RESEARCH ARTICLE

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Cytotoxic and cryopreservation effect of different cryoprotectants on human adipose tissue derived mesenchymal stem cells

ABSTRACT

Over the last decade, a number of investigations have been published, demonstrating the perspective clinical applications of mesenchymal stem cells in regenerative medicine and cell therapy. Their long-term storage, with the aim of subsequent clinical usage, is essential for good medical practice. This requires the development and optimization of cryopreservation protocols for this cell type. In our experiments, we analysed human mesenchymal stem cells obtained from adipose tissue. The isolated cultures possessed all the morphological characteristics of mesenchymal stem cells, as well as the potential to differentiate into adipocyte and osteoblast lineages. The immunophenotypic analysis showed positive expression CD73, CD90 and CD105 and absence of the hematopoietic lineage markers of CD34 and CD45. At the next stage, the cytotoxic and cryopreservation effect of several endocellular and extracellular cryoprotectants was assessed, as well as their different combinations. Our investigations pointed out, that the human adipose tissue derived mesenchymal stem cells retain highest percentage viability under program freezing with cryoprotective medium containing 8.5% dimethyl sulfoxide with hydroxyethyl starch.

Key words: mesenchymal stem cells, cytotoxicity, cryopreservation, cryoprotectant

Introduction

Cryobanking of mesenchymal stem cells (MSCs) will allow the increase of the efficiency of their application in cell therapy and tissue engineering. This requires the development and optimization of the protocols for their long-term storage.

Although cryopreservation of different types of cells is widely used in clinical practice, the stress resulting from the freezing process, such as osmotic and cold shock, can cause them irreversible damage (Kim et al., 2016). In the recent decade, stem cells emerge as a promising source for regenerative medicine, still the question whether they retain their characteristic properties after thawing is still unanswered. The loss of pluripotent markers is also associated with freezing (Yuan et al. 2016a). Most of the cryobiological research on MSCs was carried out using slow-rate cooling methods, which are often considered superior technique for preservation (Hennes et al, 2015). Other approaches, like vitrification still need further investigation (Yong et al., 2015a).

To decrease the level of cooling injury cryoprotective agents are added to the freezing medium. Cryoprotectants (CPAs) are classified as either endocellular (penetrating) or extracellular (non-penetrating) depending on their ability to pass through the plasma membrane (Marquez-Curtis et al., 2015). Endocellular CPAs, such as dimethylsulfoxide (DMSO), glycerol, ethylene glycol, propylene glycol, both decrease the freezing point and reduce intracellular crystal formation (Szurek & Eroglu, 2011). Extracellular CPAs, like sucrose, hydroxyethyl starch (HES) and Ficoll stabilize the cell surface membrane and balance the osmotic pressure (Karlsson, 2002).

The cryopreservation medium should be non-toxic, non-immunogenic, chemically inert and able to provide a high percentage of viable cells after thawing. Despite of their strongly expressed cryoprotective activity, most endocellular CPAs have cytotoxic effect, which mainly depends on their concentration, temperature and time for exposure (Davidson et al., 2015). This requires the development of protocols for preservation which focus on the implementation of suitable concentration of the cryoprotectant and optimal cooling rates, in order to minimize the cryoinjury. The combination is specific for various cell types or tissues and extensive cryobiological studies are required to develop precise protocols.

The aim of our investigation was to test the cytotoxicity and cryopreservation effect of several cryoprotectants on human MSCs isolated from adipose tissue (hAT-MSCs).

Materials and Methods

General principles and conditions

The studies were conducted in the period of 2015 – 2016. Informed consents from the patients were obtained for the usage of adipose tissue. Lipoaspirates (n = 18) were collected after liposuction.

Isolation and culture conditions of hAT-MSCs

Before processing, the lipoaspirates were held at 37 °C water bath. After washing the adipose tissue with PBS, solution of 1% collagenase type I-(Gibco) was added and the samples were incubated for 1 hour at 37 °C, 5.5% CO_2. The obtained suspensions were washed 2 times by centrifugation for 10 minutes at 1200 rpm. The supernatants were removed, and the cells were resuspended and plated at a concentration of $1x10^6$ in 24-well plates or flasks and incubated at 37 °C, 5.5% CO_2. AT-MSCs were cultured in DMEM/F-12 (Lonza) with 10% fetal bovine serum (FBS) (Lonza) and penicillin/streptomycin. The medium was replaced every 3-4 days. After reaching 80% confluence the cells were treated with 0.25% trypsin (Lonza) and subcultured.

May Grunwald – Giemsa staining

The cell morphology was analysed under light microscopy with May Grunwald – Giemsa staining used. After removal of the culture medium the cells were washed 3 times with PBS (pH 7.2) and fixed with glacial acetic acid (3%) and methanol (97%) for 2 min. Next, they were incubated with solution of May Grunwald in methanol for 3 min, stained with Giemsa for 30 min, washed and air dried.

Flowcytometry analysis

The cells were analysed with Human Mesenchymal Stem Cells Analysis Kit (BD Stemflow) including a panel of monoclonal antibodies associated with different fluorochromes - fluorescein isothiocyanate (FITC), phycoerythrin (PE) and peridinin chlorophyll protein (PerCP): CD90- FITC; CD105- PerCP-Cy5.5; CD73 -APC; CD34 - PE; CD11b - PE; CD19 - PE; CD45 - PE; HLA-DR – PE. The hAT-MSCs were incubated for 30 min on ice with the antibodies, then washed and resuspended in PBS and analyzed on flow cytometer BD LSR II (BectonDickinson). At least 10,000 cells were acquired for each sample. The assay was performed by software BD Diva (ver. 6.0.1) and WinMD (ver. 2.8).

Adipogenic and osteogenic differentiation of hAT-MSCs

For adipogenic differentiation, the hAT-MSCs after reaching 60-70% confluency were incubated in CompleteMesenCult Adipogenic Medium (Stem Cell Technologies) for about 14 days. The accumulation of lipid vacuoles in the cytoplasm was assessed by Oil Red O staining. For osteogenic differentiation, the culture medium was replaced with Complete MesenCult Osteogenic Medium (Stem Cell Technologies) after the cells were 80% confluent. The medium consisted of MesenCult Basal Meduim, 15% Osteogenic Stimulatory Supplements, 3.5 mM β-glycerophosphate, 10^{-8} M dexamethasone and 50 µg/ml ascorbic acid. The osteogenic differentiation was detected with Von Kossa staining after 5 weeks of incubation.

CPAs toxicity analysis

The effect of some endocellular – DMSO, ethylene glycol, glycerol, and extracellular – Ficoll-70 CPAs on AT-MSC was assessed. For this purpose, the cells were placed for 20-30 min at 37°C in solutions containing different concentrations (20%, 10%, 8.5%, 7%, 5%) of the respective CPAs. Cells incubated only in culture medium were used as a control. Their expansion rate after exposure and time to reach confluency were assessed.

Cell viability assay

AT-MSCs were placed for 5 min at room temperature in 0.4% solution of Trypan blue. The number of vital (unstained) cells was determined using a haemocytometer.

Cryopreservation

The program freezing was performed using Consarctic BV65 (Consarctic GmbH) equipment. AT-MSC were frozen when reaching 80% confluency. After detachment from the culture dish, the cells were centrifuged for 5 min, at 1200 rpm at room temperature. The obtained pellet was resuspended and cryopreservation medium, containing different concentration (10%, 8.5%, 7%, 5%) of dimethyl sulfoxide (DMSO) and 6% hydroxyethyl starch (HES) was added stepwise. The hAT-MSC were transferred to 2 ml cryovials and placed in the biofreezer's chamber, which was pre-cooled to 4 °C. The programme included the following stages: from 4 °C to -20 °C with a cooling rate of 1^oC/min; from -20 °C to -43 °C with 2^oC/min; from -43 °C to -120 °C with 4 °C/min; plunging into liquid nitrogen. Pre-seeding, seeding and post-seeding were performed at -6 °C.

The thawing of the samples was carried out by immersion into 37 °C water bath, serial dilution in order to remove the cryoprotectants and centrifugation.

Statistical analysis

Data are presented as means and ± standard deviations (SD) of the means of the samples. The statistical significance

was assessed using t-test. A probability of $p<0.05$ was considered significant.

Results

hAT-MSC morphology

The hAT-MSCs showed a homogenous morphology during culture. They preserved spindle-like shape and formed characteristic wave-like layers when reaching near confluency (Figure 1). The thawed and cryopreserved cell cultures were maintained through multiple passages and the characteristic fibroblast-like morphology was observed consistently.

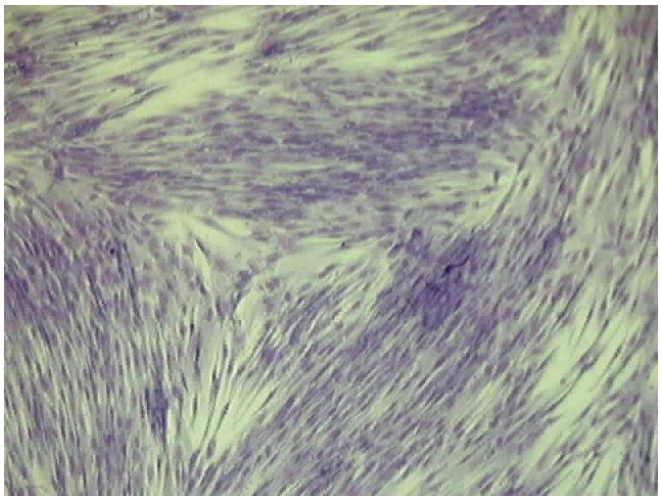

Figure 1. *May-Grunwald Giemsa staining of hAT-MSCs; 40x.*

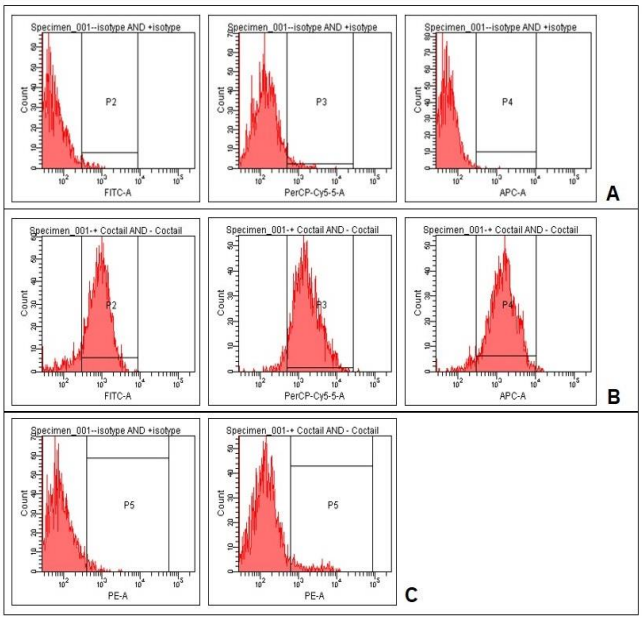

Figure 2. *Flowcytometry analysis.* ***Legend:*** *A) Isotype controls for FITC-A, PerCP and APC; B) Positive expression of CD90-FITC, CD105-PerCP and CD73-APC; C) Isotype control for PE and negative expression of the cocktail of CD34-PE, CD11b-PE, CD45-PE, HLA-DR-PE.*

Flowcytometry analysis of hAT-MSCs

The cell surface antigens of hAT-MSCs were analyzed at passage 4. The cells showed positive expression of CD73, CD90 and CD105 and negative signals for the haematopoietic lineage markers CD34 and CD45. They were also negative for CD11b, CD19, HLA-DR (Figure 2).

Examination of the differentiation potential of the hAT-MSCs

In our study we were able to show that the isolated cells could be induced to adipogenic and osteoblast phenotypes in vitro (Figure 3). The adipogenic differentiation was confirmed by the presence of neutral lipid drops in the cytoplasm of the investigated cells (stained by Oil RedO). After Von Kossa staining, the osteoblast phenotype was demonstrated by the accumulation of calcium.

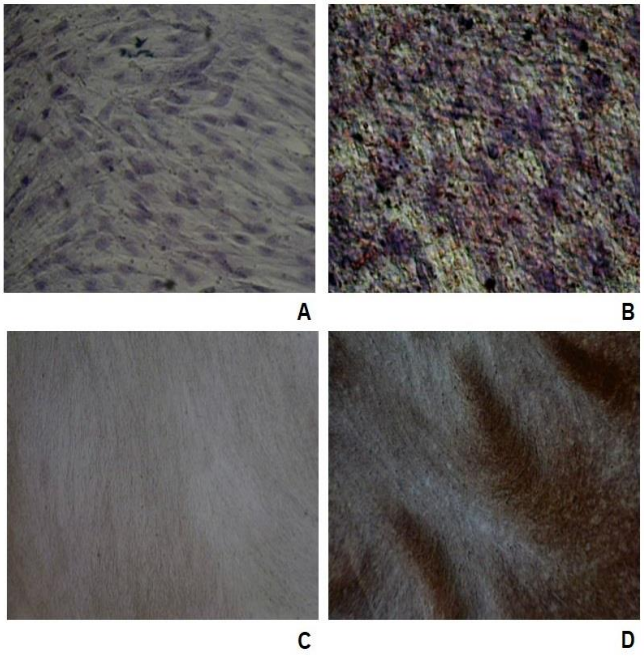

Figure 3. *Adipogenic and osteogenic differentiation of hAT-MSCs.* ***Legend:*** *A) Negative control for adipogenic differentiation of hAT-MSCs (Oil Red O staining and haematoxyllin counterstaining, 60x); B) after adipogenic differentiation (Oil Red O staining and haematoxyllin counterstaining, 60x); C) negative control for osteogenic differentiation (Von Kossa staining, 30x); D) after osteogenic differentiation (Von Kossa staining, 30x).*

Cytotoxic effect of cryoprotectants

In the present investigation we analysed the toxic effect of DMSO, glycerol, ethylene glycol and Ficoll PM70 on hAT-MSCs. Different concentrations of the above CPAs (20%, 10%, 8.5%, 7%, 5%) were tested. After the cells were incubated in the solutions, the retained vitality rate was determined by staining with Trypan Blue and by examining the population doubling times. The highest cytotoxic effect

was observed in the samples treated with DMSO. The cells showed statistically significant lower viability even when placed in 5% solution ($p < 0.05$ compared to the controls), as well as reduced expansion rate. Culture with glycerol displayed similar results. The least toxic effect was observed when the hAT-MSCs were treated with ethylene glycol. At the highest concentrations of the endocellular CPAs tested, the culture was not able to reach confluency until 14 days. The cell vitality was also reduced at 20% Ficoll PM 70 (63.8% ± 3.1), but in the other concentrations no significant differences were detected compared to the controls (Figure 4).

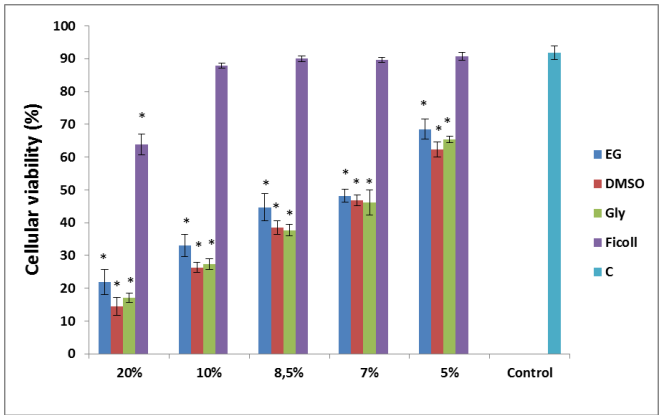

Figure 4. *Percentage vitality of hAT-MSCs after incubation with different cryoprotectants.* ***Legend:*** *EG – ethylene glycol; DMSO – dimethyl sulfoxide; Gly – glycerol; C – control; p <0.05.*

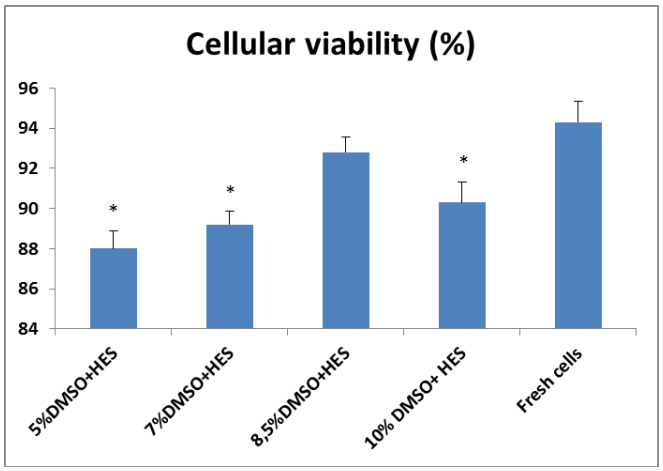

Figure 5. *Percentage recovery of viable (not stained) hAT-MSCs after programme freezing with different cryopreservation media (Trypan blue exclusion test).*

Cryopreservation effect of different cryoprotective media

In the current investigation, we analysed the effect of cryoprotective media containing different concentrations of DMSO (10%, 8.5%, 7%, 5%) in combination with 6% HES. In all the experiments, the samples were cryopreserved via programme freezing. The highest percentage recovery of hAT-MSCs was detected when using medium containing 8.5% DMSO and 6% HES (92.8% ± 0.7) (Figure. 5). Fresh (non-cryopreserved) cells were used as control.

Discussion

MSCs are considered to hold great promises for regenerative medicine due to their multilineage differentiation potential, ability to promote angiogenesis, immunomodulatory properties, unique secretome profile and potential for homing (Panchalingam et al., 2015; Perdisa et al., 2015). They can be isolated from different tissues of the adult and fetal organisms. That accounts for their relatively high population heterogeneity and the necessity for standardisation of the current protocols. In attempt to address this issue, the International Society for Cellular Therapy published the minimal criteria for defining multipotent MSCs: they have to be plastic-adherent during in vitro culture, express CD73, CD90 and CD105 and lack the hematopoietic lineage markers CD45 and CD34, as well as CD14 or CD11b, CD79 or CD19 and HLA-DR. Furthermore, MSCs must be able to differentiate into osteoblasts, adipocytes and chondroblasts in vitro (Dominici et al., 2006).

Mesenchymal stem cells derived from adipose tissue are regarded as a promising source for cell therapy and tissue engineering, also because they are relatively easily isolated and the lipoaspirates are usually discarded. In our investigation, we collected the aspirates and isolated MSCs following the previously described procedures (Yu et al., 2011). The obtained primary culture was plastic-adherent, and showed good proliferative activity in vitro, as cells got attached to the substrate during the first 24 hours and formed a confluent monolayer within 8 to 10 days. Such characteristics are described for MSCs isolated from bone marrow (Cai et al., 2015). During long-term culture, hAT-MSCs retain their typical fibroblast-like shape even after repeated passaging. On reaching full confluency, the cells displayed typical for the MSCs wave-like layers (Figire 1) (Kadivar et al., 2006). In addition, the isolated cultures possess all the morphological characteristics of MSCs, as well as the potential to differentiate into adipocyte and osteoblast lineages.

The flowcytometry analysis demonstrated that the cells have positive expression of CD73, CD90 and CD105 and miss the haematopoietic lineage markers CD34 and CD45. The hAT-MSCs were also negative for CD11b, CD19 and HLA-DR. The lack of expression of the HLA-DR antigen was observed by other investigators and may limit immune recognition (Gotherstrom et al., 2004) which is a very important feature of MSCs as potential cellular therapeutics. These results correspond with the findings of the majority of

other research groups and with the minimal criteria of the International Society for Cellular Therapy (Dominici et al., 2006).

Another important aspect for the future use of MSCs in clinical practice is the development and optimization of the cryopreservation protocols. Therefore, in the present study we aimed at investigating the cytotoxic effect of different cryoprotective agents at various concentrations. Not surprisingly, the least concentrated solutions showed the lowest toxicity levels for all of the substances studied. However, in the cryopreservation process such limited amounts are not effective to use. In our research, we used both main types of CPAs – exo- and endocellular. The hAT-MSCs retained the highest percentage viability after incubation with ethylene glycol. DMSO and glycerol showed similar results.

In long-term storage of stem cells in clinical practice, the most widely used CPA is DMSO. Our analysis pointed out, that it exhibits high cytotoxicity, but it is a preferred CPA because of its good cryoprotective activity and ability to maintain adipose MSCs viability after thawing (Yong et al., 2015a). Those results correspond with our previous experiments, where we examined the effect of 10% solutions of endocellular CPAs (DMSO, ethylene glycol and glycerol) and found that DMSO was the most effective. Ficoll-70, which does not penetrate the cell membrane showed the least cytotoxic effect, but it is not approved for medical use. For the above mentioned reasons, we decided to carry out further investigations with cryoprotective medium containing combination of DMSO as endocellular and hydroxyethyl starch as exocellular CPAs.

At the next stage of our study, we compared the effects of programme freezing under the protection of varying concentrations of DMSO in combination with HES. The largest percentage cell recovery was detected after cryopreservation with medium containing 8.5% DMSO and 6% HES. Similar results were reported for rat bone marrow derived MSCs, where the highest vitality after thawing was observed with 8% DMSO and 2% HES (Naaldijk et al., 2012). The smallest concentration of DMSO led to reduced survival rate, irrespective of its low cytotoxicity, which proves again the fact that the optimal concentration of CPAs in the medium should be balanced between their toxic effect and cryoprotective properties. Freezing under 7% and 10% DMSO in combination with HES rendered statistically significant reduced percentage vitality compared to the controls. Most of the research groups use 10% DMSO for cryopreservation of MSCs (Yong et al., 2015b), because of its high membrane permeability. However, in large concentrations it could be damaging to the cells, as has been shown in the present study as well. Also, it has to be completely removed from the suspension for administration, as it can cause adverse effects in patients (Yuan et al., 2016b). Furthermore, the addition of exocellular CPA to the medium allows the reduction of the amount of the endocellular one.

Another important aspect to be mentioned is that the investigated cells maintained their morphological characteristics and immunophenotypic profile after thawing. These results correspond with the reports from other authors, that the mesenchymal stem cells keep their properties and differentiation potential (Shivakumar et al., 2015) after long-term storage.

In conclusion, our results point out that the hAT-MSCs are cryotolerant, because they retain high survival rates after programme freezing with different combinations of CPAs. The cells cryopreserved with medium containing 8.5% DMSO and 6% HES showed the largest percentage vitality after thawing, with no statistically significant difference compared to the controls, which gives us ground to recommend this protocol for future use in clinical practice.

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