



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

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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

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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, deciduous 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 histocompatibility 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 autologous 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 procedure 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]. Polyols 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 500 μ l. Each cryovial contained 5x10⁵ 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 (5x10⁵).

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 500 μ l 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 500 μ l 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); 50 μ g/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 (0.2M Tris, 1mg/ml fast red, Sigma and 50 μ g/ml naphthol phosphate AS-BI, Sigma) for 1hr.

Adipogenic Differentiation

Adipogenic medium (10% FBS; 1% pen/strep, 10% insulin-transferrin-selenium supplement, (Sigma-Aldrich) 10⁻⁸M dexamethasone (Sigma-Aldrich); 0.5mM isobutylmethylxanthin, Sigma-Aldrich; 100 μ M 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), 10⁻⁷M dexamethasone (Sigma-Aldrich), 150 μ M ascorbic-2-phosphate (Sigma-Aldrich), 20 μ M 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 cryo-solution. 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 ($\$p \leq 0.014$) 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.

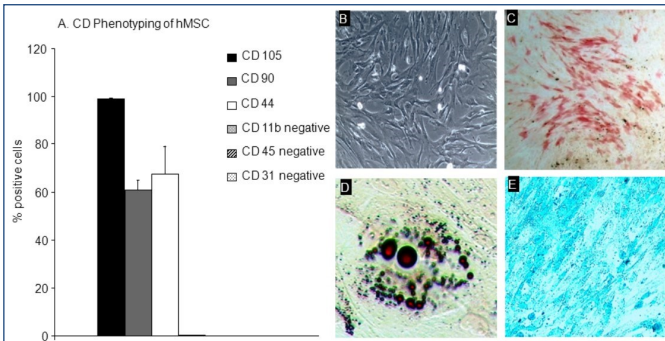


Fig. 1- MSC phenotyping. Characterization of freshly isolated MSCs from human umbilical cord. CD-phenotyping (A), Morphology of freshly isolated cells, 10X (B) ALP-activity staining in MSCs after osteogenic differentiation, 20X(C), oil red staining of differentiated MSC towards adipocyte lineage, 40X(D)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

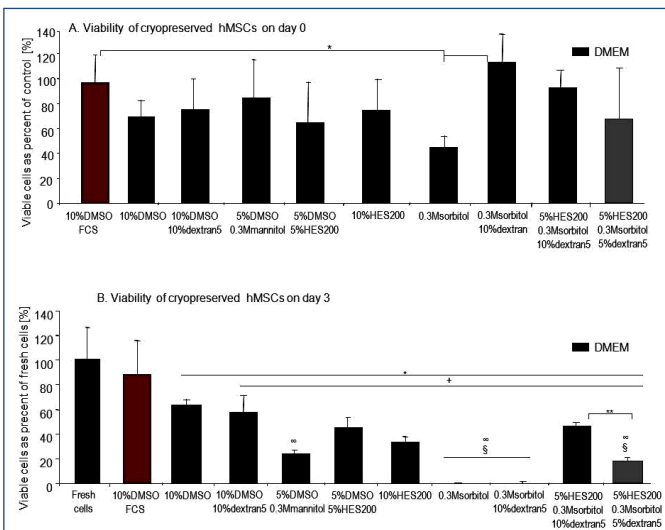


Fig. 2- Viability of cryopreserved hMSCs in comparison to fresh cells. Viability of cryopreserved MSC directly after thawing on day 0 by trypan blue (A). Determination of viability of hMSCs at day 3 after thawing using MTT-test (B). The following cryoprotectants were used: HES with MW= 200kDa, dextran with MW=5 kDa, in DMEM. * - means statistical 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 ($\$p \leq 0.012$). Similar results in cell viability were obtained for 5% DMSO in ring acetate ($\$p \leq 0.001$) 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 containing 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.

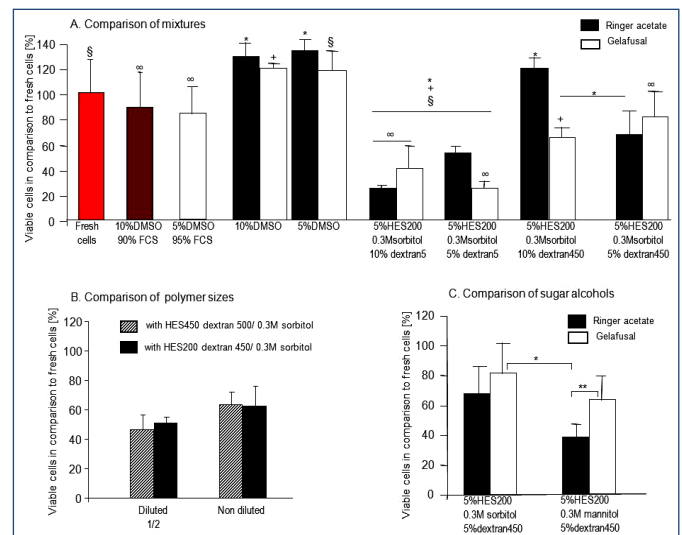


Fig. 3- Cryopreservation of hMSCs in DMSO- and FCS free- cryo-solution. Viability of hMSCs at day 3 after cryopreservation in solution containing HES, sorbitol/mannitol and dextran with MW=5 and 450 kDa in Ringer Acetate, Gelafusal or DMEM (A). Viability at day 3 of hMSCs after cryopreservation in solutions 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].

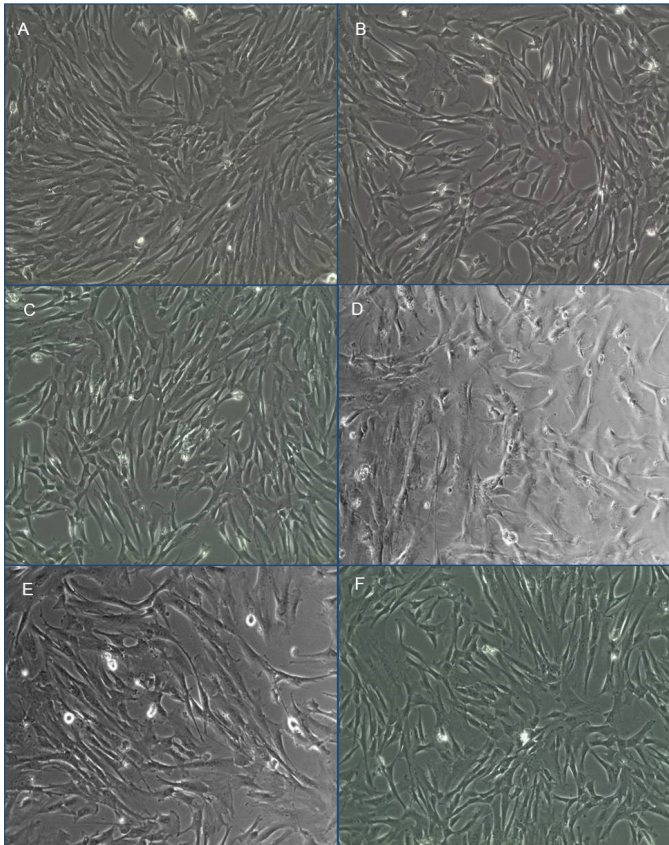


Fig. 4- Morphology of fresh and cryopreserved human MSC. Fresh MSCs at passage 3 (A) and MSCs after cryopreservation using the conventional solution with 10%DMSO and 90%FCS (B). Morphology of cryopreserved MSCs with cryopreservation with 5%DMSO and 90%FCS (C), 10%DMSO and 90% Ringer Acetate solution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

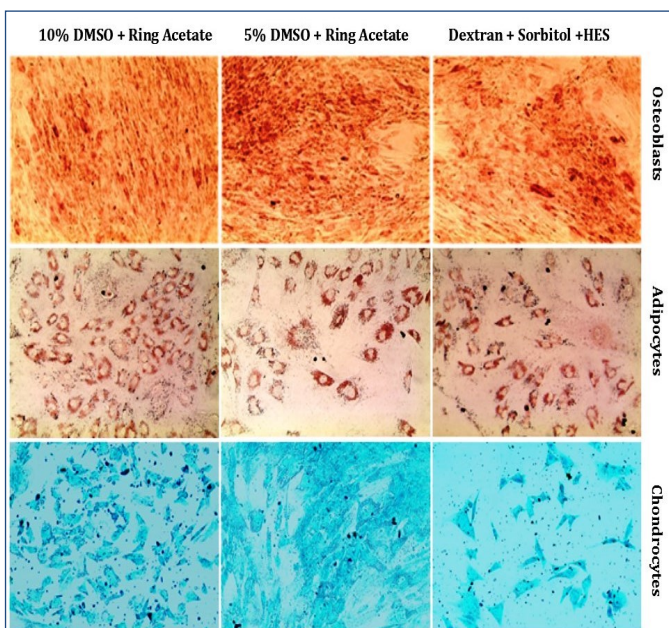


Fig. 5- Determination of differentiation capacity of cryopreserved MSC. Fresh and cryopreserved MSC in the different solutions were differentiated for 14 days into osteoblast, adipocytes and chondrocytes.

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 cryoprotective 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 mammalian 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 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.

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Conflict of Interest : None Declared

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