigh *et al.*, 1987; Phillips *et al.*, 1993; Germain *et al.*, 1995; Beele *et al.*, 2005). In the clinical setting, allogenic transplantion of fibroblasts promotes the release of cytokines and growth factors. Fibroblasts as a skin substitute promotes migration of keratinocytes to the wound area, secretion of growth factors involved in angiogenesis and responsiveness, and re-epithelialization, amongst other things (Marchese *et al.*, 2001; Werner and Smola, 2001), but can also have a detrimental role in scar formation (Lamme *et al.*, 2002; Reagan *et al.*, 1997; Hinz *et al.*, 2007). Therefore, maintenance of cellular activities and proper preservation of fibroblasts and keratinocytes are essential for their use in skin tissue engineering or for the direct clinical application of keratinocytes and fibroblasts.

There are several ways to cryopreserve cells using controlled-rate freezing protocols and vitrification. Controlled-rate freezing allows the creation of specific temperature profiles for specific cell types in order to optimize cell viability and recovery (Pegg, 2007). The advantage of using the

*Correspondence to: A. Stolzing, Fraunhofer Institute for Cell Therapy and Immunology, Perlickstrasse 1, D-04103 Leipzig, Germany. E-mail: alexandra.stolzing@izi.fraunhofer.de

controlled-rate freezing vs vitrification is that a low concentration of cryoprotectants can be used to accomplish intracellular freezing, minimizing the toxicity effects of the cryoprotectant (Meryman, 2007). Vitrification usually requires high cooling rates and high concentrations of cryopreservation agents, but avoids damage by ice crystals (Liu *et al.*, 2012; Arav *et al.*, 1993). The main constraint for cryopreservation is the low recovery rate obtained after thawing of the sample (Baust *et al.*, 2000, 2002) or the loss of proper cell function (Rosillo *et al.*, 1995; Owen *et al.*, 2007). The low recovery rate can be due to cellular damage by intracellular ice formation, osmotic pressure, apoptosis or toxicity. Since the 1990s more interest in the optimization of protocols and cryosolutions for dermal skin and cells derived from skin has arisen (Kearney, 1991; Teasdale *et al.*, 1993; Pasch *et al.*, 1999, 2000). Comparison between controlled-rate freezing and vitrification were done in most of the studies. The limitation of these publications is that few control rate freezing protocols were compared to one another.

The goal of our studies was to compare different cryopreservation protocols for fibroblasts and keratinocytes preserved as a monolayer and in suspension. In order to accomplish this, we compared controlled-rate freezing, a conventional method and vitrification. Protocols were modified based on published protocols (Pasch *et al.*, 2000; Ashwood-Smith *et al.*, 1972; Rowley *et al.*, 2003; Rodrigues *et al.*, 2008) with a spectrum from slow to fast freezing approaches. The protocols used are listed in Table 1.

We used protocol 2 (P2; 1 °C/min to −80 °C) to compare whether controlled-rate freezing is the same as the use of a conventional isopropanol-based container (P6). P1 and P3 were slightly modified from the published data, since the critical point for the formation of nucleation and ice growth is between 0 and −40 °C, and the cooling rate is performed using 10 °C/min to −40 °C. To confirm the assumption that changes after that critical range should not interfere with the cryopreservation procedure, we included P4 as a comparator.

Fast cooling protocols are not only of interest in tissue engineering to increase throughput. P5, which uses a cooling rate of 0.3 °C/min, was previously shown to be optimal for the cryopreservation of embryonic stem (ES) cells (Ware *et al.*, 2005). The choice of P6 (99 °C/min) is based on previous published data with fast freezing (> 100 °C/min) (Paynter and Fuller, 2007), where cryopreservation was successfully accomplished.

A human keratinocyte cell line, HACAT, generated by spontaneous immortalization (Boukamp *et al.*, 1988), a human fibroblast cell line, BJ (Bodnar *et al.*, 1998) and primary human fibroblasts derived from dermal skin were investigated.

We have carried out a systematic comparison of slow programmed freezing, vitrification and the use of a Nalgene freezing container ('Mr. Frosty') of human skin-specific cell types by comparing eight cryopreservation protocols. Cell morphology was studied using

Table 1. List of freezing protocols

Method	Protocols	Cooling rate	Duration	References
Controlled-rate freezing	1	Cool to 4 °C 1 °C/min to −30 °C 5 °C/min to −80 °C Transfer to −134 °C	44 min	(Rodrigues *et al.*, 2008; Rowley and Anderson, 1993; Watt *et al.*, 2007)
	2	Cool to 4 °C 1 °C/min to −80 °C Transfer to −134 °C	94 min	(Pasch *et al.*, 1999; Teasdale *et al.*, 1993; Kearney, 1991; Ji *et al.*, 2004; Stiff *et al.*, 1983)
	3	Cool to 4 °C 1 °C/min to −20 °C 5 °C/min to −40 °C 10 °C/min to −80 °C 20 °C/min to −100 °C Transfer to −134 °C	34 min	(Naaldijk *et al.*, 2012)
	4	Cool to 4 °C 1 °C/min to −6 °C 25 °C/min to −50 °C 10 °C/min to −90 °C Transfer to −134 °C	26 min	(Naaldijk *et al.*, 2012)
	5	Cool to 4 °C 0.3 °C/min to −100 °C Transfer to −134 °C	347 min	(Teasdale *et al.*, 1993; Ware *et al.*, 2005)
	6	Cool to 4 °C 99.9 °C/min to −100 °C Transfer to −134 °C	2 min	(Naaldijk *et al.*, 2012; MacFadzen, 1995)
Vitrification	7	Directly into vapour phase N_2 tank at −134 °C		(Ashwood-Smith *et al.*, 1972; Leibo and Mazur, 1971)
Conventional method	8	'Mr. Frosty' (vials) or Styrofoam box (monolayer) at 1 °C/min Transfer to −134 °C	Overnight	(Danso and Ford-Lloyd, 2011; Day and Brand, 2005; Tassone and Fidler, 2012)

DOI: 10.1002/term

light microscopy, and the viability of the tissues in culture after thawing were evaluated on day 0 and analysed 3 days after thawing.

2. Materials and methods

2.1. Cell sources

Primary human fibroblast cells were isolated from foreskin tissues obtained from the Children's Hospital in Leipzig, with ethical approval from the University of Leipzig Ethics Commission of the Medical Department. Written consent was obtained from all patients regarding sample collection.

The human keratinocyte cell line HACAT was purchased from the Cell Lines Service and the human fibroblast cell line BJ was obtained from ATCC.

2.2. Cell culture

All cell types were cultured in Dulbecco's modified Eagle's medium (DMEM, high–glucose; Gibco) supplemented with 10% fetal calf serum (FCS; Perbio) and 1% penicillin–streptomycin (Gibco). At ca. 90% confluency, the cells were trypsinized with 1× trypsin/EDTA (Gibco) and centrifuged at 1000 rpm for 5 min for subsequent subculture or cryopreservation.

2.3. Cryopreservation procedure

A controlled-rate freezing machine (Thermo Scientific Model 7452 Series) was cooled to 4 °C before starting the procedure (see Table 1 for protocols used to freeze the cells; and Figure 1 for an overview of the procedure). Protocols 1–6 (P1–P6) were performed using the same freezing machine.

Different cryosolution combinations containing DMSO (Sigma), serum, hydroxylethyl starch (Serumwerke) and/or DMEM (Gibco) were used (Table 2). The same cryosolution combinations were used for all the protocols. The average of all solutions within the protocol was compared to other protocols. In addition, the average of all protocols was compared to non-frozen cells.

- *Adherence*. One day before freezing, cells were seeded at 50 000 cells/well in a 24-well plate. The next day the medium was removed, the cells were washed once with 1× phosphate-buffered saline (PBS; Gibco) and 500 µl cryosolution was added carefully to each well.
- *Cryovials*. Trypsinized cells were counted using 0.3% Trypan blue (Merck) and a Neubauer chamber and 10^5 cells were frozen in 2 ml cryovials (Corning) in 500 µl cryosolution.
- *Freezing*. Cryovials and plates were placed on ice prior to freezing for transport into the controlled-rate freezing machine at a temperature of ca. 14.5 °C, as a starting point for the cryopreservation procedure. The starting temperature of the freezing machine was at 4 °C independently of using cryovials or plates. A temperature probe from the machine was introduced in a sample cryovial, whose temperature was ~14.5 °C, which was also transported on ice and contained cryosolution only. The temperature protocol of the machine was based on the temperature measured in the sample and not in the machine.

Table 2. Cryoprotectant solutions (cryovials and monolayer)

10% DMSO + 90% serum
5% DMSO + 95% serum
5% DMSO + 5% HES + 90% serum
10% HES + 90% serum
10% HES + 90% DMEM
10% DMSO + 90% DMEM

For cryopreservation using P7 (see Table 1), cryovials or plates were placed straight into the vapour phase of the cryotank (–134 °C).

For cryopreservation using P8, cryovials (suspended cells) were placed in a Nalgene freezing container ('Mr. Frosty') placed in a –80 °C freezer, usually overnight (exposed to a freezing rate of 1 °C/min, according to the manufacturer's instructions).

For adherent cells, 24-well plates were surrounded with cotton on all sides, placed in a Styrofoam box and transferred to a –80 °C freezer overnight. The temperature in the box was reduced at ~1 °C/min (Ebeigbe *et al.*, 1988).

All vials/plates were transferred to a liquid nitrogen tank and kept at vapour phase (–134 °C) for at least 24 h.

2.4. Post-thaw cell count

- *Thawing vials*. Vials were thawed at 37 °C in a water bath until a small piece of ice was left (~1 min) and

Figure 1. Outline of the cryopreservation framework. For better understanding of the procedures performed, we have illustrated the timeline that all protocols have in common

transferred to a 15 ml conical tube containing 1 ml prewarmed medium (at 37 °C).

- *Thawing adherent cells*. Plates were removed from the vapour-phase nitrogen tank in a sealed container and quickly transferred into a sterile hood. Under the sterile hood, the plates were thawed (~3–5 min) at room temperature (RT), the cryosolution medium was carefully removed to avoid detachment of the cells and 500 µl fresh medium was slowly added. It is important that transport of plates from the nitrogen tank to the sterile hood takes place fast, since exposure of the plates to RT will cause the lid of the plate to bend, which can cause contamination.
- *Day 0 cell count*. Cells in suspension were counted using Trypan blue solution.
- *Seeding density for the MTT test*. 24-well plates were used, 10 000 cells/well, for thawed cells previously in suspension and 50 000 cells/well for thawed monolayer cells; 1 day after thawing, the medium was changed completely. Viability at day 0 was compared to non-frozen cells.

For comparison, control cells were seeded in a culture dish for 4 h, trypsinized, collected and counted using a haemocytometer. In parallel we seeded cells into 24-well plates (10 000 for suspension; 50 000 for adherence, 24 h prior to starting), left them growing for 3 days and measured viability using MTT.

2.5. MTT assay

Three days after thawing, the 3-[4,5-dimethyltiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT; Carl Roth) reaction was performed to determine viability; 50 µl MTT (stock 5 mg/ml) was added to each well and incubated for 4 h at 37 °C. The medium was removed and 500 µl stop solution [50% dimethylformamide (VWR) in 20% SDS (Merck)] was added. The plate was incubated for 60 min and the absorbance was measured at 550 nm with a reference at 630 nm in a Tecan plate reader.

2.6. Cell morphology

Morphology of the cells was recorded 3 days later, after thawing in a light microscope. Pictures were taken at × 10 magnification, using the Leica system.

2.7. Statistics

Experiments represent the mean of $n = 3$–5. Error bars represent standard error (SE) of the mean and data were expressed as mean ± SE. Statistical analysis was performed using one-way ANOVA, followed by Turkey test, with $p < 0.05$ considered statistically significant, using Sigma Plot.

3. Results

We compared controlled-rate freezing (P1–P6), vitrification (P7) and cryopreservation using a freezing container (P8). An overview of methods and methodology is provided in Table 1 and Figure 1. We always compared frozen cells to non-frozen cultivated cells of the same passage.

3.1. Cryopreservation of keratinocytes

Significant differences between the protocols were observed at day 0 after thawing with the highest keratinocyte viabilities for P1–P3 and P7 (Figure 2a); however, all used protocols showed significantly ($p < 0.05$) lower viability directly after thawing in comparison to non-frozen cells (Figure 2b).

In addition, viability for HACAT cells was determined at 3 days after thawing (Figure 2c). Cryopreservation of HACAT in suspension using the different protocols shows significant differences ($p \leq 0.04$) between P1, P2 and P3 compared to P6 (Figure 2b), but in no other ways. Adherent HACAT frozen using P1 and P2 resulted in significantly higher cell numbers ($p \leq 0.045$) compared to P3–P7 (Figure 2e).

After 3 days of cell growth, the mean calculated cell number of frozen HACAT in suspension was significantly ($p = 0.005$) higher compared to non-frozen HACAT (Figure 2d), whereas the mean cell number of HACAT cryopreserved in monolayers was not significantly different from non-frozen HACAT (Figure 2f).

3.2. Cryopreservation of BJ cells

On day 0, BJ cells cryopreserved using P1 and P2 gave the highest survival rates. P1 showed significantly higher cell viability ($p \leq 0.002$) after thawing compared to cells frozen with P3–P8. BJ cells frozen with P2 showed significantly higher cell survival ($p \leq 0.003$) compared to cells frozen with P3, P4, P6, P7 and P8 (Figure 3a). BJ cells frozen with P5 showed significantly ($p \leq 0.029$) higher cell numbers compared to cells frozen with P6 and P8. The average cell number of all cells frozen with the various protocols was significantly lower ($p = 0.0003$) compared to non-frozen cells (Figure 3b).

For BJ numbers measured on day 3, we found that P1–P4 gave higher cell recovery than P6–P8 (Figure 3c). P1 and P2 resulted in $p \leq 0.01$ compared to P6, and P3 and P4 showed significances of $p \leq 0.039$ compared to P6–P8.

The mean viability of BJ frozen in suspension was statistically not significantly different from non-frozen BJ cells (Figure 3d).

Adherent cryopreserved BJ cell numbers measured on 3 days after freezing were not significantly different between the freezing protocols (Figure 3e), and here the numbers of BJ cells cryopreserved in monolayers were significantly lower ($p = 0.01$) compared to non-frozen BJ cells (Figure 3f).

DOI: 10.1002/term

Comparison of different cooling rates for cryopreservation

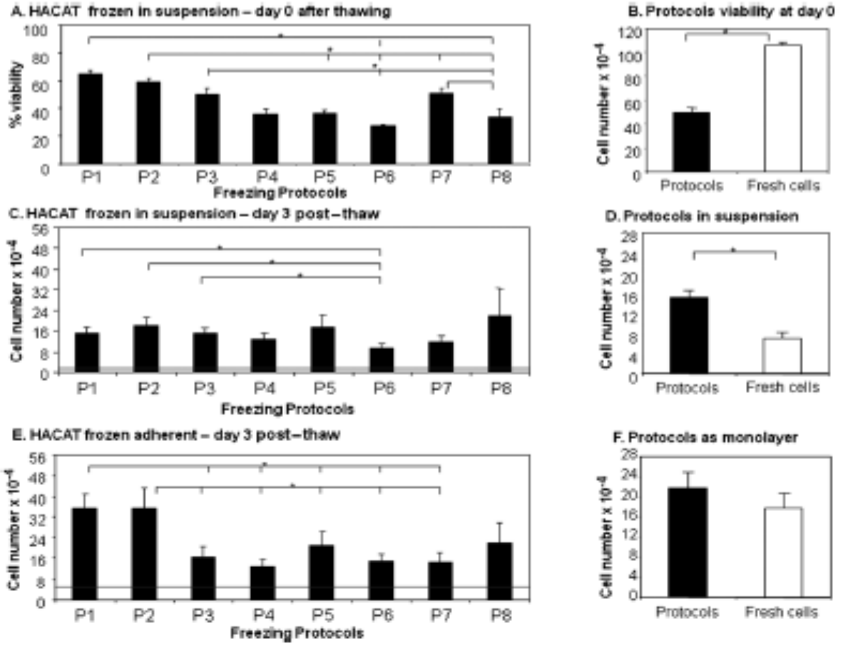


Figure 2. Cryopreservation of keratinocytes in suspension and as monolayers using different freezing protocols. Keratinocytes (HACAT) were frozen in suspension and as a monolayer using different freezing protocols. Cell viability was measured for cells preserved in suspension only at day 0 (A). Cell viability was assessed at day 3 after thawing by MTT for cells preserved in suspension (C) and as a monolayer (E). The mean of all protocols used was compared to non-frozen cells (B, D, F)

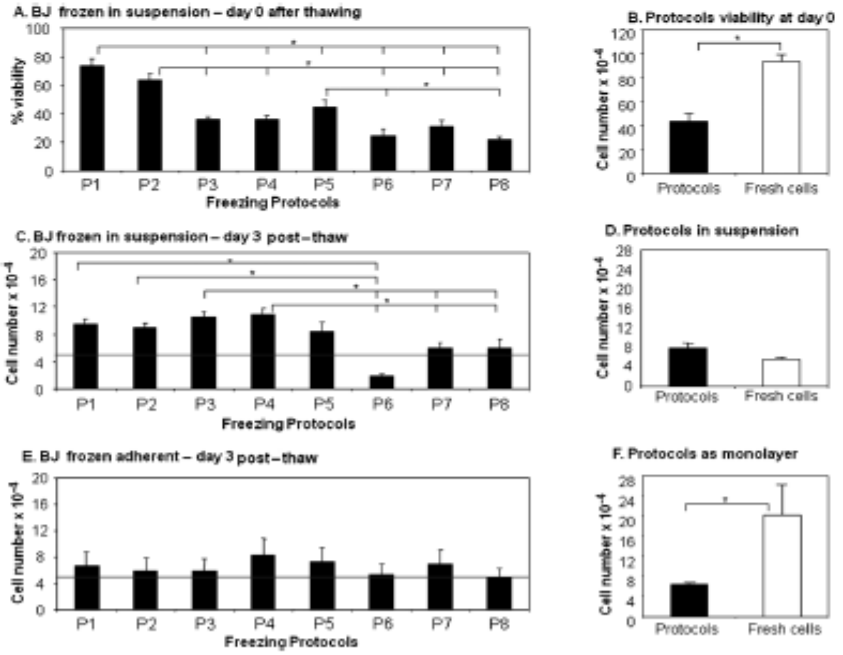


Figure 3. Cell viability of a fibroblast cell line cryopreserved using different freezing protocols. Human dermal fibroblast line (BJ) cells were frozen in suspension and as a monolayer using different freezing protocols. Cell viability was measured for cells preserved in suspension only at day 0 (A). Cell viability was assessed at day 3 after thawing by MTT for cells preserved in suspension (C) and as a monolayer (E). The mean of all protocols used was compared to non-frozen cells (B, D, F)

J Tissue Eng Regen Med (2013)
DOI: 10.1002/term

3.3. Cryopreservation of primary fibroblasts

On day 0 after thawing, primary human fibroblasts frozen using P1 showed statistically significant higher survival rates ($p \leq 0.044$) compared to fibroblasts frozen using P5, P6 and P8 (Figure 4a). The mean of all the protocols showed a significant lower viability ($p = 0.004$) of frozen fibroblasts compared to non-frozen cells (Figure 4b).

Measured 3 days after thawing, however, primary human fibroblasts cryopreservation in suspension using P5, P7 and P8 gave the highest cell viabilities compared to other protocols (Figure 4c). Primary fibroblasts frozen using P5 resulted in statistically significantly higher cell numbers ($p \leq 0.034$) compared to P3 and P6. P7 gave a higher ($p < 0.001$) yield compared to P1–P4 and P6. P8 gave a higher yield ($p \leq 0.031$) compared to P1, P3, P4 and P6 (Figure 4c). After 3 days there was no significant difference in cell numbers between non-frozen primary fibroblasts and those frozen in suspension (Figure 4d).

Cryopreservation of primary fibroblasts as a monolayer showed differences only for P8, which resulted in the highest cell numbers compared to primary fibroblasts frozen with all other protocols (Figure 4e). The cell number was significantly higher for P8 ($p \leq 0.04$) compared to P1, P2, P4 and P6. The number of fibroblasts preserved as a monolayer compared to non-frozen cells was significantly lower for all applied freezing protocols ($p < 0.05$) (Figure 4f).

3.4. Cell morphology of cryopreserved cells in suspension and monolayer

Cellular morphology was assessed by light microscopy at day 3 after thawing, and compared to the MTT results (Figure 5). No changes in cell morphology were observed during the cryopreservation of the cells in suspension, indicating that our cryopreservation protocols did not affect their morphology; on the other hand, cryopreservation of cells as a monolayer in some cases showed detachment of the cells from the plate and/or deterioration (Figure 6). The viable cells showed normal cell morphology and were able to proliferate.

4. Discussion and conclusion

Fibroblasts and keratinocytes are the 'staple crop' for tissue engineering of skin (Parenteau *et al.*, 1992; Kuroyanagi *et al.*, 1993; Horch *et al.*, 2000; Kempf *et al.*, 2011). Cryopreservation is an everyday procedure in all tissue-engineering facilities and does not get reported extensively. Tables 3 and 4 give a summary overview of reported experiences with cryopreservation of these cell types in the literature. As cells are usually required in large numbers for tissue-engineered products, we focused here on raw cell yield (Table 5).

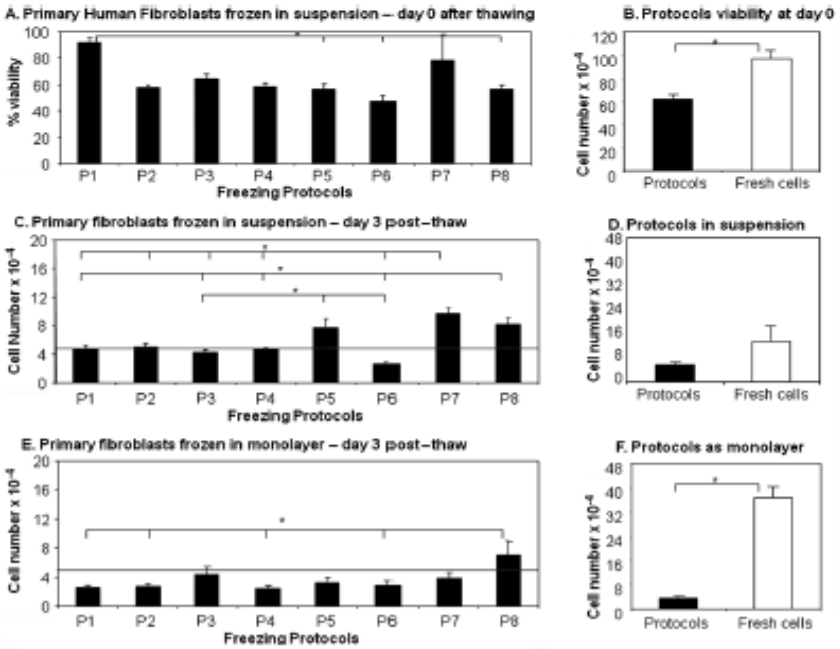


Figure 4. Primary fibroblast cells cryopreserved in cryovials and as monolayers using different freezing protocols. Primary human dermal fibroblast cells (foreskin) were frozen in suspension and as a monolayer using different freezing protocols. Cell viability was measured for cells preserved in suspension only at day 0 (A). Cell viability was assessed at day 3 after thawing by MTT for cells preserved in suspension (C) and as a monolayer (E). The mean of all protocols used was compared to non-frozen cells (B, D, F)

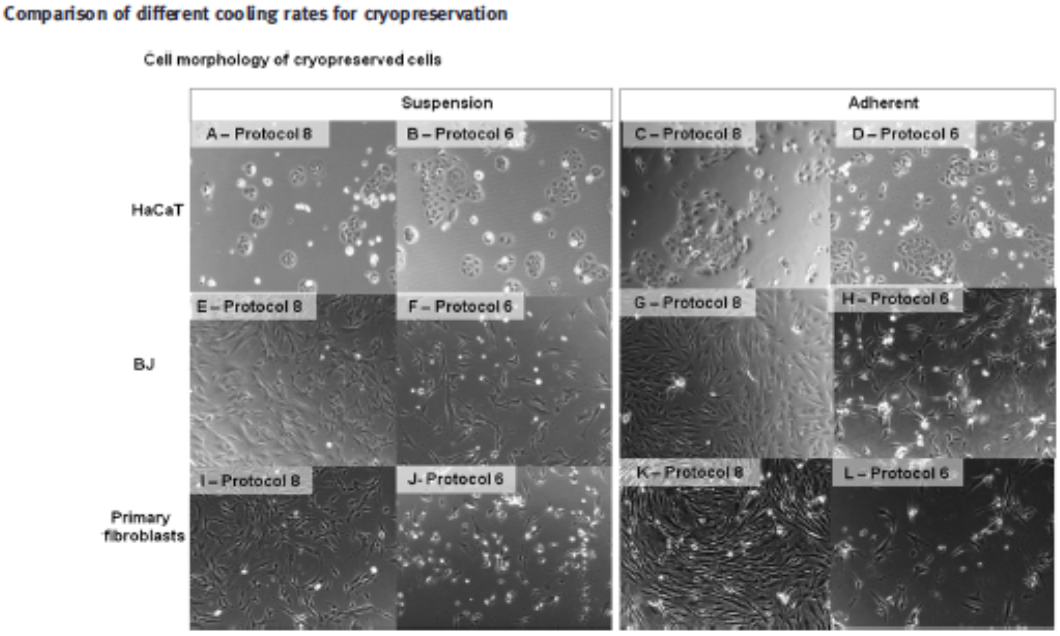


Figure 5. Cell morphology of cryopreserved cells in suspension and as a monolayer. (A) The morphology of cryopreserved keratinocytes and fibroblasts was assessed at day 3 after thawing. Protocols that gave the best cell viability (A, C, E, G, I, K) were compared to protocols which resulted in a lower cell number (B, D, F, H, J, L)

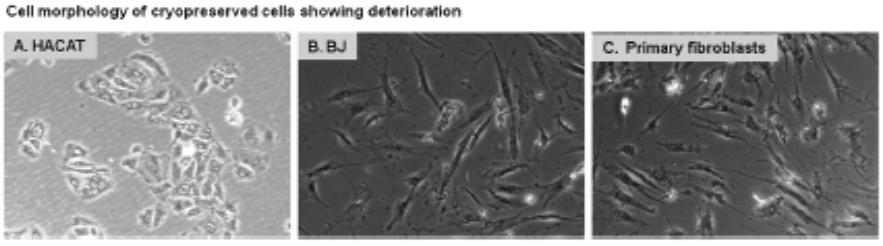


Figure 6. Cell morphology of cryopreserved cells, showing deterioration. These cells were cryopreserved using protocols that all showed low cell survival. Cells were still present after 3 days, but in a state of cell death. HaCaT (A), BJ (B) and primary fibroblasts (C) all showed signs of apoptosis and necrosis, and the cells were in a state of fragmentation

4.1. Effectiveness of cryopreservation cannot be reliably determined immediately after thawing

We measured cell viability after thawing by Trypan blue and 3 days later with MTT assay (for adherent cells, cell viability was only tested at day 3). Use of exclusion dye at day 3 might result in cell loss and miscalculation of cell viability (Baust *et al.*, 2000; Freshney, 1994).

Many of the published data on cell cryopreservation measure cell recovery after thawing (Pasch *et al.*, 1999, 2000; Rindler *et al.*, 1999), but our results clearly show that the effect of different freezing protocols takes time to observe. Freezing-associated cell death can set in 24 h after thawing (Borderie *et al.*, 1998; Saraste and Pulkki, 2000) and the observed apparent benefits associated with one freezing protocol do not correlate between the point in time immediately after thawing and observing the subsequent cell culture.

4.2. In keratinocytes, but not in fibroblasts, some cryopreservation protocols can stimulate post-thaw cell growth compared with non-frozen cells

This effect is an observable trend in cells frozen adherently, but significant for cells frozen in suspension. It might be explained by a selection of very resistant and fast-growing keratinocyte cells during the stressful cryopreservation process; similar effects have been observed for chondrocytes (Muinos-Lopez *et al.*, 2012).

4.3. Cryopreservation of fibroblasts in an adherent state greatly diminishes their subsequent growth potential

Generally (lessened by the application of the 'optimal protocol') both BJ cells and primary fibroblasts frozen in

Table 3. Fibroblast and keratinocyte cooling rates for cryopreservation in suspension

Cell type	Freezing protocol	Viability	Reference
Primary keratinocytes	−3 °C/min	+++	(Pasch *et al.*, 2000)
Primary keratinocytes	−3.5 °C/min	++	(Rindler *et al.*, 1999)
Freshly isolated and primary cultured keratinocytes	−1 °C/min in −20 °C freezer × 12 min 2 h in −80 °C, N_2 tank	++ +++	(Hirel *et al.*, 1994)
Primary keratinocytes	−0.3 °C/min −1 °C/min −10 °C/min −20 °C/min	− +++ − −	(May *et al.*, 1988)
SVK14	−1 °C/min 30 °C/min Vitrification	+++ ++ +	(Kearney, 1991)
Primary fibroblasts	1 °C/min 30 °C/min Vitrification	+++ ++ +	(Kearney, 1991)
Primary fibroblasts in collagen gel	0.5 °C/min, 1 °C/min: 20 − 70 °C 5 °C/min: −5 to −170 °C N_2 tank	+++ ++ + −	(Teasdale *et al.*, 1993)
Dermal fibroblasts	1 °C/min, −80 °C, N_2 tank	+++	(Baust *et al.*, 2007)
Lung fibroblasts	3 °C/min from RT to 4 °C 4 °C for 5 min 1 °C/min, from 4 °C to −50C 5 °C/min from −50 °C to −80 °C, N_2 tank	+++	(Murakawa *et al.*, 2000)
I-cell disease fibroblasts	4 °C for 30 min, −70 °C freezer (5 °C/min), N_2 tank	++	(Buck *et al.*, 1981)

Table 4. Cooling rates for cryopreservation of fibroblast and keratinocytes as monolayer

Cell type	Freezing protocol	Viability	Reference
Primary keratinocytes	1 °C/min 3 °C/min cryomicroscope 10 °C/min	++ +++ +	(Pasch *et al.*, 1999)
Primary keratinocytes HaCaT	3.3 °C/min in −85 °C freezer Styrofoam box in −70 °C	88% +++	(Pasch *et al.*, 2000) (Corsini *et al.*, 2002)
Dermal skin fibroblast slices	0.5 °C/min, 2 °C/min, 1 °C/min: −4 to −60 °C, N_2 tank	++ +++ +	(Wang *et al.*, 2007)
Dermal fibroblasts in PGA scaffold	0.5 °C/min, 2 °C/min, 1 °C/min: −4 to −60 °C, N_2 tank	+++ +++ ++	(Wang *et al.*, 2007)

Table 5. 'Optimal' protocol

	Protocol no.	
Cell type	Suspension	Adherent
HACAT	8 (1)	1
BJ	4	4
Fibroblasts	7(5)	8 (7)

The first number identifies the protocol number with the respective highest yield.
The alternative second number in brackets is provided where required, where a controlled-rate protocol is of the essence (loosely factoring in operating considerations such as time and consistency).

an adherent manner clearly 'suffered' from freezing compared to the growth rate of non-frozen cells. In primary fibroblasts, P8 sustained some cell recovery. This was not observed when freezing in suspension or using HaCaT.

Cell preservation as a monolayer may be dependent on the storage temperature. At −196 °C cell detachment and loss of membrane integrity was observed compared to −80 °C when endothelial cells on glass coverslips were frozen (Ebertz and McGann, 2004).

To explain the differences with HACAT cells, we can assume that cell size matters a lot. HACAT cells are much smaller (~30 μm) (Biggs *et al.*, 2012) compared

DOI: 10.1002/term

to fibroblasts (~50 µm) (Salem *et al.*, 2002). Cells shrink when frozen (Mazur, 1984), which impacts on their focal adhesion point and thereby delivers mechanical stress to the cell. Mechanical stress would be higher with large cells, as cell shrinkage is more extensive, and it is known that mechanical stress induces apoptosis. It is known from other reports that adherent cells can be more vulnerable to cryoinjury (Teasdale *et al.*, 1993; Xu *et al.*, 2012). Nonetheless, one cannot jump to the conclusion that freezing in suspension is *always* inadvisable.

Choi and Bischof (2011) describe a correlation between cell recovery, intracellular ice formation (IIF) and cooling rate in the cryopreservation of fibroblasts. Their findings support our results regarding a too slow cooling rate for cryopreserved fibroblasts as monolayers and the increase in cell recovery.

4.4. Adherent freezing can be broadly as effective as freezing in suspension for small cells

Adherent cell culture is not widely used for cell cryopreservation, but these results suggest that it is a viable alternative for freezing in suspension, with the notable advantages of being quicker and less costly. While we can demonstrate that freezing in monolayer is effective for the HACAT cell line, further studies will need to be conducted on freezing as a monolayer for other small cell types (notably, human ES cell viability was higher in preservation as a monolayer compared to cryopreservation in suspension; Ji *et al.*, 2004).

4.5. The 'optimal' thawing protocol depends on cell type and intended use

In summary, if pressed for a recommendation for the single most effective solution across the board, the comparatively 'uncontrolled' freezing container method could be recommended for all three cell types, except that a superior protocol exists for BJ cells in suspension. In addition, primary fibroblasts are more sensitive to cryo-injury than BJ cells.

Whereas we can confirm the results by Baust *et al.* (2007) that primary fibroblasts preserved with –1 °C/min to –80 °C after 3 days show roughly equivalent cell yield compared to non-frozen cells, we find that P8 gave significantly better cell recovery than P2 in this setting. Both protocols have a reported cooling rate of 1 °C/min until –80 °C, but they differ in the method of accomplishing this cooling rate: controlled-rate machine-based freezing vs a –80 °C freezer. Further research will need to be conducted to explore the details of freezing dynamics in these different environments.

It should also be highlighted that a 'straight plunge' approach was generally not inferior to the much more time-intensive controlled-rate methods (in fact, for primary fibroblasts frozen in suspension, it was the most effective approach). In contrast, Kearney (1991) could recover only low numbers of cells after thawing using a 'straight freeze' approach.

Many laboratories have reported good results using a controlled-rate freezing approach (Borderie *et al.*, 1998; Balint *et al.*, 2006); however, a 'quick freeze' (often, but not always correctly, equated with 'vitrification') has been demonstrated to be at least as effective (Cetinkaya and Arat, 2011; Tan *et al.*, 2007) or superior in terms of cell quality (Kuleshova *et al.*, 2009; Berz *et al.*, 2007). Cells frozen using P7 showed no vitrified glass state by visual inspection.

We can show, however, that rapidity as such does not correlate with cell yield: in primary suspension-frozen fibroblasts, for example, the 'quickest' controlled-rate protocol (P6) was significantly less effective than both 'slow' P1/P2 protocols and a 'straight plunge' (P7). In mesenchymal stem cells, fast cooling (5–10 °C/min) disrupted the cytoskeleton more than slow cooling (1 °C/min) (Xu *et al.*, 2012). One possible explanation is that the benefit is only associated with 'ultrafast' cooling. Based on our observations, such a 'straight plunge' approach (into the vapour phase) may be a convenient and effective alternative to even rapid or easy controlled-rate methods.

However, for some clinical protocols and in tissue-engineering manufacturing, a closely monitored freezing protocol might be preferred in a Good Manufacturing Practice context.

This is the first extensive comparison of published and new cryopreservation protocols applied to the optimization of fibroblasts and keratinocytes cryopreservation. We can recommend adherent cryopreservation for keratinocytes but freezing in suspension for fibroblasts. In general, a quick 'straight freeze' cryopreservation approach remains a viable solution in most situations and, overall, machine-based controlled-rate freezing had no advantages over the commonly used 'informal' slow freezing method.

Acknowledgements

This study was supported in part by Serumwerke Bernburg, a manufacturer of hydroxyethyl starch, and the project 'Automated Tissue Engineering on Demand' from the Stiftungsprojekt of the Fraunhofer Society.

Author contributions

YN and AFS performed experiments, consolidated data and provided the first draft of the manuscript; SS assisted with manuscript review and analysis; and AS planned the experiments, secured the funding and revised the manuscript.

Conflict of interest

The authors declare no conflicts of interest.

References

Arav A, Shehu D, Mattioli M. 1993; Osmotic and cytotoxic study of vitrification of immature bovine oocytes. *J Reprod Fertil* **99**: 353–358.

Ashwood-Smith MJ, Warby C *et al.* 1972; Low-temperature preservation of mammalian cells in tissue culture with polyvinylpyrrolidone (PVP), dextrans, and hydroxyethyl starch (HES). *Cryobiology* **9**: 441–449.

Balint B, Puanivic D, Vucetic D *et al.* 2006; Controlled-rate versus uncontrolled-rate freezing as predictors for platelet cryopreservation efficacy. *Transfusion* **46**: 230–235.

Baust JM, Van B, Baust JG. 2000; Cell viability improves following inhibition of cryopreservation-induced apoptosis. *In Vitro Cell Dev Biol Anim* **36**: 262–270.

Baust JM, Van Buskirk R, Baust JG. 2002; Modulation of the cryopreservation cap: elevated survival with reduced dimethyl sulfoxide concentration. *Cryobiology* **45**: 97–108.

Baust JM, Vogel MJ, Snyder KK *et al.* 2007; Activation of mitochondrial-associated apoptosis contributes to cryopreservation failure. *Cell Preserv Technol* **5**: 155–164.

Beele H, De la Brassine M, Lambert J *et al.* 2005; A prospective multicenter study of the efficacy and tolerability of cryopreserved allogenic human keratinocytes to treat venous leg ulcers. *Int J Low Extrem Wounds* **4**: 225–233.

Berz D, McCormack EM, Winer ES *et al.* 2007; Cryopreservation of hematopoietic stem cells. *Am J Hematol* **82**: 463–472.

Biggs LC, Rhea L, Schutte BC *et al.* 2012; Interferon regulatory factor 6 is necessary, but not sufficient, for keratinocyte differentiation. *J Invest Dermatol* **132**: 50–58.

Bodnar AG, Ouellette M, Frolkis M *et al.* 1998; Extension of life-span by introduction of telomerase into normal human cells. *Science* **279**: 349–352.

Borderie VM, Lopez M, Lombet A *et al.* 1998; Cryopreservation and culture of human corneal keratocytes. *Invest Ophthalmol Vis Sci* **39**: 1511–1519.

Boukamp P, Petrussevska RT, Breitkreutz D *et al.* 1988; Normal keratinization in a spontaneously immortalized aneuploid human keratinocyte cell line. *J Cell Biol* **106**: 761–771.

Buck DW, Hanssens LK, Kennett RH *et al.* 1981; Successful cryopreservation of tissue and skin fibroblast cultures from patients with mucolipidosis II (I-cell disease). *Cytogenet Cell Genet* **29**: 9–15.

Cetinkaya G, Arat S. 2011; Cryopreservation of cartilage cell and tissue for biobanking. *Cryobiology* **63**: 292–297.

Choi JAB, Bischof JC. 2011; Cooling rate dependent biophysical and viability response shift with attachment state in human dermal fibroblast cells. *Cryobiology* **63**: 285–291.

Corsini J, Maxwell F, Maxwell IH. 2002; Storage of various cell lines at –70°C or –80°C in multi-well plates while attached to the substratum. *Biotechniques* **33**: 42, 44, 46.

Danso KE, Ford-Lloyd BV. 2011; Cryopreservation of cassava micropropagules using simple slow freezing and vitrification techniques. *Biotechnology* **10**: 415–420.

Day JG, Brand JJ. 2005; Cryopreservation methods for maintaining microalgal cultures. In Algal Culturing Techniques, Anderson R (ed.). Academic Press: New York.

Ebeigbe AB, Muller-Schweinitzer E, Vogel A. 1988; Effects of calcium channel blockade in canine saphenous veins after storage at –190°C. *Br J Pharmacol* **94**: 381–388.

Ebertz SL, McGann LE. 2004; Cryoinjury in endothelial cell monolayers. *Cryobiology* **49**: 37–44.

Freshney RI. 1994; Culture of Animal Cells: A Manual of Basic Techniques. Academic Press: New York.

Germain L, Guignard R, Rouabhia M *et al.* 1995; Early basement membrane formation following the grafting of cultured epidermal sheets detached with thermolysin or dispase. *Burns* **21**: 175–180.

Griffith LG, Naughton G. 2002; Tissue engineering – current challenges and expanding opportunities. *Science* **295**: 1009–1014.

Hinz B, Phan SH, Thannickal VJ *et al.* 2007; The myofibroblast: one function, multiple origins. *Am J Pathol* **170**: 1807–1816.

Hirel B, Chesne C, Pailheret JP *et al.* 1994; Expression of differentiation markers in human adult keratinocytes cultured in submerged conditions. *In Vitro Cell Dev Biol Anim* **30A**: 372–378.

Horch RE, Debus M, Wagner G *et al.* 2000; Cultured human keratinocytes on type I collagen membranes to reconstitute the epidermis. *Tissue Eng* **6**: 53–67.

Ji L, De Pablo JJ, Palecek SP. 2004; Cryopreservation of adherent human embryonic stem cells. *Biotechnol Bioeng* **88**: 299–312.

Kearney JN. 1991; Cryopreservation of cultured skin cells. *Burns* **17**: 380–383.

Kempf M, Miyamura Y, Liu PY *et al.* 2011; A denatured collagen microfiber scaffold seeded with human fibroblasts and keratinocytes for skin grafting. *Biomaterials* **32**: 4782–4792.

Kuleshova LL, Tan FC, Magalhaes R *et al.* 2009; Effective cryopreservation of neural stem or progenitor cells without serum or proteins by vitrification. *Cell Transpl* **18**: 135–144.

Kuroyanagi Y, Kenmochi M, Ishihara S *et al.* 1993; A cultured skin substitute composed of fibroblasts and keratinocytes with a collagen matrix: preliminary results of clinical trials. *Ann Plast Surg* **31**: 340–349; discussion, 349–351.

Lamme EN, Van Leeuwen RT, Mekkes JR *et al.* 2002; Allogenic fibroblast in dermal substitute induce inflammation and scar formation. *Wound Repair Regen* **10**: 152–160.

Leibo SP, Mazur P. 1971; The role of cooling rates in low-temperature preservation. *Cryobiology* **8**: 447–452.

Leigh I, Wojnarowska F, Burge S *et al.* 1987; Use of cultured epithelial cells, including keratinocytes, for the detection of antinuclear antibodies. *Ann Rheum Dis* **46**: 929–933.

Liu J, Phy J, Yeomans E. 2012; Theoretic considerations regarding slow cooling and vitrification during cryopreservation. *Theriogenology* **78**: 1641–1652.

Macfadzen I. 1995; *Cryopreservation of* Crassostrea gigas. Humana: Totowa, NJ.

Marchese C, Felici A, Visco V *et al.* 2001; Fibroblast growth factor 10 induces proliferation and differentiation of human primary cultured keratinocytes. *J Invest Dermatol* **116**: 623–628.

May SR, Rudis SK, Rubin CA. 1988; Optimum cooling and warming rate for the cryopreservation of cultured human keratinocytes and mouse L cells. *Cryobiology* **25**: 516.

Mazur P. 1984; Freezing of living cells: mechanisms and implications. *Am J Physiol* **247**: C125–C142.

Meryman HT. 2007; Cryopreservation of living cells: principles and practice. *Transfusion* **47**: 935–945.

Muinos-Lopez E, Rendal-Vazquez ME, Hermida-Gomez T *et al.* 2012; Cryopreservation effect on proliferative and chondrogenic potential of human chondrocytes isolated from superficial and deep cartilage. *Open Orthop J* **6**: 150–159.

Murakawa T, Nakajima J, Ono M *et al.* 2000; Allogenicity of cryopreserved human fibroblasts: cryopreservation does not downregulate the allogenicity of fibroblasts making up the matrices of allografts. *J Thorac Cardiovasc Surg* **120**: 712–719.

Naaldijk Y, Staude M, Fedorova V *et al.* 2012; Effect of different freezing rates during cryopreservation of rat mesenchymal stem cells using combinations of hydroxyethyl starch and dimethylsulfoxide. *BMC Biotechnol* **12**: 49–59.

Owen RE, Sindair E, Emu B *et al.* 2007; Loss of T cell responses following long-term cryopreservation. *J Immunol Methods* **326**: 93–115.

Parenteau NL, Bilbo P, Nolte CJ *et al.* 1992; The organotypic culture of human skin keratinocytes and fibroblasts to achieve form and function. *Cytotechnology* **9**: 163–171.

Pasch J, Schiefer A, Heschel I *et al.* 2000; Variation of the HES concentration for the cryopreservation of keratinocytes in suspensions and in monolayers. *Cryobiology* **41**: 89–96.

Pasch J, Schiefer A, Heschel I *et al.* 1999; Cryopreservation of keratinocytes in a monolayer. *Cryobiology* **39**: 158–168.

Paynter SJ, Fuller BJ. 2007; Cryopreservation of mammalian oocytes. In Cryopreservation and Freeze-drying Protocols, Day JG, Stacey GN (ed.). Humana: Totowa, NJ.

Pegg DE. 2007; Principles of cryopreservation. *Methods Mol Biol* **368**: 39–57.

Phillips TJ, Provan A, Colbert D *et al.* 1993; A randomized single-blind controlled study of cultured epidermal allografts in the treatment of split-thickness skin graft donor sites. *Arch Dermatol* **129**: 879–882.

Reagan BJ, Madden MR, Huo J *et al.* 1997; Analysis of cellular and decellular allogeneic dermal graft for the treatment of full-thickness wounds in a porcine model. *J Trauma* **43**: 458–466.

Rindler V, Pasch J, Heschel I *et al.* 1999; Cryopreservation of suspended keratinocytes with hydroxyethyl starch. *Cryo Lett* **20**: 3–12.

Rodrigues JP, Paraguassu-Braga FH, Carvalho L *et al.* 2008; Evaluation of trehalose and sucrose as cryoprotectants for hematopoietic stem cells of umbilical cord blood. *Cryobiology* **56**: 144–151.

Rosillo MC, Ortuno F, Rivera J *et al.* 1995; Cryopreservation modifies flow-cytometric analysis of hemopoietic cells. *Vox Sang* **68**: 210–214.

Rowley SD, Anderson GL. 1993; Effect of DMSO exposure without cryopreservation on hematopoietic progenitor cells. *Bone Marrow Transpl* **11**: 389–393.

Rowley SD, Feng Z, Chen L *et al.* 2003; A randomized phase III clinical trial of autologous

blood stem cell transplantation comparing cryopreservation using dimethylsulfoxide vs dimethylsulfoxide with hydroxyethyl starch. *Bone Marrow Transpl* **31**: 1043–1051.

Salem AK, Stevens R, Pearson RG *et al.* 2002; Interactions of 3T3 fibroblasts and endothelial cells with defined pore features. *J Biomed Mater Res* **61**: 212–217.

Saraste A, Pulkki K. 2000; Morphologic and biochemical hallmarks of apoptosis. *Cardiovasc Res* **45**: 528–537.

Stiff PJ, Murgo AJ, Zaroulis CG *et al.* 1983; Unfractionated human marrow cell cryopreservation using dimethylsulfoxide and hydroxyethyl starch. *Cryobiology* **20**: 17–24.

Tan FC, Lee KH, Gouk SS *et al.* 2007; Optimization of cryopreservation of stem cells cultured as neurospheres: comparison between vitrification, slow-cooling and rapid cooling freezing protocols. *Cryo Lett* **28**: 445–460.

Tassone G, Fidler SJ. 2012; Separation and cryopreservation of lymphocytes from spleen and lymph node. *Methods Mol Biol* **882**: 351–357.

Teasdale B, Sieber VK, Riches DJ *et al.* 1993; Cryopreservation of cultured dermal fibroblast impregnated collagen gels. *Burns* **19**: 406–410.

Wang X, Hua TC, Sun DW *et al.* 2007; Cryopreservation of tissue-engineered dermal replacement in Me_2SO: toxicity study and effects of concentration and cooling rates on cell viability. *Cryobiology* **55**: 60–65.

Ware CB, Nelson AM, Blau CA. 2005; Controlled-rate freezing of human ES cells. *Biotechnology* **38**: 879–883.

Watt SM, Austin E, Armitage S. 2007; Cryopreservation of Hematopoietic Stem/Progenitor Cells for Theraupertic Use. *Methods Mol Biol* **368**: 237–259.

Werner S, Smola H. 2001; Paracrine regulation of keratinocyte proliferation and differentiation. *Trends Cell Biol* **11**: 143–146.

Xu X, Liu Y, Cui Z *et al.* 2012; Effects of osmotic and cold shock on adherent human mesenchymal stem cells during cryopreservation. *J Biotechnol* **162**: 224–231.

4.4 Cryopreservation of human umbilical cord-derived mesenchymal stem cells in complex sugar based cryoprotective solutions

Journal of Biotechnology Letters
ISSN: 0976-7045 & E-ISSN: 0976-7053, Volume 4, Issue 2, 2013, pp.-95-99.
Available online at http://www.bioinfopublication.org/jouarchive.php?opt=&jouid=BPJ0000250

CRYOPRESERVATION OF HUMAN UMBILICAL CORD-DERIVED MESENCHYMAL STEM CELLS IN COMPLEX SUGAR BASED CRYOPROTECTIVE SOLUTIONS

NAALDIJK Y.[1,2], FEDOROVA V.[1], STOLZING A.[1,2]*

[1]Fraunhofer Institute for Cell Therapy and Immunology, Perlickstrasse 1, 04103 Leipzig, Germany.
[2]Translational Centre for Regenerative Medicine, University of Leipzig, 04103 Leipzig, Germany.
*Corresponding Author: Email- alexandra.stolzing@izi.fraunhofer.de

Received: June 25, 2013; Accepted: July 24, 2013

Abstract- Mesenchymal stem cells (MSCs) are able to differentiate in vivo and in vitro giving rise to different cell types including osteoblasts, adipocytes, chondrocytes and neuronal cells, providing a valuable source for treatment of degenerative and age-associated diseases. Improvement of protocols and procedures for human MSCs cryopreservation will contribute significantly to the development of cell replacement therapies. We developed an alternative cryopreservation solutions for stem cell cryopreservation. Most cryoprotectants need to be removed from the cells by washing after thawing, a procedure that can lead to a loss of precious stem cells. Additionally, the procedure is time and cost-consuming. In our study we used a combination of transfusable and non-toxic substances such as hydroxyethylstarch, sorbitol and dextran replacing DMSO and FCS. We found that a cryosolution containing 5% HES, 0.3M sorbitol and 5% dextran provide successful protection for human umbilical cord derived mesenchymal stem cells. These MSC retain a high viability and show multilineage differentiation.

Keywords- mesenchymal stem cells, DMSO, differentiation, viability, sugar, HES

Citation: Naaldijk Y., Fedorova V., Stolzing A. (2013) Cryopreservation of Human Umbilical Cord-Derived Mesenchymal Stem Cells in Complex Sugar Based Cryoprotective Solutions. Journal of Biotechnology Letters, ISSN: 0976-7045 & E-ISSN: 0976-7053, Volume 4, Issue 2, pp.-95-99.

Introduction

Mesenchymal stem cells (MSCs) have been isolated from a broad range of tissues including bone marrow, peripheral blood, umbilical cord blood and the umbilical cord itself, adipose tissue, periosteum, deciduos teeth, amniotic fluid and membranes and fetal tissues [1,2]. Isolated MSCs from all these sources are able to differentiate towards cell types of the mesodermal lineage. The immune phenotype of MSCs, lacking expression of main histocompartibility complex II and costimulatory molecules, are regarded as immunosuppressive [3]. All these characteristics make MSCs an indispensable source for cell replacement therapy of degenerative age-dependent or trauma-associated conditions [4]. Banking of MSCs provides an opportunity to have controlled MSC batches ready for transplantation. Umbilical cords represent a readily available source of MSCs which is usually discarded directly after birth. The umbilical cord contains MSCs with a higher proliferation capacity compared to bone marrow derived MSCs, especially when isolated from aged donors.

DMSO is the most commonly used cryoprotectant because of its high-membrane permeability. Fetal bovine serum (FBS) is used in combination with DMSO in order to stabilise the cell membrane and to adjust osmotic pressure [5-8]. These two components are the most common cryoprotectants for MSCs, however, both have disadvantages. DMSO is known to cause various side-effects including neurological damage, gastrointestinal effects and mutations, among others [9-12]. Bovine serum is also undesirable as it carries the risk of transmitting viral diseases and it may initiate immune responses; however, the alternative use of autologus serum is time-consuming.

The aim of our study was to find an alternative cryoprotection solution by replacing DMSO and serum in the cryopreservation solution while providing high viability and recovery rate of hMSCs. We choose hydroxyethyl starch (HES), sorbitol and dextran as alternative cryoprotectants as they are already approved for medical use. Former experiments of our group using only HES as a sole cryoprotectant for cryopreservation of rat MSCs were not successful in terms of achieving an acceptable level of cryopreservation efficacy [13]. We therefore sought to improve the proceduere by including additional substances. Sorbitol is an organic osmolyte which has shown protective functions for cryopreservation of red blood cells preventing hyper-and hypo-osmotic cell damage [14]. Polyalcohols have shown to provide successful cryoprotection for oocytes and embryos [15]. Some approved and commonly used polymers such as dextran have been already successfully recruited as alternative cryoprotectants for freezing different cell types [16,17]. We analysed the identity of the isolated MSCs and tested growth characteristics and differentiation potential before and after cryopreservation with different combinations of the cryoprotectants.

Material and Methods

Isolation and Culture of Mesenchymal Stem Cells

MSCs were isolated from umbilical cords (UCs). The material was collected from the Saint Elisabeth hospital using consent forms. UCs were stored in PBS and processed within 8h after delivery. UCs were washed with PBS and transferred to petri dishes, cut into sections of approximately 1cm in diameter. Sections from 1 UC were then transferred to 2-3 culture dishes and left without medium for 10-15 min to attach. After that time, 20ml medium (Dulbecco's modified Eagle's medium (DMEM, 1 g/L D-Glucose; Invitrogen) containing 10% fetal bovine serum (FBS; Hyclone) and 1% penicillin/streptomycin (Invitrogen) was added to each culture dish. Medium was changed every 2 days until outgrowth from the sections were visible. The sections were transferred to a new culture dish. Up to 3 transfers can be performed. Culture dishes with outgrowth were left for ~1 week until expansion of the cells was observed, this step was counted as passage 0. For the cryopreservation experiment MSCs on passage 1 - 3 were used.

Cryopreservation

MSCs were frozen when they reached 80% confluence. Cell number was determined by trypan blue staining using a Neubauer hemocytometer. Final volume of cells with cryoprotectant was 500µl. Each cryovial contained $5x10^5$ MSC. HES with molecular weight (MW) 200 and 450 kDa, sorbitol, mannitol and dextran with MW=5, 450 and 500 kDA were obtained from Serumwerke Bernburg GmBH. All components were diluted in either ringer acetate solution (RAS), Gelafusal or DMEM. Cells were frozen using the programmed rate freezer (Thermo Scientific). Cooling started at 4°C with a cooling rate of 1°C/min until -30°C, then at a rate of 5°C/min until -80°C then the MSCs were transferred into the gas phase of the liquid nitrogen tank. Thawing was performed in the water bath, at 37°C.

Viability Assessment

After thawing, cells were stained with trypan blue and counted with a Neubauer chamber. Percent of viable cell cells was calculated as a ratio of live unstained cells to number of frozen cells ($5x10^5$).

Phenotyping of Mesenchymal Stem Cells

MSCs were stained for CD90 using Alexa488-conjugated AB at a 1:10 dilution (Abcam), CD105 using Alexa647-conjugated AB (Serotec) at a 1:100 dilution, CD44 using AF488-conjugated AB (Abcam) at a 1:100 dilution, CD31 using Cy3-conjugated AB (Abcam) at a 1:50 dilution, CD45 using FITC-conjugated AB (Abcam) at 1:10 dilution and CD11b using FITC-conjugated AB (Abcam) at a 1:10 dilution, for 1 h at 4°C in the dark followed by 3-time washing with PBS. Stained cells were analyzed using FACScalibur flow cytometer (BD).

MTT Assay

The MTT assay was performed 3 days after thawing. Each well was filled with 500µl of media containing the MTT-reagent, consisting of 5mg/ml MTT (Carl Roth) in PBS. After incubation for 4h at 37°C, medium was removed and 500µl stop-solution (10% SDS (Merck) and 50% dimethylformamide, (VWR International)) was added. The cells were incubated overnight at 37°C and absorbance was measured using a microplate reader (TECAN) at 550nm and 630nm as reference wavelength.

Osteogenic Differentiation

The day after thawing, medium was changed to osteo-inductive medium (low Glucose DMEM; 10% FBS; 1% pen/strep; 10nm dexamethasone, (Sigma-Aldrich); 50µg/ml ascorbic acid 2-phosphate, (Sigma-Aldrich). Differentiation media was changed every 2 days for a period of 14 days. For qualitative analysis of osteogenic differentiation, cells were fixed in 70% ethanol for 15 min and washed once with ddH_2O. After washing, cells were stained with ALP buffer pH 8.5 (0.2M Tris, 1mg/ml fast red, Sigma and 50µg/ml naphtol phosphate AS-BI, Sigma) for 1hr.

Adipogenic Differentiation

Adipogenic medium (10% FBS; 1% pen/strep, 10% insulin-transferrin-selenium supplement, (Sigma-Aldrich) 10^{-8}M dexamethasone (Sigma-Aldrich); 0.5mM isobutylmethylxanthin, Sigma-Aldrich; 100µM indomethacin, Sigma-Aldrich) was added the day after thawing. The media was changed every 2 days. After 14 days cell phenotype was analyzed by Oil Red O (Sigma-Aldrich) staining.

Chondrogenic Differentiation

Chondrogenic media (10% FBS; 1% pen/strep 1% insulin-transferrin-selenium supplement (Sigma-Aldrich), 10^{-7}M dexamethasone (Sigma-Aldrich), 150µM ascorbic-2-phosphate (Sigma-Aldrich), 20µM linoic acid (Sigma-Aldrich) and 0.1ng/ml TGF-β (Oncogenic Sciences) was added the day after thawing. After 2 weeks, cells were stained with Alcian Blue (Sigma-Aldrich).

Cell Morphology

Morphology of the cells was analyzed 3 days after thawing under a light microscope. Pictures were taken at 10x magnification with the Leica system.

Statistics

All experiments were repeated at least three times with three different donors. Statistical analysis was performed using ANOVA followed by Tukey test, with $p < 0.05$ considered statistically significant using Sigma Plot.

Results

The MSCs derived from the umbilical tissue showed fibroblast-like morphology [Fig-1B] and were positive for the surface markers CD44, CD90, CD105 and negative for CD11b, CD31, and CD45 [Fig-1A]. Following cultivation in osteo-inductive, chondro-inductive and adipo-inductive medium, cells differentiate into osteoblasts, chondrocytes and adipocytes, respectively proving that they are truly mesenchymal stem cells [Fig-1C], [Fig-1D] and [Fig-1E].

MSCs were frozen with different solutions to identify the best cryosolution. Cell viability was measured on day 0 and day 3. A higher significant cell viability was found with the solutions containing 0.3M sorbitol / 10% dextran in DMEM and 10% DMSO in serum ($p \leq 0.039$) compared to 0.3M sorbitol in DMEM [Fig-2A]. The other solutions tested show no significant differences in cell viability. However significant cell loss is observed on day 3 after thawing for nearly all cryosolutions, showing that cell death occurs during the 24h after thawing. In all later experiments we only used day 3 data [Fig-2B].

The solution that (without DMSO) provided the highest viability contained 5% HES200, 0.3M sorbitol and 10% dextran5 compared to 10% DMSO in DMEM [Fig-2B]. Significances in cell viability at day 3 were determined for 10% DMSO in serum ($+p \leq 0.001$) compared

to solutions containing, DMSO, sorbitol, dextran, mannitol and/or HES in DMEM. The cryosolution of 10% DMSO in DMEM showed significant ($\infty p \leq 0.033$) increase in cell viability when compared to 5% DMSO/mannitol,sorbitol, sorbitol/dextran5 and HES/sorbitol/5% dextran5. In addition, 10% DMSO/10% dextran5, 5% DMSO/5% HES and 5% HES/sorbitol/5% dextran5 showed statistically significant increase in cell viability ($\S p \leq 0.014$) compared to sorbitol and sorbitol/10% dextran 5. Differences were observed between the use of 5 and 10% dextran5 with HES and sorbitol. Dextran5 at 5% concentration ($p = 0.02$) showed a decrease in cell viability compared to 10% dextran5.

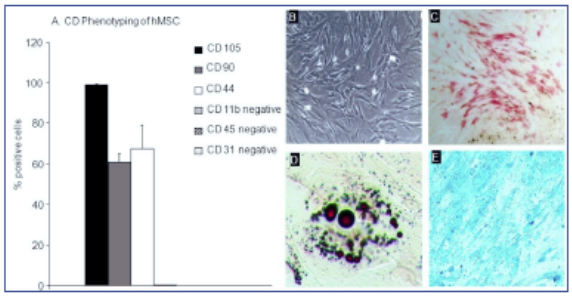


Fig. 1- MSC phenotyping. Characterization of freshly isolated MSCs from human umbillical cord. CD-phenotyping (**A**), Morphology of freshly isolated cells, 10X (**B**) ALP-activity staining in MSCs after osteogenic differentiation, 20X(**C**), oil red staining of differentiated MSC towards adipocyte lineage, 40X(**D**) and alcian blue staining after chondrogenic differentiation 20X (**E**).

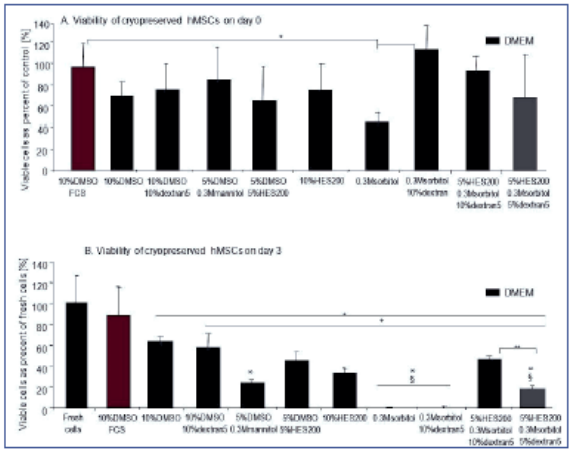


Fig. 2- Viability of cryopreserved hMSCs in comparison to fresh cells. Viability of cryopreserved MSC directly after thawing on day 0 by trypan blue (**A**). Determination of viability of hMSCs at day 3 after thawing using MTT-test (**B**). The following cryoprotectans were used: HES with MW= 200kDa, dextran with MW=5 kDa, in DMEM. * - means statistically significances in comparison to fresh cells.

In the next experiments we tested a smaller dextran (dextran5) versus dextran450 at two different concentrations [Fig-3A] while keeping the beneficial sorbitol and HES200. We found that 10% dextran450 in ring acetate and 5-10% DMSO in ring acetate were significantly better ($^*p < 0.01$) compared to dextran5 and 5% dextran450 [Fig-3A]. Usage of 5% dextran450 in gelafusal and 5-10% DMSO in serum showed a statistical increase ($\infty p \leq 0.038$) in cell viability compared to 10% dextran5. In addition, higher cell viability was observed for 10% DMSO in gelafusal ($+p \leq 0.001$) compared to dextran5 and 10% dextran450 in gelafusal. Cell viability of cryopreserved cells in the different solutions were compared to fresh cells and we found lower cell viability for solutions containing dextran5 ($\S p \leq 0.012$). Similar results in cell viability were obtained for 5% DMSO in ring acetate ($\S p \leq 0.001$) compared to dextran5.

In a further attempt to increase cell viability, we tested if a lower sorbitol concentration, 0.15M, is as effective as 0.3M sorbitol [Fig-3B] and found no significant difference. We therefore used 0.3M sorbitol in subsequent experiments. Comparison of solutions contaiing sorbitol, HES and dextran showed significant decrease in cell viability compared to DMSO in ring acetate [Fig-3B]. In addition, we tested different HES/dextran combinations with different osmolarity and found no difference either, there was however a trend that the HES 200/dextran450 solution was leading to better cell viability [Fig -3B]. Therefore, dextran450 was chosen for further improvements with the goal to replace the medium containing serum and switch to a fully defined buffer system.

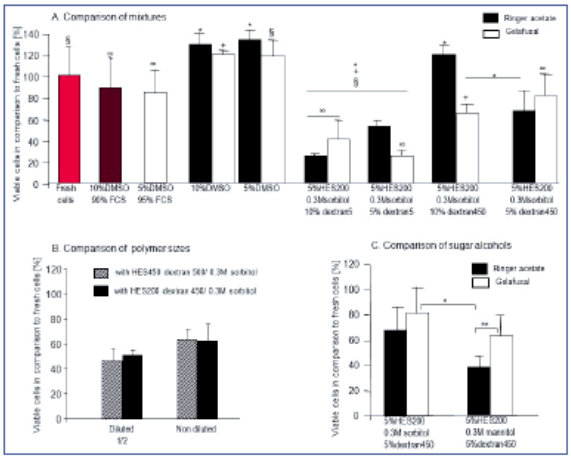


Fig. 3- Cryopreservation of hMSCs in DMSO- and FCS free- cryosolution. Viability of hMSCs at day 3 after cryopreservation in solution containing HES, sorbitol/mannitol and dextran with MW=5 and 450 kDa in Ringer Acetate, Gelafusal or DMEM (**A**). Viability at day 3 of hMSCs after cryopreservation in solutions containing HES200 and dextran450 or HES450 and dextran500 prepared in Ringer acetate solution (**B**). Effect of sorbitol in comparison to mannitol, in combination with HES200 and dextran450 (**C**).

For the next step, we compared a mixture containing HES200 and 0.3M sorbitol concentration with an alternative to sorbitol, mannitol, in two different buffer systems: ringer acetate and gelafusal [Fig-3C]. Solutions containing sorbitol in gelafusal showed higher cell viability ($p = 0.011$) compared to mannitol in ring acetate. Differences in cell viability were determined for mannitol in gelafusal compared to ring acetate. A significant decrease in cell viability was determined for mannitol in ring acetate ($p = 0.05$) compared to gelafusal.

The MSC morphology was not affected in the serum free cryosolutions containing HES200, sorbitol and dextran450 in ring acetate [Fig-4]. In addition we differentiated the MSCs with the HES/sorbitol and dextran solution after cryopreservation and found that the differentiation capacity was preserved [Fig-5].

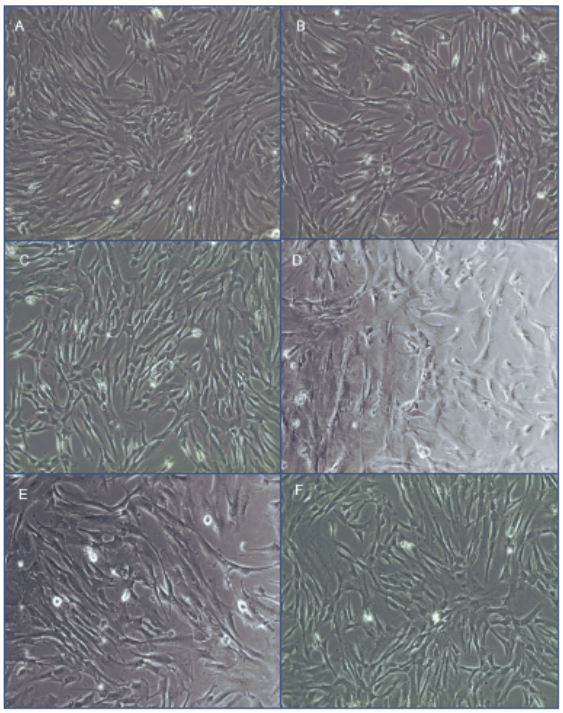


Fig. 4- Morphology of fresh and cryopreserved human MSC. Fresh MSCs at passage 3 (**A**) and MSCs after cryopreservation using the conventional solution with 10%DMSO and 90%FCS (**B**). Morphology of cryopreserved MSCs with cryopreservation with 5%DMSO and 90%FCS (**C**), 10%DMSO and 90% Ringer Acetate solution (D), 5%DMSO and 90%Ringer Acetate solution (**E**) and 5% HES450+0.3M Sorbitol+ 5%Dextran 500 (**F**).

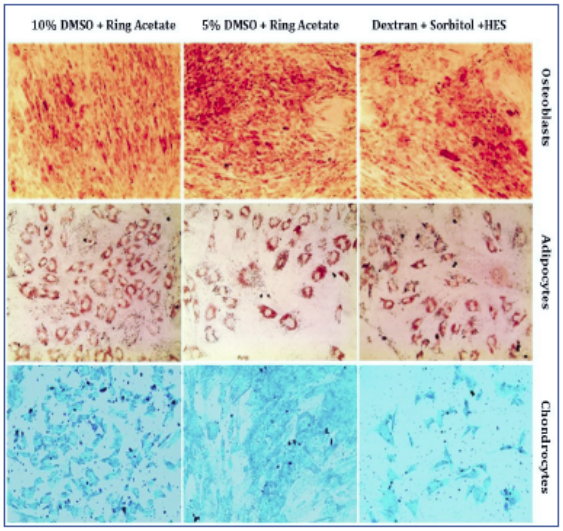


Fig. 5- Determination of differentiation capacity of cryopreserved MSC. Fresh and cryopreserved MSC in the different solutions were differentiated for 14 days into osteoblast, adipocytes and chondrocytes.

Discussion

MSCs are very promising for clinical therapy because of their accessibility, multilineage potentials and immune-suppression activities [18,19]. The placenta and umbilical cord are attractive sources of MSCs. Placentas are discarded at birth, providing an ample tissue source, and in our experience, represent a relatively large reservoir of MSCs, minimising the expansion required to obtain the required numbers of cells for research and possibly clinical applications. Placental MSCs show classical MSC surface phenotype, differentiation potential and potent immunosuppressive properties [20,21].

Many reasons exist to cryopreserve stem cells: multiple cell transplantations, allogeneic applications and gene therapy approaches using stem cells. There is a growing concern about the affect of DMSO and serum in the process of human cell cryopreservation, banking and consequent use of cryopreserved cells in transplantation [22]. Possible complications after transplantation of DMSO-cryopreserved cells represent a serious cause for substitution of these cryoprotectants with less harmful and dangerous components. The present study focuses on the possibility of replacing serum and DMSO in cryopreservation medium for human MSCs.

The solution tested here contains HES, sorbitol and dextran which have been used by other researchers to increase cell cryopreservation efficiency and to reduce the concentration of DMSO in cryosolutions, but in all those experiments these components have always been used together with additional cryopotective agents – usually DMSO, serum or albumin [23-25]. In our study we have shown that neither HES, nor sorbitol or dextran are able to provide efficient cryoprotection when used alone as a single cryoprotectant in serum -free solution even in presence of DMSO [Fig-2B]

We combined these three components, HES, sorbitol and dextran, in order to achieve high cell viability and recovery rates of human MSCs after thawing [Fig-3A], [Fig-3B]. The mechanism by which HES, sorbitol and dextran positively affect cryopreservation efficacy are not clearly understood. HES and dextran both serve as organic osmolytes preventing hyper- and hypo-osmotic cell damage and intracellular ice formation upon the cooling process [26]. Large molecules like HES and dextran increase the viscosity of the solution and decrease the cooling rate required for optimal survival during vitrification, while simultaneously increase the tendency of supercooling and kinetically inhibit ice formation [27,28].

There is no consensus in the literature about the extent of *sorbitol* permeability through *mammalian cell* membranes. According to Alvarez and Storey, sorbitol penetrates the cellular membrane and is therefore considered to be an intracellular cryoprotectant [29]. Other scientists consider the sorbitol molecule to be non-penetrating for the majority of cells under normal conditions [30]. Kracke et al. concluded that there is a specific sorbitol transport pathway, at least in human erythrocytes, similar to the sorbitol permease in renal epithelial cells [31]. As the organic osmolyte, sorbitol prevents osmotic damage in red blood cells [14] and early mammalial embryos [15]. Here we show that sorbitol and mannitol, both being polyols, act in a similar way when utilized in a cryoprotective solution. There is a non-significant tendency towards higher values of cell viability for sorbitol compared to to mannitol. We compared the efficacy of solutions for cryopreservation of MSCs containing HES 200 and Dextran 450 to those containing HES 450 and Dextran 500. The cryopreservation efficiency for MSCs was unaffected by the molecular weight of polymeric HES and Dextran. This obser-

vation may be due to the fact that all compounds tested belong to the high molecular weight polymers and differences in molecular weight do not contribute significantly to their physical properties. Differences in the concentration of HES and dextran, 5 and 10%, similar to the average weight of the polymers, do not have a statistically significant affect on cell viability on day 3.

In the present study we have demonstrated that both DMSO and serum can be replaced using a combination of substances which provide good cell viability after cryopreservation. Human MSCs after cryopreservation using a combination of HES, sorbitol and dextran retain their characteristics and ability to differentiate towards adipogenic, osteogenic and chondrogenic lineages.

Acknowledgments

The work presented on this paper was made possible by fundings from SerumWerk GmbH, the Sächsische-Anhalt Aufbaubank and the German Federal Ministry of Education and Research (BMBF 1315883). We thank Dr. Andreas Schubert for the human cord samples

Conflict of Interest : None Declared

References

[1] Mafi R., Hindocha S., Mafi P., Griffin M. and Khan W.S. (2011) *Open Orthop. J.,* 5(2), 242-248.

[2] Hass R., Kasper C., Bohm S. and Jacobs R. (2011) *Cell Commun. Signal.,* 9, 12.

[3] Abumaree M., Al Jumah M., Pace R.A. and Kalionis B. (2012) *Stem Cell Rev.,* 8, 375-392.

[4] Chamberlain G., Fox J., Ashton B. and Middleton J. (2007) *Stem Cells,* 25, 2739-2749.

[5] Pegg D.E. (2007) *Cryopreservation and Freeze-Drying Protocols*, Humana Press, 39-57.

[6] Lovelock J.E. and Bishop M.W. (1959) *Nature,* 183, 1394-1395.

[7] Ragab A.H., Gilkerson E. and Myers M. (1977) *Cryobiology* 14, 125-134.

[8] Mazur P. (1970) *Science* 168, 939-949.

[9] Sulzberger M.B., Cortese T.A., Fishman L., Wiley H.S. and Peyakovich P.S. (1967) *Ann. N. Y. Acad. Sci.,* 141, 437-450.

[10] Davis J., Rowley S.D. and Santos G.W. (1990) *Prog. Clin. Biol. Res.,* 333, 531-540.

[11] Zambelli A., Poggi G., Da Prada G., Pedrazzoli P., Cuomo A., Miotti D., Perotti C., Preti P. and Robustelli della Cuna G. (1998) *Anticancer Res.,* 18, 4705-4708.

[12] Mueller L.P., Theurich S., Christopeit M., Grothe W., Muetherig A., Weber T., Guenther S. and Behre G. (2007) *Eur. J. Haematol.,* 78, 527-531.

[13] Naaldijk Y., Staude M., Fedorova V. and Stolzing A. (2012) *BMC Biotechnology*, 12, 49.

[14] Moeckel G.W., Shadman R., Fogel J.M. and Sadrzadeh S.M. (2002) *Life Sci.,* 71, 2413-2424.

[15] Kuleshova L.L., MacFarlane D.R., Trounson A.O. and Shaw J.M. (1999) *Cryobiology,* 38, 119-130.

[16] Quan G.B., Han Y., Liu M.X. and Gao F. (2009) *Cryobiology,* 59, 258-267.

[17] Pellerin-Mendes C., Million L., Marchand-Arvier M., Labrude P. and Vigneron C. (1997) *Cryobiology,* 35, 173-186.

[18] Yeo T.P. (2004) *J. Cardiovasc. Nurs.,* 19, 396-403.

[19] Kassem M., Kastrup J., Olsen G. and Johnsen H.E. (2003) *Ugeskr Laeger,* 165, 4852-4855.

[20] Jones B.J., Brooke G., Atkinson K. and McTaggart S.J. (2007) *Placenta,* 28, 1174-1181.

[21] Barlow S., Brooke G., Chatterjee K., Price G., Pelekanos R., Rossetti T., Doody M., Venter D., Pain S., Gilshenan K. and Atkinson K. (2008) *Stem Cells Dev.,* 17, 1095-1107.

[22] Ruiz-Delgado G.J., Mancias-Guerra C., Tamez-Gomez E.L., Rodriguez-Romo L.N., Lopez-Otero A., Hernandez-Arizpe A., Gomez-Almaguer D. and Ruiz-Arguelles G.J. (2009) *Acta Haematol.,* 122, 1-5.

[23] Ashwood-Smith M.J., Warby C., Connor K.W. and Becker G. (1972) *Cryobiology,* 9, 441-449.

[24] Kenmochi T., Asano T., Maruyama M., Saigo K., Akutsu N., Iwashita C., Ohtsuki K., Suzuki A. and Miyazaki M. (2008) *Cell Transplant.,* 17, 61-67.

[25] Maruyama M., Kenmochi T., Sakamoto K., Arita S., Iwashita C. and Kashiwabara H. (2004) *Transplant Proc.,* 36, 1133-1134.

[26] Connor W. and Ashwood-Smith M.J. (1973) *Cryobiology,* 10, 488-496.

[27] Lehle K., Hoenicka M., Jacobs V.R., Schmid F.X. and Birnbaum D.E. (2005) *Cryobiology,* 50, 154-161.

[28] Thomas M.J., Parry E.S., Nash S.G. and Bell S.H. (1996) *Transfus. Sci.,* 17, 385-396.

[29] Alvarez J.G. and Storey B.T. (1993) *J. Androl.,* 14, 199-209.

[30] O'Brien M.M., Schofield P.J. and Edwards M.R. (1983) *Biochem. J.,* 211, 81-90.

[31] Kracke G.R., Preston G.G. and Stanley T.H. (1994) *Am. J. Physiol.,* 266, C343-350.

5. Discussion

The development of an effective cryopreservation protocol without the loss of cell functionality, viability and differentiation is a critical requirement for the application of tissue engineering and cell-based technologies which are required for further clinical applications. In most situations cryopreservation involves the addition of cytotoxic and xenogenous compounds in order to enhance effectiveness. The long-term cryogenic storage of cells and tissue is not very well investigated nor adopted for different products. The use of alternative cryosolutions and the titration of DMSO concentration have been investigated in many different cell types with various degrees of success.
The studies presented in this thesis emphasize the reduction of DMSO from the cryosolution and the use of alternative non-toxic cryoprotectant agents such as sugars and starches for mesenchymal stem cell cryopreservation. In addition, we showed a complete comparison of 3 freezing methods: controlled rate freezing, vitrification and the use of an isopropanol-based freezing container. No data is available where these methods were fully compared for the cryopreservation of fibroblasts and keratinocytes in suspension and as adherent cells, and to some extent for mesenchymal stem cells.

5.1 Sugars and starches as substitute for DMSO in the cryoprotectant solution

5.1.1 DMSO in cryopreservation

DMSO is extensively used as a cryoprotectant agent since favorable cryopreservation is mostly achieved with high standards for cell recovery after thawing. During the cryopreservation process DMSO diffuses into the cells, maintaining osmotic balance and replacing water and reducing the formation of intracellular ice.
The use of DMSO as a cryoprotectant or solvent has negative side-effects at the biological and clinical levels. DMSO can decrease membrane thickening and induces temporary water pore formation when used at lower concentrations. At higher concentrations it induces disintegration of the bilayer structure of the lipid membrane [37]. At the cellular level, DMSO induces changes in morphology, adhesion, growth, cytoskeleton distribution, decreases the number of stress fibers and can cause apoptosis [160-166].
In human embryonic stem cells, exposure to DMSO diminishes stemness and differentiation potential [167, 168] while their cryopreservation with DMSO reduces the expression of Oct-4, a pluripotent marker [169]. Similar results were obtained for human pluripotent stem cells where exposure to DMSO reduces plating efficiency [170].
In the clinical setting, the effect of DMSO in topical application has been tested as well as in transplantation. *In vivo*, DMSO acts as a histamine releaser. Application of DMSO into human skin causes whealing, swelling, erythema, a strong garlic taste in the mouth and pruritus, among other effects [171-173]. Long-term exposure of DMSO affects the cyclic AMP (cAMP) system in epidermal keratinocytes in pig skin dermis [174] and degeneration and disintegration of the epidermis [175].
At the moment skin tissue, red blood cells and hematopoietic progenitor cells are stored using dimethyl sulfoxide, which can cause severe side-effects in patients such as neurological damage, gastroinstestinal problems and loss of consciousness, among others effects [176-179]. Therefore removal of DMSO by washing is usually necessary after thawing

of the cells meaning loss of time and cells which are important as only low amounts of stem cell are available.
Given that DMSO has various adverse effects, it is important to have a DMSO-free cryosolutions for the cryopreservation of cells. As substitutes for DMSO, sugars and starch can be used as cryoprotectants. They are not toxic to cells and can protect the cell membranes thereby providing an alternative or addition to classical cryopreservation solutions. Combinations of sugars and low concentration of DMSO have been shown to be successful in the cryopreservation of mouse oocytes [180], hematopoietic stem cells isolated from umbilical cord [181], and human fetal liver hematopoietic stem/progenitor stem cells [182].
Therefore, reduction or elimination of DMSO from the cryosolution is necessary. Data presented in this dissertation clearly demonstrate that DMSO can be substituted by sugars and starches in the cryopreservation of mesenchymal stem cells as describe in the sections below.

5.1.2 Hydroxyethyl starch in the cryopreservation of mesenchymal stem cells

HES is widely applied in the clinical setting as a plasma volume expander due to its colloidal osmotic pressure to allow delivery of oxygen by red blood cells, in hemodilution treatment to enhance microcirculation, and for peripheral arterial stenosis treatment [97]. Since serious side-effects are not often reported and are less harmful compared to others plasma expander, pruritus (itching) is known to occur after intravenous HES administration. The reason for the development of pruritus is not clear but recent publications suggest that uptake of HES by mononuclear cells, keratinocytes, and Langerhans cells, among other cells, are responsible for the itching effect observed after HES administration, as seen by biopsy specimens and by *in vitro studies* [183-187].
Autologous re-infusion of cryopreserved red blood cells showed that HES is nontoxic and is safe in clinical practice. No adverse reactions after reinfusion of cryopreserved RBC with or without post-thaw washing step were observed.[188].
It was shown that successful non-penetrating cryoprotectors such as Ficoll, PVP and dextran have glass-transition temperature (Tg) around -20°C and 20% HES solution which allows successful cryopreservation of human monocytes also has a Tg close to -20°C [101]. At temperatures lower than -20° C, the formation of glass avoids further osmotic stress and cells are isolated from the extracellular ice reducing water loss by the cells [101].
There are several publications that illustrate the use of HES in cell cryopreservation [146, 150, 189-195]. It is suggested that HES protects by reducing the intracellular ice formation since HES limits crystal growth [99, 100, 196]. HES has been used to preserve keratinocytes [146, 150], human islets [197], red blood cells [191], and human peripheral blood stem cells [195, 198], among others. The addition of HES to the cryoprotectants solution increases the recovery and viability rate after freezing [146]. Moreover, Singbartl et al showed that cryopreservation of human erythrocytes using HES resulted in transient rheological alterations in patients after transplantations [199]. Therefore, like any other cryoprotectants, the effectiveness of HES in cryopreservation might depend on the used freezing protocols, techniques and materials, as well as on cell type**.**

5.1.2.1 HES as an alternative to DMSO in MSC cryopreservation

Since HES is a good candidate and possibly reduction of DMSO concentration from the cryosolution can be possible, we analyzed stemness, differentiation potential and viability

after mesenchymal stem cell cryopreservation with different molecular weight of HES and titration of HES: DMSO ratio.

DMSO was substituted with HES 450 –molecular weight of 450 kDa - showing no negative effect on MSCs CD markers – CD90 and CD44 – and on cell viability (more than 85%) after thawing. In prolonged cultures of cryopreserved MSC, CD phenotype does not change in agreement with previous findings by others [200, 201] but cell viability is dramatically reduced at day 3. The decrease in cell recovery suggests that a penetrating cryoprotectant, e.g. DMSO, is still necessary for survival. HES is known to be a non-penetrating cryoprotectant and there is no evidence whether MSCs are able to uptake HES by pinocytosis like other cell types [187].In addition, a decrease in cell viability can be interpreted as cryo-injury due to formation of intracellular ice crystals during the freezing or thawing process, osmotic changes or apoptosis. Cell death and necrosis associated with cryopreservation after 24 hours of thawing have been shown previously [202-204] which can explain the reduced viability. Given this, determination of cell viability after thawing cannot represent reliable criteria for estimation of cryopreservation efficacy.

The cell viability of cryopreserved MSC was dependent on DMSO:HES 450 concentration. Reduction of DMSO concentration lower than 4% showed detrimental cell recovery since less intracellular cryoprotection is available increasing the likelihood of cellular damage. Ginis et al reported reduced cell viability for cryopreserved BM-MSC with 2% DMSO compared to 5% and 10% DMSO [205].

Analysis of stemness after cryopreservation showed that osteogenic differentiation is DMSO dose-dependent. At higher DMSO concentrations, reciprocal HES 450 concentration decreases, and slight reduction in ostegenic capacity was observed for cryopreserved MSC. DMSO is known to affect differentiation of stem cells by altering the gene expression [169, 170, 206] but some publications have found no differences in osteogenic differentiation after cryopreservation with 5% and 10% DMSO [155, 207, 208], but only for 20% DMSO [208]. In addition, cryopreserved embryonic stem cells with DMSO tend to differentiate immediately after been thawed [209].

Findings, including our own, on stem cells might indicate that stress induced during cryopreservation and/or DMSO toxicity interferes with the differentiation capacity of MSCs. It is know that differentiation of ESC is affected by oxidative stress [210] and impaired differentiation of endothelial progenitor cells was observed after cryopreservation due to an increase in cell death [211]. Therefore, stress induced by cryopreservation seems play an essential role in balancing the survival and differentiation of the cells.

To summarize the findings discussed in this section partial replacement of DMSO by HES 450 for mesenchymal stem cells cryopreservation is admissible. Differentiation potential of MSC is affected by DMSO concentration but the use of solely HES clearly showed an improvement in differentiation potential. Viability is dependent on DMSO since HES by itself cannot sustain cell recovery after cryopreservation.

In conclusion, if viability is required then partial reduction of DMSO is possible but if differentiation potential is the main goal HES alone can be used; however, it will require a higher number of cryopreserved cells to compensate for the cell loss after thawing.

5.1.2.2 Comparison of different HES molecular weight in cryopreservation of MSC

In the cryopreservation field, different molecular weights of HES have been used ranging from 150 to 450 kDa [146, 150, 190, 191, 212] making it difficult to determine whether HES MW has an effect as a cryoprotectant. Different MW ranging from 109 to 609 kDa with the same molar substitution were tested in MSC cryopreservation in absence of DMSO. HES molecular weight does not have an effect on differentiation and cell viability within the different HES MW. Cell viability was lower compared to DMSO indicating that HES alone does not support cryopreservation of MSCs. Our findings contradict Pasch et al which found that cryopreservation of keratinocytes result in viability around 80% for HES alone [146]. We must take in consideration that Pasch findings consist of higher standard deviations indicating great variations within the experiments probably due to HES viscosity or cell death associated to the freezing/thawing process.
In summary, HES molecular weight has no effect on cryopreservation efficiency for mesenchymal stem cells but the question of whether different molar substitutions on HES can decrease or increase the quality of the cryopreserved cells must be investigated.
In conclusion if viability and stemness are both necessary for MSC cryopreservation a solution based on 6:5 (HES:DMSO) can be used. It was shown by others that cryopreservation of bone marrow and stem cells with 6% HES & 5% DMSO provides successful cryopreservation [192, 198, 212, 213]. At 6% HES the viscosity in the extracellular environment of the cell increases and the diffusion capability decreases reducing shrinkage of the cells during the cooling process decreasing ice formation.[96, 100, 101].

5.1.3 Cryosolutions based on dextran, sorbitol and hydroxyethyl starch in the cryopreservation of mesenchymal stem cells

In nature, survival of certain animals such as frogs in cold temperature is based on the accumulation of sugars in their bodies indicating that sugars can act as CPA.
Sugars have a high glass transition temperature compared to DMSO which makes sugars a good candidate for cryopreservation. It was shown that cryopreservation of oocytes [180], cornea endothelial cells [214], and several cancer cell lines, e.g HeLa, HaCaT, and NIH3T3 cells, among others [215] could be improved by using sugars (e.g. glucose, trehalose, d-allose).
Trehalose as a complex sugar is a good candidate in cryopreservation since it provides protection to cell membranes and proteins [69], but efforts have been made to introduce trehalose inside cells [68, 70, 71] making the process time consuming and the results may vary within the experiments. As an alternative to trehalose, glucose is also used as cryoprotectant. Glucose is involved in various biological processes - e.g. glycolysis - which might cause undesired side-effects to the cryopreservation process, such as protein glycations.
Dextran, like HES, is used in the clinical setting as a plasma expander and it is rapidly removed by the kidneys [89, 216]. As a cryoprotectant, dextran is a colloid that is well tolerated in the cryopreservation of organs [217, 218] without increasing the viscosity of the cryosolution [219] and in combination with low-potassium the solution can act as a free radical scavenger [220, 221]. Although, sorbitol is a polyoalcohol which displays good

cryoprotective activity it is usually used in the freeze-drying of proteins [222], lipososomes [223] and chromatins [224] and in the vitrification of mammalian gametes [85]. It is unclear whether sorbitol is a non-penetrating or penetrating cryoprotectant. Two publications argue that sorbitol acts as an intracellular cryoprotectant since is able to penetrate the cell membrane [88] and cells have a sorbitol transport mechanism [225] while others consider sorbitol non-penetrating for most of the cells under normal conditions [87].
Given this, we decided to use the sugars sorbitol and dextran in combination with HES for the cryopreservation of mesenchymal stem cells. Previously, we described the use of HES 450 as cryoprotectant, but for the continuing experiments HES 200 was used. As we demonstrated previously HES molecular weight has no effect as cryoprotectant [226] and HES 200 is commonly used medically as a volume expander [227]. In the context of the study presented in this thesis sorbitol was selected to replace DMSO in the cryosolution. Therefore, a combination of sorbitol, dextran and HES were used as cryoprotectants replacing DMSO and serum from the cryosolution in the cryopreservation of MSCs. Stemness and viability were analyzed after cryopreservation. As basal media, ringer acetate or DMEM was used. Ringer acetate is an electrolyte solution, clinically acceptable, which is employed as a carrier solution or as volume replacement.
Analysis of sorbitol, dextran and HES independently showed no efficient cryopreservation of MSC in a serum-free solution - DMEM. This can be explained by the lack of an intracellular cryoprotectant or serum leading to changes in osmosis, formation of ice and apoptosis. Addition of DMSO to any one of the independent solutions did not increase the cryopreservation efficiency which was not expected due to the effective cryoprotective role of DMSO. Others have been able to enhance cell recovery or reduce DMSO concentration when sorbitol, dextran or HES were used but in combinations with DMSO and/or serum [190, 197, 228]. Serum is known to provide protection during the cryopreservation process but is still a xenogenous component that must be replaced for cell transplantation.
The combination of sorbitol, dextran and HES in DMEM showed viability similar to DMSO in DMEM but still lower compared to instances where serum was used.
Substitution of DMEM by ringer acetate clearly improves cell viability comparable to fresh control. Cryopreserved MSC in sorbitol, dextran and HES in ringer acetate were able to differentiate into osteogenic, adipogenic and chrondrogenic lineages showing that stemness was not affected by the cryopreservation process. The mechanism of action from these substances is not clearly understood. However, HES and dextran are able to reduce intracellular ice and both of them prevent hyperosmotic and hyposmotic damage into the cells [229].
Furthermore, electrolyte solutions, e.g. ring acetate, can act as a substitute for cell culture media [230, 231]. DMEM as basal media for cryosolution contains several components such as amino acids, inorganic salts, vitamins and glucose which can be beneficial or detrimental for cryopreservation. Previous findings show that addition of amino acids to the cryoprotectant improved post-thaw motility and velocity of cryopreserved sperm [232-234] but at higher concentration increases the osmotic pressure [233]. DMEM uses have not been applied for therapeutic purposes and no study on human safety is available [235].
To summarize these findings we demonstrated that DMSO, serum and DMEM can be successfully replaced by non-toxic and clinically approved solutions without interfering with the cellular functions and minimizing cell loss. A new cryosolution formulation based on sorbitol, dextran and HES in Ringer Acetate for the cryopreservation of MSC was developed.

5.2 The effect of cooling rates in the cryopreservation of cells

During cryopreservation various factors are involved than can interfere with the proper cryopreservation of cells, one of them being the cooling rate of the sample. Various methods are available in the cryopreservation field where the temperature of cooling is defined to prevent and reduce intracellular ice formation such as controlled rate freezing, vitrification and the conventional method. The different methods must be optimized for the different cell types, tissues or organs to be cryopreserved since the same cooling rate cannot be applied for the different purposes.

Controlled rate freezing allows us to manipulate and to control the temperature in which cells can be cryopreserved in a consistent way. It is known that slow freezing results in a higher cellular damage since an increase in electrolytes to a toxic level will be present as ice is formed extracellularly [236], and dehydration of the cells can also be observed [237]. Rapid freezing – vitrification - is suggested as a better choice for cryopreservation since intracellular water is retained preventing dehydration and freezing will occur intracellular and extracellular [2]. Additionally, the conventional method, known as Mr. Frosty Nalgene container, provides a constant freezing rate of 1°C/min.

5.2.1 Cooling rate for Mesenchymal Stem Cells

Mesenchymal stem cells were cryopreserved with 7 different freezing protocols: 6 protocols from a controlled rate freezing machine and one protocol for “vitrification” which is directly placing cryovials into a vapour phase nitrogen tank. Comparison of the cooling effect between the different freezing protocols was performed by analysing cell viability after thawing and differentiation potential.

Cell viability was analysed at day 0 and 3 after thawing. At day 0 cryopreserved MSC showed more than 80% cell recovery for all the protocols tested. Determination of cell viability at day 0 is very common in cryopreservation but it does not provide with the proper estimation of cell recovery for optimal cryopreservation efficiency as described in the previous section. Therefore, is crucial to analyse cell recovery over a period of time in culture.

The cryopreserved MSC were allowed to recover for 3 days and cell viability was dependent of the cooling rate. The cell viability decreased compared to day 0 indicating that during the recovery period of 3 days cell loss was evident. The decrease in cell number can be explained due to apoptosis or necrosis events which have been reported to occur after 6 hours of cells being thawed [139, 202, 238, 239]. A freezing protocol consisting of a constant cooling rate of 1°C/min until -30°C followed by 5°C/min until -80°C and protocols with a fast cooling rate (> 99°C/min) are the most suitable for the cryopreservation of MSC. Our results are consistent with findings by others in which cryopreservation of MSC were performed with slow cooling rates or directly into the liquid nitrogen tank [154, 157, 226] but full comparison between the different cryopreservation methods were not performed.

A crucial range during cooling where nucleation and crystal ice growth exists is between 0°C to -40°C. Cooling of cells to -40°C without formation of intracellular ice can be accomplished only with cooling rates below 10°C/min [11]. To overcome this strict temperature range the use of high cooling rate (> 99°C/min) may well avoid

intracellular ice formation. However, a low cooling rate, which is found in most cases suggested for proper cryopreservation, allows for partial cell dehydration which can also prevent intracellular ice formation [240].
In summary, cooling rate has an effect on cell recovery and differentiation of cryopreserved MSC. Ice nucleation and intracellular ice formation are both dependent on the cooling rate showing the importance of determining the proper freezing rate and release of heat from the cryosolution.
In the studies presented here we concluded that slow (0.3°C - 1°C/min to -30°C) being below -30°C the critical point and rapid cooling (> 99°C/min) can be used to preserve viability and stemness of MSC.

5.2.2 Cooling rates for fibroblasts and keratinocytes cryopreservation in suspension and adherent

Reports available on the cryopreservation of fibroblasts and keratinocytes have not shown comparison between the freezing methods and cooling rates for the cryopreservation of these cells in suspension and as adherent cells. Therefore, fibroblasts and keratinocytes were cryopreserved in suspension and as adherents with 8 different freezing protocols: 6 of them controlled-rate freezing, directly into vapor phase of liquid nitrogen and by the conventional method.
We found that viability at day 0 for the cryopreservation of human fibroblast cells and keratinocytes was dependent of the freezing protocol, slow cooling or placing them directly into the nitrogen tank being the most suitable. Given that viability at day 0 is not a reliable for determining proper cryopreservation, cell recovery was determined at day 3 after thawing. Keratinocytes showed higher cell viability with conventional method followed by controlled rate freezing. An enhancement in cell proliferation was observed for some freezing protocols. Increases in cell numbers have been reported for heart cells and chondrocytes after cryopreservation [241, 242]. During the stressful process of cryopreservation alteration within the cell might occur in which insusceptible populations of keratinocytes cells were selected during the cryopreservation process and cultivation period. Liu et al observed a temporary up-regulation of stress proteins, heat shock protein and growth factors after thawing thereby demonstrating the activation of stress response and inducers of proliferative capacity [120]. In our studies, keratinocytes cells were able to be cryopreserved in suspension with a cooling rate more than 100°C/min. Similar efforts were performed by others with no success of recovery [111, 149].
Furthermore, keratinocytes cryopreserved as adherent cells showed higher cell viability at day 3 with slow cooling (1°C/min). The use of fast cooling (> 100°C/min) is possible but cell recovery is lower. Previous attempts to cryopreserve keratinocytes as adherent cells showed that viability is dependent on cooling rate: 3°C/min > 1°C/min > 10°C/min [150]. Ji demonstrated cryopreservation of adherent human embryonic stem cells [209], another cell type that resemble keratinocytes in cell size, but not effort has been made to determine whether “vitrification” is possible for keratinocyte adherent cultures.
The results clearly demonstrated differences in the cryopreservation of keratinocyte cells in suspension and as adherent cells which have never been clearly demonstrated before.
In the studies presented here we compared cryopreservation of a fibroblast cell line versus primary fibroblasts culture. Viability determined at day 0 (directly after thawing) was protocol dependent for fibroblasts cells. The fibroblast cell line preferred slow cooling compared to

primary fibroblasts where slow and rapid cooling resulted in higher viability. Analysis of cell recover at day 3 showed that cooling rate of 0.5°C/min, conventional method and vitrification (straight freeze) for primary fibroblasts resulted in higher cell number compared to the fibroblast cell line. Cryopreservation of fibroblasts cells using slow cooling rates < 1°C/min have been accomplished by others but the use of vitrification showed no success [111, 138]. The results obtained showed differences in cryopreservation of primary fibroblasts and fibroblasts cell line in suspension. A possible explanation for the differences is that a cell line is an immortalized cell that has an unlimited life span and growth capacity therefore they can behave distinctively than primary fibroblasts.

Fibroblasts cryopreserved as adherent cells showed unexpected results. The proliferative capacity after thawing was greatly diminished for both primary fibroblasts and fibroblast cell lines. In some protocols cell detachment or deterioration was observed during the period of 3 days in culture. Available data showed that cells preserved as adherent cells showed loss of metabolic activities and membrane integrity [243]. In addition, preservation of endothelial cells as adherent cells seems to be dependent on the cooling temperature in which at -196°C cell detachment and loss of membrane integrity was observed as compared to -80°C [244]. Cryopreservation of cells as adherent cells is still in development and more studies are necessary to determine the suitable cell culture plate and freezing protocols.

In the freezing protocols used in this study, 2 protocols share a cooling rate of 1°C/min but the way in which this rate is accomplished differs. One protocol is based on the use of a controlled rate freezing machine while the other uses an isopropanol-based container named Mr Frosty. While we expected to obtain the same results for the cryopreservation of keratinocytes and fibroblasts differences were observed. Keratinocyte cells showed better cryopreservation with controlled rate freezing while fibroblasts preferred the use of the Mr. Frosty container demonstrating dissimilarity between the cell types. One can argue that the use of controlled rate freezing machine provides a more accurate and precise cooling than a freezing container which has as a constraint the “accuracy” temperature of a -80°C freezer. At the moment no one has reported whether variations within experiments are possible with controlled rate freezing machine since the cooling rate can be dependent on the sample or the machine. Researchers rely on records from the freezing programs always being available and that minimal variations can easily be noticed and interpreted.

I suggest that fibroblast cells and keratinocytes can be cryopreserved using a slow freezing protocol for cryovials and that only keratinocytes be cryopreserved as adherent cells. Usage of slow freezing protocols have been shown to be beneficial for the storage of myoblasts [245], human embryonic stem cells [246], and cells derived from hematopoietic origins [247], among others.

To summarize the findings the use of the Nalgene container Mr. Frosty and vitrification are the most convenient and cost-effective methods for the cryopreservation of fibroblasts and keratinocytes either in suspension or as adherent cells. Cryopreservation protocols cannot be applicable to all cell types as seen for primary fibroblasts and fibroblast cell lines, indicating that cooling rates are cell-dependent. In addition, we accomplished to cryopreserve fibroblasts and keratinocytes as adherent cells which can be further translated in the field of skin tissue engineering.

6. Summary

Fibroblasts, keratinocytes and mesenchymal stem cells are a useful tool in the field of regenerative medicine and tissue engineering due to their ability of being functional cell types of mesodermal lineage. Storing and banking of these cells can provide every potential patient with a source of allogeneic or autologous cells and tissues which are an important factor for cell replacement therapies and tissue production.
The most effective way to preserve cells and tissues is by cryopreservation. It usually involves the use of compounds that can be cytotoxic (e.g. DMSO) or xenogeneous (e.g. serum). The perfect cryoprotectant agent is non-toxic, non-antigenic, chemically inert and provides a high viability rate. Given that, sugars and starches which are often used as natural cryoprotectors can be used as a cryoprotectant agent, thereby minimizing the adverse effects due to the presence of DMSO and/or serum in the cryosolution.
Here I showed the development of a novel DMSO- and serum-free cryosolution for the cryopreservation of stem cells based on HES, sorbitol and dextran in Ringer Acetate. MSC differentiation potential and viability were not affected after cryopreservation demonstrating that these cells are still functional and that transplantation into the patient without negative effects and washing steps is hypothetically possible.
Furthermore, several attempts in the cryopreservation field to find the perfect cooling rate and method are still in process. Full comparison of different freezing rates and methods were performed in this thesis for MSC, fibroblasts and keratinocyte cryopreservation. The three cell types can be cryopreserved with slow cooling and vitrification. The use of a controlled rate freezing machine which is expensive and time consuming is unnecessary. Cooling rate can affect cell viability and differentiation when cryo-injury is allowed to occur thereby affecting cell recovery after thawing. There is not one common freezing rate that can be applicable for the cryopreservation of MSC, fibroblasts and keratinocytes cells as observed in these studies.
Furthermore, I clearly demonstrated that primary and cell line fibroblasts, being both the same cell type, differ in cryopreservation efficiency in suspension and as adherent cells, and being this thesis the first report to show that freezing protocols cannot be applicable for primary cells and cell lines.

Zusammenfassung

Fibroblasten, Keratinozyten und mesenchymale Stammzellen (MSC) sind Zellen mesenchymalen Ursprungs und stellen ein nützliches Werkzeug im Bereich der regenerativen Medizin und *Tissue engineering* (Gewebekonstruktion). Die Langerung dieser Zellen würde für potentielle Patienten eine Quelle für allogene oder autologe Zellen und Gewebe darstellen, dies wäre ein wichtiger Faktor für Zelltherapien und die Herstellung von Gewebe. Der effektivste weg, um Zellen und Gewebe zu konservieren, ist die

Kryokonservierung. Ein großer Nachteil ist, dass die verwendeten Komponenten zur Kryokonservierung, zytotoxisch (z.B. DMSO) oder xenogen (z.B. Serum) sind.
Die perfekten Kryoportektaten darstellen, zur Kryokonservierung genutzt werden und dadurch den schädlichen Effekt durch die Verwendung von DMSO und/or Serum in der Kryolösung verringern.
MSC sind empfindlicher gegenüber der Kryolösung als vergleichsweise Fibroblasten und Keratinozyten; eine Beeinträchtigung der Stammzelleingenschaften der MSC führt zu einer Verringerung des Differenzierungspotentials der MSC und der Expression der Marker. Aus diesem Grund wird in der vorliegenden Arbeit die Entwicklung einer neuen, DMSO- und Serumfreien Kryolösung auf der Basis von HES, Sorbitol und Dextran in der Ringer-Lösung die Kryokonservierung von MSC beschrieben. Das Differenzierungspotential und die Vitalität von MSC wurden nicht beeinflusst durch die Kryokonservierung. Dies zeigt, dass diesen Zellen immer noch funktionell sind und eine Transplantation in den Patienten wäre ohne negative Effekte und Waschschritte möglich.
Darüber hinaus werden derzeit in der Kryokonservierung verschiedene Ansätze erforscht, um die perfekte Kühlrate und Methoden zu etablieren. Es wurden in dieser Arbeit verschiedene Kühlraten und Methoden für die Kryokonservierung von MSC, Fibroblasten und Keratinozyten miteinander verglichen. Diese Zelltypen können mit langsamer Kühlrate (1°C/min) und Vitrifikation (>100°C/min) kryokonserviert werden. Die Verwendung von Maschine mit kontrollierter Einfrierrate, die teuer und aufwendig sind, erwies sich als unnötig. Die Einfrierrate wirkt sich auf die Erholung von MSC, Fibroblasten und Keratinozyten über einen längeren Kultivierungszeitraum aus. Zusätzlich wurde eine Verbesserung der Proliferationskapazität von Keratinozyten beobachtet. Das Differenzierungspotential von MSC war abhängig von der Einfrierrate. Diese Studien zeigen, dass es keine allgemein anwendbare Einfrierrate für MSC, Fibroblasten und Keratinozyten gibt.
Zusätlich konnte ich zeigen, dass sich die Effizienz bei der Kryokonservierung von primäre und sekundäre (Zelllinie) Fibroblasten in Suspension und als anhaftenden unterscheidet, obgleich beide den gleichen Zelltyp darstellen. In dieser Arbeit wurde damit zum ersten Mal gezeigt, dass die Protokolle zur Kryokonservierung für primäre Zellen und sekundäre Zellen nicht gleichermaßen anwendbar sind.

7. References:

1. Polge C, Smith AU, Parkes AS. Revival of spermatozoa after vitrification and dehydration at low temperatures. **Nature**. 1949;164:666.
2. Fowler A, Toner M. Cryo-injury and biopreservation. **Annals of the New York Academy of Sciences**. 2005;1066:119-135.
3. Mazur P, Leibo SP, Chu EH. A two-factor hypothesis of freezing injury. Evidence from Chinese hamster tissue-culture cells. **Experimental cell research**. 1972;71:345-355.
4. Muldrew K AJ, Elliot J AW, McGann LE. The Water to Ice Transition: Implications for Living Cells. In: Fuller B.J. LN, Benson E.E., ed. Life in the Frozen State: CRC Press; 2004:67-103.
5. Mazur P. Freezing of living cells: mechanisms and implications. **The American journal of physiology**. 1984;247:C125-142.
6. Baust JG, Gao D, Baust JM. Cryopreservation: An emerging paradigm change. **Organogenesis**. 2009;5:90-96.
7. Oezkavucku S EE. Cryopreservation: Basic Knowledge and Biophysical Effects. **J of Ankara Medical School**. 2002;24:187-196.
8. Irimia D. KJOM. Kinetics and mechanism of intercellular ice propagation in a micropatterned tissue construct. **Biophys**. 2002;82:1858-1868.
9. Acker JP, Larese A, Yang H et al. Intracellular ice formation is affected by cell interactions. **Cryobiology**. 1999;38:363-371.
10. Berger WK, Uhrik B. Freeze-induced shrinkage of individual cells and cell-to-cell propagation of intracellular ice in cell chains from salivary glands. **Experientia**. 1996;52:843-850.
11. Karlsson JO, Cravalho EG, Borel Rinkes IH et al. Nucleation and growth of ice crystals inside cultured hepatocytes during freezing in the presence of dimethyl sulfoxide. **Biophysical journal**. 1993;65:2524-2536.
12. Mazur P. The role of cell membranes in the freezing of yeast and other single cells. **Annals of the New York Academy of Sciences**. 1965;125:658-676.
13. Arav A, Zeron Y, Leslie SB et al. Phase transition temperature and chilling sensitivity of bovine oocytes. **Cryobiology**. 1996;33:589-599.
14. Lyons JM RJ. A temperature-induced transition in mitochondria oxidation: Contrast between cold and warm blooded animals. **Comp Biochem Physiol**. 1970;37:405-411.
15. Raison JK. The influence of temperature-induced phase changes on the kinetics of respiratory and other membrane-associated enzyme systems. **Journal of bioenergetics**. 1973;4:285-309.
16. Ricciutti MA. Lysosomes and myocardial cellular injury. **The American journal of cardiology**. 1972;30:498-502.
17. Zeron Y, Pearl M, Borochov A et al. Kinetic and temporal factors influence chilling injury to germinal vesicle and mature bovine oocytes. **Cryobiology**. 1999;38:35-42.
18. Clarke DM, Baust JM, Van Buskirk RG et al. Addition of anticancer agents enhances freezing-induced prostate cancer cell death: implications of mitochondrial involvement. **Cryobiology**. 2004;49:45-61.
19. Jennings RB, Ganote CE. Structural changes in myocardium during acute ischemia. **Circulation research**. 1974;35 Suppl 3:156-172.
20. Jennings RB, Ganote CE. Mitochondrial structure and function in acute myocardial ischemic injury. **Circulation research**. 1976;38:I80-91.

21. Liu X, Engelman RM, Rousou, JA, Das, DK. Myocardial reperfusion injury during adult cardiac surgery. Boca Raton: CRC Press; 1993.
22. Weglicki WB, Owens K, Ruth RC et al. Activity of endogenous myocardial lipases during incubation at acid pH. **Cardiovascular research**. 1974;8:237-242.
23. White BC, Grossman LI, Krause GS. Brain injury by global ischemia and reperfusion: a theoretical perspective on membrane damage and repair. **Neurology**. 1993;43:1656-1665.
24. Brinkley BR, Cartwright J, Jr. Cold-labile and cold-stable microtubules in the mitotic spindle of mammalian cells. **Annals of the New York Academy of Sciences**. 1975;253:428-439.
25. Overstrom EW P-PD, Toner M and Cravacho EG. Cryoprotectant and thermal effect on cytoskeletal organization and IVF rate of mouse oocytes. **Biol Reprod**. 1990;42 175 abstr.
26. Russotti G, Brieva TA, Toner M et al. Induction of tolerance to hypothermia by previous heat shock using human fibroblasts in culture. **Cryobiology**. 1996;33:567-580.
27. Stefanovich P, Ezzell RM, Sheehan SJ et al. Effects of hypothermia on the function, membrane integrity, and cytoskeletal structure of hepatocytes. **Cryobiology**. 1995;32:389-403.
28. Taylor MJ, Song, Y.C. and Brockbank, K. G. M. Vitrification in Tissue Preservation: New Developments. In: Benson E, Fuller, B. and Lane, N, ed. Life in Frozen State. London: CRC Press LLC; 2004:603-641.
29. Kuwayama M. Highly efficient vitrification for cryopreservation of human oocytes and embryos: the Cryotop method. **Theriogenology**. 2007;67:73-80.
30. Kuwayama M, Vajta G, Kato O et al. Highly efficient vitrification method for cryopreservation of human oocytes. **Reproductive biomedicine online**. 2005;11:300-308.
31. Fan WX, Ma XH, Liu TQ et al. Vitrification of corneal endothelial cells in a monolayer. **J Biosci Bioeng**. 2008;106:610-613.
32. Rall WF, Fahy GM. Ice-free cryopreservation of mouse embryos at -196 degrees C by vitrification. **Nature**. 1985;313:573-575.
33. Meryman HT. Cryopreservation of living cells: principles and practice. **Transfusion**. 2007;47:935-945.
34. Butler SaP, S. Precision in Cryopreservation - Equipment and Control: InTech; 2012.
35. Pegg DE. Principles of cryopreservation. **Methods in molecular biology**. 2007;368:39-57.
36. Song YC, Khirabadi BS, Lightfoot F et al. Vitreous cryopreservation maintains the function of vascular grafts. **Nature biotechnology**. 2000;18:296-299.
37. Gurtovenko AA, Anwar J. Modulating the structure and properties of cell membranes: the molecular mechanism of action of dimethyl sulfoxide. **The journal of physical chemistry B**. 2007;111:10453-10460.
38. Leekumjorn S, Sum AK. Molecular study of the diffusional process of DMSO in double lipid bilayers. **Biochimica et biophysica acta**. 2006;1758:1751-1758.
39. Lovelock JE, Bishop MW. Prevention of freezing damage to living cells by dimethyl sulphoxide. **Nature**. 1959;183:1394-1395.
40. Buchanan SS, Gross SA, Acker JP et al. Cryopreservation of stem cells using trehalose: evaluation of the method using a human hematopoietic cell line. **Stem cells and development**. 2004;13:295-305.

41. Anchordoguy TJ, Cecchini CA, Crowe JH et al. Insights into the cryoprotective mechanism of dimethyl sulfoxide for phospholipid bilayers. **Cryobiology**. 1991;28:467-473.
42. Arakawa T, Kita Y, Timasheff SN. Protein precipitation and denaturation by dimethyl sulfoxide. **Biophysical chemistry**. 2007;131:62-70.
43. Arakawa T CJ, Kita YA, Crowe JH. The basis for toxicity of certain cryoprotectants: A hypothesis. **Cryobiology**. 1990;27:401-415.
44. Ahkong QF, Fisher D, Tampion W et al. Mechanisms of cell fusion. **Nature**. 1975;253:194-195.
45. Jacob SW, Herschler R. Pharmacology of DMSO. **Cryobiology**. 1986;23:14-27.
46. Lyman GH, Preisler HD, Papahadjopoulos D. Membrane action of DMSO and other chemical inducers of Friend leukaemic cell differentiation. **Nature**. 1976;262:361-363.
47. Mazur P. Cryobiology: the freezing of biological systems. **Science**. 1970;168:939-949.
48. Ragab AH, Gilkerson E, Myers M. Factors in the cryopreservation of bone marrow cells from children with acute lymphocytic leukemia. **Cryobiology**. 1977;14:125-134.
49. Shahdadfar A, Fronsdal K, Haug T et al. In vitro expansion of human mesenchymal stem cells: choice of serum is a determinant of cell proliferation, differentiation, gene expression, and transcriptome stability. **Stem cells**. 2005;23:1357-1366.
50. NA M. **Berichte der Deutschen botanischen Gesellschaft**. 1912;30.
51. Storey KB. Life in a frozen state: adaptive strategies for natural freeze tolerance in amphibians and reptiles. **The American journal of physiology**. 1990;258:R559-568.
52. Z H. Protectants used in the cryopreservation of microorganism. **Cryobiology**. 2003;46:205-229.
53. Crowe JH, Crowe LM, Chapman D. Preservation of membranes in anhydrobiotic organisms: the role of trehalose. **Science**. 1984;223:701-703.
54. Fernandez O, Bethencourt L, Quero A et al. Trehalose and plant stress responses: friend or foe? **Trends in plant science**. 2010;15:409-417.
55. Crowe LM, Crowe JH. Anhydrobiosis: a strategy for survival. **Advances in space research : the official journal of the Committee on Space Research**. 1992;12:239-247.
56. Potts M. Desiccation tolerance of prokaryotes. **Microbiological reviews**. 1994;58:755-805.
57. Crowe JH, Leslie SB, Crowe LM. Is vitrification sufficient to preserve liposomes during freeze-drying? **Cryobiology**. 1994;31:355-366.
58. Leslie SB, Israeli E, Lighthart B et al. Trehalose and sucrose protect both membranes and proteins in intact bacteria during drying. **Applied and environmental microbiology**. 1995;61:3592-3597.
59. Crowe JH, Crowe LM, Carpenter JF et al. Stabilization of dry phospholipid bilayers and proteins by sugars. **The Biochemical journal**. 1987;242:1-10.
60. Crowe JH, Crowe LM, Carpenter JF et al. Interactions of sugars with membranes. **Biochimica et biophysica acta**. 1988;947:367-384.
61. Chen T, Fowler A, Toner M. Literature review: supplemented phase diagram of the trehalose-water binary mixture. **Cryobiology**. 2000;40:277-282.
62. Levine H SL. Water as a plasticizer: physico-chemical aspects of low-moisture

polymeric systems. Cambridge, UK: Cambridge Unversity Press; 1988.
63. Campbell LH, Brockbank, K.G.M. Cryopreservation of Adherent Smooth Muscle and Endothelial Cells with Disaccharides. In: Katvok PI, ed. Current Frontiers in Cryopreservation: InTech; 2012:17-36.
64. Jiang L, Shen C, Dai J et al. Di-rhamnolipids improve effect of trehalose on both hypothermic preservation and cryopreservation of rat hepatocytes. **Applied microbiology and biotechnology**. 2013;97:4553-4561.
65. Lerbret A, Bordat P, Affouard F et al. How homogeneous are the trehalose, maltose, and sucrose water solutions? An insight from molecular dynamics simulations. **The journal of physical chemistry B**. 2005;109:11046-11057.
66. Crowe JH CL. Lyotropic effects of water on phospholipids. Cambridge, England: Cambridge University Press; 1990.
67. Rudolph AS, Crowe JH. Membrane stabilization during freezing: the role of two natural cryoprotectants, trehalose and proline. **Cryobiology**. 1985;22:367-377.
68. Campbell LH, Brockbank KG. Comparison of electroporation and Chariot for delivery of beta-galactosidase into mammalian cells: strategies to use trehalose in cell preservation. **In vitro cellular & developmental biology Animal**. 2011;47:195-199.
69. Eroglu A, Russo MJ, Bieganski R et al. Intracellular trehalose improves the survival of cryopreserved mammalian cells. **Nature biotechnology**. 2000;18:163-167.
70. Eroglu A, Toner M, Toth TL. Beneficial effect of microinjected trehalose on the cryosurvival of human oocytes. **Fertility and sterility**. 2002;77:152-158.
71. Holovati JL, Gyongyossy-Issa MI, Acker JP. Effects of trehalose-loaded liposomes on red blood cell response to freezing and post-thaw membrane quality. **Cryobiology**. 2009;58:75-83.
72. Balduini CL, Mazzucco M, Sinigaglia F et al. Cryopreservation of human platelets using dimethyl sulfoxide and glycerol-glucose: effects on "in vitro" platelet function. **Haematologica**. 1993;78:101-104.
73. Hunt CJ. Cryopreservation of Human Stem Cells for Clinical Application: A Review. **Transfusion medicine and hemotherapy : offizielles Organ der Deutschen Gesellschaft fur Transfusionsmedizin und Immunhamatologie**. 2011;38:107-123.
74. Kotelba-Witkowska B, Schiffer CA. Cryopreservation of platelet concentrates using glycerol-glucose. **Transfusion**. 1982;22:121-124.
75. Quan G, Zhang L, Guo Y et al. Intracellular sugars improve survival of human red blood cells cryopreserved at -80 degrees C in the presence of polyvinyl pyrrolidone and human serum albumin. **Cryo letters**. 2007;28:95-108.
76. Storey KB, Mommsen TP. Effects of temperature and freezing on hepatocytes isolated from a freeze-tolerant frog. **The American journal of physiology**. 1994;266:R1477-1482.
77. King PA, Rosholt MN, Storey KB. Adaptations of plasma membrane glucose transport facilitate cryoprotectant distribution in freeze-tolerant frogs. **The American journal of physiology**. 1993;265:R1036-1042.
78. Suzuki T, Komatsu H, Miyajima K. Effects of glucose and its oligomers on the stability of freeze-dried liposomes. **Biochimica et biophysica acta**. 1996;1278:176-182.
79. Plachinta M, Zhang T, Rawson DM. Studies on cryoprotectant toxicity to zebrafish (Danio rerio) oocytes. **Cryo letters**. 2004;25:415-424.

80. Bunn HF, Higgins PJ. Reaction of monosaccharides with proteins: possible evolutionary significance. **Science**. 1981;213:222-224.
81. Guimaraes EL, Empsen C, Geerts A et al. Advanced glycation end products induce production of reactive oxygen species via the activation of NADPH oxidase in murine hepatic stellate cells. **Journal of hepatology**. 2010;52:389-397.
82. Morita M, Yano S, Yamaguchi T et al. Advanced glycation end products-induced reactive oxygen species generation is partly through NF-kappa B activation in human aortic endothelial cells. **Journal of diabetes and its complications**. 2013;27:11-15.
83. Rosenthal AK, Gohr CM, Mitton E et al. Advanced glycation end products increase transglutaminase activity in primary porcine tenocytes. **Journal of investigative medicine : the official publication of the American Federation for Clinical Research**. 2009;57:460-466.
84. Shimizu F, Sano Y, Haruki H et al. Advanced glycation end-products induce basement membrane hypertrophy in endoneurial microvessels and disrupt the blood-nerve barrier by stimulating the release of TGF-beta and vascular endothelial growth factor (VEGF) by pericytes. **Diabetologia**. 2011;54:1517-1526.
85. Kuleshova LL, MacFarlane DR, Trounson AO et al. Sugars exert a major influence on the vitrification properties of ethylene glycol-based solutions and have low toxicity to embryos and oocytes. **Cryobiology**. 1999;38:119-130.
86. Moeckel GW, Shadman R, Fogel JM et al. Organic osmolytes betaine, sorbitol and inositol are potent inhibitors of erythrocyte membrane ATPases. **Life Sci**. 2002;71:2413-2424.
87. O'Brien MM, Schofield PJ, Edwards MR. Polyol-pathway enzymes of human brain. Partial purification and properties of sorbitol dehydrogenase. **Biochem J**. 1983;211:81-90.
88. Alvarez JG, Storey BT. Evidence that membrane stress contributes more than lipid peroxidation to sublethal cryodamage in cryopreserved human sperm: glycerol and other polyols as sole cryoprotectant. **J Androl**. 1993;14:199-209.
89. Leonsins AJ. Dextran; a valuable plasma volume expander. **South African medical journal = Suid-Afrikaanse tydskrif vir geneeskunde**. 1952;26:546-549.
90. Boldt J. Modern rapidly degradable hydroxyethyl starches: current concepts. **Anesth Analg**. 2009;108:1574-1582.
91. Kozek-Langenecker SA. Effects of hydroxyethyl starch solutions on hemostasis. **Anesthesiology**. 2005;103:654-660.
92. Treib J, Baron JF, Grauer MT et al. An international view of hydroxyethyl starches. **Intensive Care Med**. 1999;25:258-268.
93. Quan GB, Han Y, Liu MX et al. Effects of pre-freeze incubation of human red blood cells with various sugars on postthaw recovery when using a dextran-rapid cooling protocol. **Cryobiology**. 2009;59:258-267.
94. Pellerin-Mendes C, Million L, Marchand-Arvier M et al. In vitro study of the protective effect of trehalose and dextran during freezing of human red blood cells in liquid nitrogen. **Cryobiology**. 1997;35:173-186.
95. Lehle K, Hoenicka M, Jacobs VR et al. Cryopreservation of human endothelial cells for vascular tissue engineering. **Cryobiology**. 2005;50:154-161.
96. Thomas MJ, Parry ES, Nash SG et al. A method for the cryopreservation of red blood cells using hydroxyethyl starch as a cryoprotectant. **Transfus Sci**.

1996;17:385-396.
97. Stolzing A, Naaldijk Y, Fedorova V et al. Hydroxyethylstarch in cryopreservation - mechanisms, benefits and problems. **Transfusion and apheresis science : official journal of the World Apheresis Association : official journal of the European Society for Haemapheresis**. 2012;46:137-147.
98. Dieterich HJ, Kraft D, Sirtl C et al. Hydroxyethyl starch antibodies in humans: incidence and clinical relevance. **Anesthesia and analgesia**. 1998;86:1123-1126.
99. Korber C, Scheiwe MW. The cryoprotective properties of hydroxyethyl starch investigated by means of differential thermal analysis. **Cryobiology**. 1980;17:54-65.
100. McGann LE. Differing actions of penetrating and nonpenetrating cryoprotective agents. **Cryobiology**. 1978;15:382-390.
101. Takahashi T, Hirsh A, Erbe E et al. Mechanism of cryoprotection by extracellular polymeric solutes. **Biophys J**. 1988;54:509-518.
102. http://skincare.dermis.net/content/e01aufbau/e583/index_eng.html.
103. http://www.drmhijazy.com/english/chapters/chapter02.htm.
104. Alt E, Yan Y, Gehmert S et al. Fibroblasts share mesenchymal phenotypes with stem cells, but lack their differentiation and colony-forming potential. **Biology of the cell / under the auspices of the European Cell Biology Organization**. 2011;103:197-208.
105. Eckert RL. Structure, function, and differentiation of the keratinocyte. **Physiological reviews**. 1989;69:1316-1346.
106. Eckert RL, Rorke EA. Molecular biology of keratinocyte differentiation. **Environmental health perspectives**. 1989;80:109-116.
107. Watt FM. Role of integrins in regulating epidermal adhesion, growth and differentiation. **The EMBO journal**. 2002;21:3919-3926.
108. Hafemann B, Hettich R, Ensslen S et al. Treatment of skin defects using suspensions of in vitro cultured keratinocytes. **Burns : journal of the International Society for Burn Injuries**. 1994;20:168-172.
109. Rennekampff HO, Hansbrough JF, Kiessig V et al. Wound closure with human keratinocytes cultured on a polyurethane dressing overlaid on a cultured human dermal replacement. **Surgery**. 1996;120:16-22.
110. Corsini J, Maxwell F, Maxwell IH. Storage of various cell lines at -70 degrees C or -80 degrees C in multi-well plates while attached to the substratum. **Biotechniques**. 2002;33:42, 44, 46.
111. Kearney JN. Cryopreservation of cultured skin cells. **Burns**. 1991;17:380-383.
112. Eaglstein WH, Iriondo M, Laszlo K. A composite skin substitute (graftskin) for surgical wounds. A clinical experience. **Dermatologic surgery : official publication for American Society for Dermatologic Surgery [et al]**. 1995;21:839-843.
113. Eaglstein WH, Falanga V. Tissue engineering and the development of Apligraf a human skin equivalent. **Advances in wound care : the journal for prevention and healing**. 1998;11:1-8.
114. Eaglstein WH, Falanga V. Tissue engineering for skin: an update. **Journal of the American Academy of Dermatology**. 1998;39:1007-1010.
115. http://embryo.ib.amwaw.edu.pl/invittox/prot/I_1320000608en00010089.pdf. Skin Corrosion, Rat Skin TER and Human Skin Model Assay.
116. http://iccvam.niehs.nih.gov. Interagency Coordinating Committe on the

Validation of Alternative Methods.
117. Bernhofer LP, Seiberg M, Martin KM. The influence of the response of skin equivalent systems to topically applied consumer products by epithelial--mesenchymal interactions. **Toxicology in vitro : an international journal published in association with BIBRA**. 1999;13:219-229.
118. Wong T, McGrath JA, Navsaria H. The role of fibroblasts in tissue engineering and regeneration. **The British journal of dermatology**. 2007;156:1149-1155.
119. Phillips TJ, Manzoor J, Rojas A et al. The longevity of a bilayered skin substitute after application to venous ulcers. **Archives of dermatology**. 2002;138:1079-1081.
120. Liu K, Yang Y, Mansbridge J. Comparison of the stress response to cryopreservation in monolayer and three-dimensional human fibroblast cultures: stress proteins, MAP kinases, and growth factor gene expression. **Tissue engineering**. 2000;6:539-554.
121. Friedenstein AJ. Precursor cells of mechanocytes. **International review of cytology**. 1976;47:327-359.
122. Dominici M, Le Blanc K, Mueller I et al. Minimal criteria for defining multipotent mesenchymal stromal cells. The International Society for Cellular Therapy position statement. **Cytotherapy**. 2006;8:315-317.
123. Ball LM, Bernardo ME, Roelofs H et al. Cotransplantation of ex vivo expanded mesenchymal stem cells accelerates lymphocyte recovery and may reduce the risk of graft failure in haploidentical hematopoietic stem-cell transplantation. **Blood**. 2007;110:2764-2767.
124. Newman RE, Yoo D, LeRoux MA et al. Treatment of inflammatory diseases with mesenchymal stem cells. **Inflammation & allergy drug targets**. 2009;8:110-123.
125. Rodrigo SF, van Ramshorst J, Beeres SL et al. Intramyocardial injection of bone marrow mononuclear cells in chronic myocardial ischemia patients after previous placebo injection improves myocardial perfusion and anginal symptoms: an intra-patient comparison. **American heart journal**. 2012;164:771-778.
126. Sykova E, Jendelova P, Urdzikova L et al. Bone marrow stem cells and polymer hydrogels--two strategies for spinal cord injury repair. **Cellular and molecular neurobiology**. 2006;26:1113-1129.
127. Can A, Karahuseyinoglu S. Concise review: human umbilical cord stroma with regard to the source of fetus-derived stem cells. **Stem cells**. 2007;25:2886-2895.
128. Kraus KH, Kirker-Head C. Mesenchymal stem cells and bone regeneration. **Veterinary surgery : VS**. 2006;35:232-242.
129. Bifari F, Lisi V, Mimiola E et al. Immune Modulation by Mesenchymal Stem Cells. **Transfusion medicine and hemotherapy : offizielles Organ der Deutschen Gesellschaft fur Transfusionsmedizin und Immunhamatologie**. 2008;35:194-204.
130. Salem HK, Thiemermann C. Mesenchymal stromal cells: current understanding and clinical status. **Stem cells**. 2010;28:585-596.
131. Quevedo HC, Hatzistergos KE, Oskouei BN et al. Allogeneic mesenchymal stem cells restore cardiac function in chronic ischemic cardiomyopathy via trilineage differentiating capacity. **Proceedings of the National Academy of Sciences of the United States of America**. 2009;106:14022-14027.
132. Grove JE, Bruscia E, Krause DS. Plasticity of bone marrow-derived stem cells.

Stem cells. 2004;22:487-500.
133. Banfi A, Muraglia A, Dozin B et al. Proliferation kinetics and differentiation potential of ex vivo expanded human bone marrow stromal cells: Implications for their use in cell therapy. **Experimental hematology**. 2000;28:707-715.
134. Bonab MM, Alimoghaddam K, Talebian F et al. Aging of mesenchymal stem cell in vitro. **BMC cell biology**. 2006;7:14.
135. Sethe S, Scutt A, Stolzing A. Aging of mesenchymal stem cells. **Ageing research reviews**. 2006;5:91-116.
136. Stolzing A, Jones E, McGonagle D et al. Age-related changes in human bone marrow-derived mesenchymal stem cells: consequences for cell therapies. **Mechanisms of ageing and development**. 2008;129:163-173.
137. Stolzing A, Scutt A. Age-related impairment of mesenchymal progenitor cell function. **Aging cell**. 2006;5:213-224.
138. Teasdale B, Sieber VK, Riches DJ et al. Cryopreservation of cultured dermal fibroblast impregnated collagen gels. **Burns**. 1993;19:406-410.
139. Baust JMV, M.J; Snyder, K.K; Van Buskirk, R.G. and Baust, J.G. Activation of Mitochondrial-Associated Apoptosis Contributes to Cryopreservation Failure **Cell Preservation Technology**. 2007;5:155-164.
140. Murakawa T, Nakajima J, Ono M et al. Allogenicity of cryopreserved human fibroblasts: cryopreservation does not downregulate the allogenicity of fibroblasts making up the matrices of allografts. **J Thorac Cardiovasc Surg**. 2000;120:712-719.
141. Buck DW, Hanssens LK, Kennett RH et al. Successful cryopreservation of tissue and skin fibroblast cultures from patients with mucolipidosis II (I-cell disease). **Cytogenet Cell Genet**. 1981;29:9-15.
142. Sui L. NR, Dong Y., Yamaguchi F., Izumori K., Tokuda M. Cryoprotective effects of d-allose on mammalian cells. **Cryobiology**. 2007;55:87-92.
143. Wang X, Hua TC, Sun DW et al. Cryopreservation of tissue-engineered dermal replacement in Me2SO: Toxicity study and effects of concentration and cooling rates on cell viability. **Cryobiology**. 2007;55:60-65.
144. Ujihira M. IA, Aoki K., Omaki S., Goto E., Mabuchi K. Cryoprotective effect of low-molecular-weight hyaluronan on human dermal fibroblast monolayers. **Cryo Letters**. 2010;3:101-111.
145. Choi J. B, J.C. Cooling rate dependent biophysical and viability response shift with attachment state in human dermal fibroblast cells. **Cryobiology**. 2011;63:285-291.
146. Pasch J, Schiefer A, Heschel I et al. Variation of the HES concentration for the cryopreservation of keratinocytes in suspensions and in monolayers. **Cryobiology**. 2000;41:89-96.
147. Rindler V. PJ, Heschel, I.and Rau, G. Cryopreservation of suspended keratinocytes with hydroxyethyl starch. **Cryo-Letters**. 1999;20:3–12.
148. Hirel B, Chesne C, Pailheret JP et al. Expression of differentiation markers in human adult keratinocytes cultured in submerged conditions. **In Vitro Cell Dev Biol Anim**. 1994;30A:372-378.
149. May SRR, S.K.; Rubin, C.A. Optimum cooling and warming rate for the cryopreservation of cultured human keratinocytes and mouse L cells. **Cryobiology**. 1988;25:516.
150. Pasch J, Schiefer A, Heschel I et al. Cryopreservation of keratinocytes in a monolayer. **Cryobiology**. 1999;39:158-168.
151. Hanna J, Hubel A. Preservation of stem cells. **Organogenesis**. 2009;5:134-

137.
152. Lee MW, Choi J, Yang MS et al. Mesenchymal stem cells from cryopreserved human umbilical cord blood. **Biochemical and biophysical research communications**. 2004;320:273-278.
153. Balci D, Can A. The assessment of cryopreservation conditions for human umbilical cord stroma-derived mesenchymal stem cells towards a potential use for stem cell banking. **Current stem cell research & therapy**. 2013;8:60-72.
154. Liu Y, Xu X, Ma X et al. Cryopreservation of human bone marrow-derived mesenchymal stem cells with reduced dimethylsulfoxide and well-defined freezing solutions. **Biotechnology progress**. 2010;26:1635-1643.
155. Kotobuki N, Hirose M, Machida H et al. Viability and osteogenic potential of cryopreserved human bone marrow-derived mesenchymal cells. **Tissue engineering**. 2005;11:663-673.
156. Naaldijk Y, Staude M, Fedorova V et al. Effect of different freezing rates during cryopreservation of rat mesenchymal stem cells using combinations of hydroxyethyl starch and dimethylsulfoxide. **BMC biotechnology**. 2012;12:49.
157. Renzi S. LT, Dotti S., Dessi S.S., De Blasio, P. and Ferrari, M. Mesenchymal Stromal Cell Cryopreservation. **Biopreservation and Biobanking**. 2012;10:276-289.
158. Xu X, Liu Y, Cui Z et al. Effects of osmotic and cold shock on adherent human mesenchymal stem cells during cryopreservation. **Journal of biotechnology**. 2012;162:224-231.
159. Liu G, Zhou H, Li Y et al. Evaluation of the viability and osteogenic differentiation of cryopreserved human adipose-derived stem cells. **Cryobiology**. 2008;57:18-24.
160. Santos NC, Figueira-Coelho J, Martins-Silva J et al. Multidisciplinary utilization of dimethyl sulfoxide: pharmacological, cellular, and molecular aspects. **Biochem Pharmacol**. 2003;65:1035-1041.
161. Ishiguro K, Sartorelli AC. Activation of transiently transfected reporter genes in 3T3 Swiss cells by the inducers of differentiation/apoptosis--dimethylsulfoxide, hexamethylene bisacetamide and trichostatin A. **European journal of biochemistry / FEBS**. 2004;271:2379-2390.
162. Qi W. DD, Salvi R.J. Cytotoxic effects of dimethyl sulphoxide (DMSO) on cochlear organotypic cultures. **Hearing Research**. 2008;236:52-60.
163. Sakuragawa N. SM, Yoshida Y., Kamo I., Arima M., Satoyoshi E. Effects of dimethylsulfoxide on sphingomyelinase in culture human fibroblasts and correction of sphingomyelinase deficiency in fibroblasts from Niemann-Pick patients. **Biochemical and biophysical research communications**. 1985;126:756-762.
164. Yamamoto N. Effect of dimethyl sulfoxide on cytosolic ionized calcium concentration and cytoskeletal organization of hepatocytes in a primary culture. **Cell Struct Funct**. 1989;14:75-85.
165. Lampugnani M.G. PM, Niewiarowski A., Casali B., Donati M.G., Corbascio G.C., Marchisio P.C. Effects of dimethyl sulfoxide (DMSO) on microfilament organization, cellular adhesion, and growth of cultured mouse B16 melanoma cells. **Experimental cell research**. 1987;172:385-396.
166. Hegner B, Weber M, Dragun D et al. Differential regulation of smooth muscle markers in human bone marrow-derived mesenchymal stem cells. **Journal of hypertension**. 2005;23:1191-1202.

167. Adler S, Pellizzer C, Paparella M et al. The effects of solvents on embryonic stem cell differentiation. **Toxicology in vitro : an international journal published in association with BIBRA**. 2006;20:265-271.
168. Pal R, Mamidi MK, Das AK et al. Diverse effects of dimethyl sulfoxide (DMSO) on the differentiation potential of human embryonic stem cells. **Archives of toxicology**. 2012;86:651-661.
169. Katkov, II, Kim MS, Bajpai R et al. Cryopreservation by slow cooling with DMSO diminished production of Oct-4 pluripotency marker in human embryonic stem cells. **Cryobiology**. 2006;53:194-205.
170. Katkov, II, Kan NG, Cimadamore F et al. DMSO-Free Programmed Cryopreservation of Fully Dissociated and Adherent Human Induced Pluripotent Stem Cells. **Stem cells international**. 2011;2011:981606.
171. Brayton C. Dimethyl sulfoxide (DMSO): a review. **Cornell Vet**. 1986;76:61-90.
172. (R) M. Healthcare service. Vol 120; 2004.
173. www.mskcc.org/mskcc/html/69205.cfm.
174. Watanabe M, Iizuka H. Dimethyl sulfoxide-induced augmentation of adenosine-adenylate cyclase response of pig skin epidermis. **Arch Dermatol Res**. 1986;278:470-473.
175. Astley JP, Levine M. Effect of dimethyl sulfoxide on permeability of human skin in vitro. **J Pharm Sci**. 1976;65:210-215.
176. Higman MA, Port JD, Beauchamp NJ, Jr. et al. Reversible leukoencephalopathy associated with re-infusion of DMSO preserved stem cells. **Bone marrow transplantation**. 2000;26:797-800.
177. Mueller LP, Theurich S, Christopeit M et al. Neurotoxicity upon infusion of dimethylsulfoxide-cryopreserved peripheral blood stem cells in patients with and without pre-existing cerebral disease. **Eur J Haematol**. 2007;78:527-531.
178. Zambelli A, Poggi G, Da Prada G et al. Clinical toxicity of cryopreserved circulating progenitor cells infusion. **Anticancer Res**. 1998;18:4705-4708.
179. Schlegel PG, Wolfl M, Schick J et al. Transient loss of consciousness in pediatric recipients of dimethylsulfoxide (DMSO)-cryopreserved peripheral blood stem cells independent of morphine co-medication. **Haematologica**. 2009;94:1473-1475.
180. Eroglu A, Bailey SE, Toner M et al. Successful cryopreservation of mouse oocytes by using low concentrations of trehalose and dimethylsulfoxide. **Biol Reprod**. 2009;80:70-78.
181. Rodrigues JP, Paraguassu-Braga FH, Carvalho L et al. Evaluation of trehalose and sucrose as cryoprotectants for hematopoietic stem cells of umbilical cord blood. **Cryobiology**. 2008;56:144-151.
182. Petrenko YA, Jones DR, Petrenko AY. Cryopreservation of human fetal liver hematopoietic stem/progenitor cells using sucrose as an additive to the cryoprotective medium. **Cryobiology**. 2008;57:195-200.
183. Jurecka W, Szepfalusi Z, Parth E et al. Hydroxyethylstarch deposits in human skin--a model for pruritus? **Arch Dermatol Res**. 1993;285:13-19.
184. Nohe B, Burchard M, Zanke C et al. Endothelial accumulation of hydroxyethyl starch and functional consequences on leukocyte-endothelial interactions. **Eur Surg Res**. 2002;34:364-372.
185. Sirtl C, Laubenthal H, Zumtobel V et al. Tissue deposits of hydroxyethyl starch (HES): dose-dependent and time-related. **Br J Anaesth**. 1999;82:510-515.
186. Stander S, Szepfalusi Z, Bohle B et al. Differential storage of hydroxyethyl starch (HES) in the skin: an immunoelectron-microscopical long-term study.

Cell Tissue Res. 2001;304:261-269.
187. Szepfalusi Z, Parth E, Jurecka W et al. Human monocytes and keratinocytes in culture ingest hydroxyethylstarch. **Arch Dermatol Res**. 1993;285:144-150.
188. Sputtek A, Horn EP, Schulte Am Esch J et al. [Cryopreservation of red blood cells using hydroxyethyl starch (HES) - From laboratory investigations to clinical application]. **Anasthesiol Intensivmed Notfallmed Schmerzther**. 2001;36 Suppl 2:S162-164.
189. Scheiwe MW, Nick HE, Korber C. An experimental study on the freezing of red blood cells with and without hydroxyethyl starch. **Cryobiology**. 1982;l9:461-477.
190. Ashwood-Smith MJ, Warby C, Connor KW et al. Low-temperature preservation of mammalian cells in tissue culture with polyvinylpyrrolidone (PVP), dextrans, and hydroxyethyl starch (HES). **Cryobiology**. 1972;9:441-449.
191. Allen ED, Weatherbee L, Spencer HH et al. Large unit red cell cryopreservation with hydroxyethyl starch. **Cryobiology**. 1976;13:500-506.
192. Stiff PJ, Koester AR, Weidner MK et al. Autologous bone marrow transplantation using unfractionated cells cryopreserved in dimethylsulfoxide and hydroxyethyl starch without controlled-rate freezing. **Blood**. 1987;70:974-978.
193. Kim H, Tanaka S, Une S et al. A comparative study of the effects of glycerol and hydroxyethyl starch in canine red blood cell cryopreservation. **J Vet Med Sci**. 2004;66:1543-1547.
194. Lionetti FJ, Hunt SM, Gore JM et al. Cryopreservation of human granulocytes. **Cryobiology**. 1975;12:181-191.
195. Clapisson G, Salinas C, Malacher P et al. Cryopreservation with hydroxyethylstarch (HES) + dimethylsulfoxide (DMSO) gives better results than DMSO alone. **Bull Cancer**. 2004;91:E97-102.
196. McGann LE. Optimal temperature ranges for control of cooling rate. **Cryobiology**. 1979;16:211-216.
197. Maruyama M, Kenmochi T, Sakamoto K et al. Simplified method for cryopreservation of islets using hydroxyethyl starch and dimethyl sulfoxide as cryoprotectants. **Transplant Proc**. 2004;36:1133-1134.
198. Stiff PJ, Murgo AJ, Zaroulis CG et al. Unfractionated human marrow cell cryopreservation using dimethylsulfoxide and hydroxyethyl starch. **Cryobiology**. 1983;20:17-24.
199. Singbartl K, Langer R, Henrich A. Altered membrane skeleton of hydroxyethylstarch-cryopreserved human erythrocytes. **Cryobiology**. 1998;36:115-123.
200. Martinello T, Bronzini I, Maccatrozzo L et al. Canine adipose-derived-mesenchymal stem cells do not lose stem features after a long-term cryopreservation. **Res Vet Sci**.91:18-24.
201. Moon JH, Kwak SS, Park G et al. Isolation and characterization of multipotent human keloid-derived mesenchymal-like stem cells. **Stem Cells Dev**. 2008;17:713-724.
202. Baust JM, Van B, Baust JG. Cell viability improves following inhibition of cryopreservation-induced apoptosis. **In Vitro Cell Dev Biol Anim**. 2000;36:262-270.
203. Frim J, Snyder RA, McGann LE et al. Growth kinetics of cells following freezing in liquid nitrogen. **Cryobiology**. 1978;15:502-516.

204. Heng BC, Ye CP, Liu H et al. Loss of viability during freeze-thaw of intact and adherent human embryonic stem cells with conventional slow-cooling protocols is predominantly due to apoptosis rather than cellular necrosis. **J Biomed Sci**. 2006;13:433-445.
205. Ginis I, Grinblat B, Shirvan MH. Evaluation of bone marrow-derived mesenchymal stem cells after cryopreservation and hypothermic storage in clinically safe medium. **Tissue engineering Part C, Methods**. 2012;18:453-463.
206. Iwatani M. IK, Kremenska Y., Hattori N., Tanaka S., Yagi S., Shiota K. Dimethyl sulfoxide has an impact on epigenetic profile in mouse embryoid body. **Stem cells**. 2006;24:2549-2556.
207. Carvalho KA, Cury CC, Oliveira L et al. Evaluation of bone marrow mesenchymal stem cell standard cryopreservation procedure efficiency. **Transplantation proceedings**. 2008;40:839-841.
208. Ock SA, Rho GJ. Effect of dimethyl sulfoxide (DMSO) on cryopreservation of porcine mesenchymal stem cells (pMSCs). **Cell transplantation**. 2011;20:1231-1239.
209. Ji L, de Pablo JJ, Palecek SP. Cryopreservation of adherent human embryonic stem cells. **Biotechnol Bioeng**. 2004;88:299-312.
210. Yanes O, Clark J, Wong DM et al. Metabolic oxidation regulates embryonic stem cell differentiation. **Nature chemical biology**. 2010;6:411-417.
211. Lu X, Proctor SJ, Dickinson AM. The effect of cryopreservation on umbilical cord blood endothelial progenitor cell differentiation. **Cell transplantation**. 2008;17:1423-1428.
212. Luo K, Wu G, Wang Q et al. Effect of dimethylsulfoxide and hydroxyethyl starch in the preservation of fractionated human marrow cells. **Cryobiology**. 1994;31:349-354.
213. Rowley SD, Feng Z, Chen L et al. A randomized phase III clinical trial of autologous blood stem cell transplantation comparing cryopreservation using dimethylsulfoxide vs dimethylsulfoxide with hydroxyethylstarch. **Bone Marrow Transplant**. 2003;31:1043-1051.
214. Fan WX, Ma XH, Ge D et al. Cryoprotectants for the vitrification of corneal endothelial cells. **Cryobiology**. 2009;58:28-36.
215. Sui L, Nomura R, Dong Y et al. Cryoprotective effects of D-allose on mammalian cells. **Cryobiology**. 2007;55:87-92.
216. Davies JW, Ricketts CR, Williams BN. Plasma Volume Expansion by Rapid Infusion of a Low Molecular Weight Dextran. **British journal of pharmacology and chemotherapy**. 1963;21:220-225.
217. Ar'Rajab A, Ahren B, Sundberg R et al. A new dextran 40-based solution for liver preservation. **Transplantation**. 1992;53:742-745.
218. Fasola CG, Kim YS, Dunning M et al. Successful canine small bowel preservation in simple cold storage for 48 hours using a dextran-40-based solution. **Transplantation proceedings**. 1993;25:1576-1579.
219. Brockbank KGaTJM. Tissue Preservation. In: Baust JGaBJM, ed. Advances in Biopreservation. Bocan Raton, Fl: CRC Press; 2007:157-186.
220. de Hoyos AL, Patterson GA, Maurer JR et al. Pulmonary transplantation. Early and late results. The Toronto Lung Transplant Group. **The Journal of thoracic and cardiovascular surgery**. 1992;103:295-306.
221. Torres RL, Belo-Klein A, Andrade CF et al. Effect of systemically administered low potassium dextran solution on oxidative stress in a rat model of lung

ischemia. **Interactive cardiovascular and thoracic surgery**. 2009;8:3-6.
222. Liao YH, Brown MB, Quader A et al. Protective mechanism of stabilizing excipients against dehydration in the freeze-drying of proteins. **Pharmaceutical research**. 2002;19:1854-1861.
223. Cortesi R, Esposito E, Nastruzzi C. Effect of DNA complexation and freeze-drying on the physicochemical characteristics of cationic liposomes. **Antisense & nucleic acid drug development**. 2000;10:205-215.
224. Gilles R, Bourdouxhe-Housiaux C, Colson P et al. Effect of compensatory organic osmolytes on resistance to freeze-drying of L929 cells and of their isolated chromatin. **Comparative biochemistry and physiology Part A, Molecular & integrative physiology**. 1999;122:145-155.
225. Kracke GR, Preston GG, Stanley TH. Identification of a sorbitol permease in human erythrocytes. **Am J Physiol**. 1994;266:C343-350.
226. Naaldijk Y, Staude, M., Fedorova, V. and Stolzing, A. Effect of different freezing rates during cryopreservation of rat mesenchymal stem cells using combinations of hydroxyethyl starch and dimethylsulfoxide. **BMC Biotechnology**. 2012.
227. Haass A, Kroemer H, Jager H et al. [Dextran 40 or HES 200/0.5? Hemorheology of the long-term treatment of ischemic cerebral attacks]. **Deutsche medizinische Wochenschrift**. 1986;111:1681-1686.
228. Kenmochi T, Asano T, Maruyama M et al. Cryopreservation of human pancreatic islets from non-heart-beating donors using hydroxyethyl starch and dimethyl sulfoxide as cryoprotectants. **Cell Transplant**. 2008;17:61-67.
229. Connor W, Ashwood-Smith MJ. Cryoprotection of mammalian cells in tissue culture with polymers; possible mechanisms. **Cryobiology**. 1973;10:488-496.
230. Areman EM, Dickerson SA, Bender JG et al. Use of licensed electrolyte solutions and anticoagulant citrate dextrose for bone marrow collection. **Progress in clinical and biological research**. 1992;377:353-359.
231. Areman EM, Dickerson SA, Kotula PL et al. Use of a licensed electrolyte solution as an alternative to tissue culture medium for bone marrow collection. **Transfusion**. 1993;33:562-566.
232. Kundu CN, Das K, Majumder GC. Effect of amino acids on goat cauda epididymal sperm cryopreservation using a chemically defined model system. **Cryobiology**. 2001;42:21-27.
233. Trimeche A, Yvon JM, Vidament M et al. Effects of glutamine, proline, histidine and betaine on post-thaw motility of stallion spermatozoa. **Theriogenology**. 1999;52:181-191.
234. Renard P, Grizard G, Griveau JF et al. Improvement of motility and fertilization potential of postthaw human sperm using glutamine. **Cryobiology**. 1996;33:311-319.
235. Hubel A. Preservation of Cellular Therapies: Gene Therapy, Cellular, and Metabolic Engineering. In: Baust JGaBJM, ed. Advances in Biopreservation. Boca Raton, Fl: CRC Press; 2007:143-152.
236. Lovelock JE. The mechanism of the protective action of glycerol against haemolysis by freezing and thawing. **Biochim Biophys Acta**. 1953;11:28-36.
237. P. M. Principles of cryobiology. In: Fuller B.J. LN, Benson E.E., ed. Life in frozen state: CRC Press; 2004:5-55.
238. Borderie VM, Lopez M, Lombet A et al. Cryopreservation and culture of human corneal keratocytes. **Invest Ophthalmol Vis Sci**. 1998;39:1511-1519.
239. Baust JM, Van Buskirk R, Baust JG. Modulation of the cryopreservation cap:

elevated survival with reduced dimethyl sulfoxide concentration. **Cryobiology**. 2002;45:97-108.
240. Karlsson JO, Toner M. Long-term storage of tissues by cryopreservation: critical issues. **Biomaterials**. 1996;17:243-256.
241. Yokomuro H, Shiono N, Ozawa T et al. Effect of cryopreservation on cell proliferation and immunogenicity of transplanted human heart cells. **Ann Thorac Cardiovasc Surg**. 2010;16:105-112.
242. Muinos-Lopez E. R-VME, Hermida-Gomez T., Fuentes-Boquete I., Diaz-Prado S., Blanco F.J. Cryopreservation effect on proliferative and chrondrogenic potential of human chrondrocyte isolated from superficial and deep cartilage. **Open Orthop J**. 2012;6:150.
243. Malpique R, Ehrhart F, Katsen-Globa A et al. Cryopreservation of Adherent Cells: Strategies to Improve Cell Viability and Function after Thawing. **Tissue Eng Part C Methods**. 2009.
244. Ebertz SL, McGann LE. Cryoinjury in endothelial cell monolayers. **Cryobiology**. 2004;49:37-44.
245. Murua A, Orive G, Hernandez RM et al. Cryopreservation based on freezing protocols for the long-term storage of microencapsulated myoblasts. **Biomaterials**. 2009;30:3495-3501.
246. Valbuena D, Sanchez-Luengo S, Galan A et al. Efficient method for slow cryopreservation of human embryonic stem cells in xeno-free conditions. **Reproductive biomedicine online**. 2008;17:127-135.
247. Conscience JF, Fischer F. An improved preservation technique for cells of hemopoietic origin. **Cryobiology**. 1985;22:495-498.

8. Eigenständigkeitserklärung

I hereby declare that the work presented on this thesis I independently wrote and use solely the specified resources. The work of others, wording or the meanings used was specified as secondary literature. The thesis presents no conflict of interest with third parties.

The preparation of the thesis was based according to the guidelines and rules of the Biologisch-Pharmazeutischen Fakultät der Friedrich-Schiller, Universität Jena.

_____

Yahaira M Naaldijk

22.07.2013

9. Curriculum Vitae

Curriculum Vitae

Personal Details

Name	Yahaira M Naaldijk
Birthdate	June 2, 1979
Birthplace	San Juan, Puerto Rico (USA)
Nationality	American and Dutch

Education

Promotion Student in Faculty of Biology and Pharmacy, Institute of Nutrition, present

Friedrich-Schiller University Jena

Master of Science in Biochemistry and Molecular Biology, **May, 2008**
Indiana University, Indianapolis, Indiana

- Minor in Cancer Biology

Bachelor of Science in Cell and Molecular Biology, May 2001
Universidad Metropolitana, Cupey, Puerto Rico

Working Experiences

Since 2011	Translational Centre for Regenerative Medicine (TRM) Scientist Working Title: Use of Microglia Derived from iPS-cells for a Replacement Therapy of Alzheimer Disease
Since 2010	Fraunhofer Institute of Immunology and Cell Therapy (IZI) PhD Student Working Title: Application of sugars and starch in the cryopreservation of cells.
2008-2010	Fraunhofer Institute of Immunology and Cell Therapy (IZI) Technical Assistant Performance of cell and molecular experiments focusing on cryopreservation of cells and cell fusion
2005-2008	Indiana University School of Medicine, Indiana Master student Working Title: Knockdown of *CUL-4A* gene using siRNA induces cell death by apoptosis
2002-2005	Working title: To study whether *CUL-4A* haploinsufficiency and/or deficiency interferes with *in vitro* hematopoietic differentiation into the different hematopoietic lineages using Embryonic Stem (ES) mouse
2001	Indiana University School of Medicine, Indiana Student trainee Working title: Determine whether or not the **m**ouse **P**elle-**L**ike Protein **K**inase (mPLK) molecules oligomerize
2001	University of Puerto Rico Medical Science Campus Student trainee Learn new techniques related to cell cultures using Jurkat cells
2000	College of Veterinary and Biomedical Science, Colorado State University Summer Internship Working title: To construct single-stranded IGF-I and IGF-II anti-sense probes suitable to study IGF gene expression in *cynomologus* monkey tissues.
1998-2000	Universidad Metropolitana, Puerto Rico Student trainee Working title: To study the effect of the NBQ-91 compound (topoisomerase inhibitor) in the presence and absence of galactose in bovine lens epithelial cells

Awards

- ImmunoTools Award 2013

- 7th International Symposium on Neuroprotection and Neurorepair (ISN) Scholarship in May 2012 held in Potsdam
- Minority Predoctoral Fellowship Program NIH 1F31HL079889-01 at IUPUI in 2005-07

Patent filed

Cryopreservation solution using complex carbohydrates (2012) EP12189243

Publications

- Hodzic M., **Naaldijk Y**. and Stolzing A. (2013): Regulation of aging in adult stem cells by microRNA. Zeitschrift für Gerontologie und Geriatrie. Accepted for publication.
- **Naaldijk, Y**, Staude M., Fedorova V, Stolzing A (2012): Effect of different freezing rates during cryopreservation of rat mesenchymal stem cells using combinations of hydroxyethyl starch and dimethylsulfonide. *BMC Biotechnology*, Vol 12, Issue 1, pp49-59
- Stolzing A, **Naaldijk** Y, Fedorova V, Sethe S (2012): Hydroxyethylstarch in cryopreservation - Mechanisms, benefits and problems. *Transfus Apher Sci,* Vol 46 Issue 2, pp 137-47
- Arnold, A., **Naaldijk, Y**., Fabian, C., Wirth, H., Binder, H., Armstrong, L., Stolzing, A. (2012): Generation of disease-specific human pluripotent stem cells using mRNA. Regenerative Medicine. *ISCR Cell Biology*, Vol 2012, pp 1-12
- Waning D, Li, B., Jia, N., **Naaldijk, Y**., Hogenesch, A., Goebl, W.S., and Chun, K.T. (2008) Cul-4A is required for hematopoietic cell viability and its deficiency leads to apoptosis. *Blood* Vol 110, Issue 7, pp 2704-07

Oral presentations

- Stolzing A. and **Naaldijk, Y.** (2012) Differentiation of patient-specific human iPS cells into microglia. 7th Annual Congress of the German Society for Stem Cell Research 29-30.11.12, Leipzig

Posters presentations

2012

- **Naaldijk, Y** Jäger, C., Tennemann, , Arnold, A. and Stolzing, A. (2012) Microglia derivation from human induced pluripotent stem cells as a therapy for Alzheimer's disease. 11th Leipzig Research Festival for Life Sciences 14.12.12, Leipzig
- Jäger, C., **Naaldijk, Y.,** Böhme, J., Fabian, C., Tennemann, , Friedrich-Stöckgit, A and Stolzing, A. (2012) Microglia derivation from human induced pluripotent stem cells as a therapy for Alzheimer's disease. 11th Leipzig Research Festival for Life Sciences 14.12.12, Leipzig
- Rohani, L, Arnold, A, Fabian C, **Naaldijk, Y** and Stolzing, A. (2012) Integration-free mRNA reprogramming of human fibroblast11th Leipzig Research Festival for Life Sciences 14.12.12, Leipzig
- **Naaldijk, Y** Jäger, C., Tennemann, , Arnold, A. and Stolzing, A. (2012) Microglia derivation from human induced pluripotent stem cells as a therapy for Alzheimer's disease. 7th Annual Congress of the German Society for Stem Cell Research 29-30.11.12, Leipzig
- Jäger, C., **Naaldijk, Y.,** Böhme, J., Fabian, C., Tennemann, , Friedrich-Stöckgit, A and Stolzing, A. (2012) Microglia derivation from human induced pluripotent stem cells as

a therapy for Alzheimer's disease. 7th Annual Congress of the German Society for Stem Cell Research 29-30.11.12, Leipzig

- Rohani, L, Arnold, A, Fabian C, **Naaldijk, Y** and Stolzing, A. (2012) Integration-free mRNA reprogramming of human fibroblast. 7th Annual Congress of the German Society for Stem Cell Research 29-30.11.12, Leipzig
- Rohani, L, **Naaldijk Y**, Arnold, A, Fabian C, and Stolzing, A. (2012) DNA repair capacity and mitochondrial activity analysis in human reprogrammed induced pluripotent stem cells. 7th Annual Congress of the German Society for Stem Cell Research 29-30.11.12, Leipzig
- **Naaldijk, Y** and Stolzing A**.** (2012): Human iPS cells differentiated into microglia as a therapy for Alzheimer´s disease. Saxon Biotechnology Symposium 2012. 20.06.12, Leipzig
- **Naaldijk, Y** and Stolzing A. (2012): Use of Microglia Derived from iPS-cells for a Replacement Therapy of Alzheimer Disease. 7th International Symposium on Neuroprotection and Neurorepair (ISN). 2-5.05.12, Potsdam, Germany

2011

- **Naaldijk, Y**., Meisel, J. and Stolzing, A (2011): Different responses to DNA damage and repair in Mesenchymal Stem Cells during Ageing. 10th Leipziger Research Festival, 16.12.11, Leipzig, Germany
- **Naaldijk, Y**., Meisel, J. and Stolzing, A (2011): Different responses to DNA damage and repair in ADSC in presence of UV Light and H_2O_2. World Conference of Regenrative Medicine. 2-4.11.11, Leipzig, Germany
- **Naaldijk, Y**., Meisel, J. and Stolzing, A (2011): Differential gene expression of Adipose-derived Mesenchymal Stem Cells during Ageing. Alterstagung: Prevention and Interventions, Martin Luther Universität Halle. 16-18.09.11, Halle, Germany

2010

- **Naaldijk Y**, Meisel, J and Stolzing A (2010): Effect of Ageing on Adipose-derived Mesenchymal Stem Cells. 9th Leipziger Research Festival. 12.10, Leipzig, Germany
- A. Arnold, **Y. Naaldijk**, C. Fabian, T. Piroth, G. Nikkhah, A. Stolzing (2010): Huntington-specific human mRNA-iPS and their potential for cellular senescence. 3rd International Congress on Stem Cells and Tissue Formation, 11-14.07.2010, Dresden

2009

- A. Arnold, **Y. Naaldijk**, C. Fabian, S. Sauerzweig, A.Stolzing (2009): Basic assistive factors in cellular reprogramming. Human Pluripotent Stem Cells Symposium, 22. 04-24.04 2009,Dublin, Ireland
- A. Arnold, C. Fabian, S. Sauerzweig, **Y. Naaldijk**, U. Brinckmann, U. G. Liebert, A. Stolzing (2009): iPS cells as model system for human virus diseases. 19th Annual Meeting of the Society for Virology, 18. 03-21.03. 09, Leipzig; 8. Biotechnologietag 26.05.2009, Leipzig

2008

- Helbi, A**, Naaldijk, Y** and Stolzing A (2008): Cryopreservation of stem cells. Novel Cryobiotechnology for Solving Fundamental and Applied Task in Medicine 11.08, Kharkov, Ukraine

10. Acknowledgement

I would like to thank to my mentor Dr. Alexandra Stolzing for giving me the great opportunity to do my gradute studies in her laboratory, for her guidance and support during this past years.

To Viktoriya. Marek and James for working together as a team in the project and for helping me, especially to Viktoriya for your great insight in the project.

In addition, to my lab collegues for giving me their assistances, support and motivation during this whole time. To the girls, Dorota, Antje, Joana, Leili, Franzi and Anja for the good times.

To Annett for being there for me through the whole time, for listening to all my problems and situations and for your helpful advise. Thanks to Dr. Dr. Johannes Boltze for allowing to be a member on his department and for his great support and advice.

To my two good best friends, Javi and Yari, I still take both of you with me whenever I go and I am very happy that you are part of my life. Mandy, you have been there always for me giving me your love, support and understanding and you will always have the same from me.

I want to thank to my family for their support and understading especially to my mom for having great patience through this journey and being always there for me.