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Brief Communication

The effects of cryopreservation on cells isolated from adipose, bone marrow and dental pulp tissues

O.G. Davies *, A.J. Smith, P.R. Cooper, R.M. Shelton, B.A. Scheven

School of Dentistry, University of Birmingham, St Chads Queensway, Birmingham B4 6NN, UK

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ABSTRACT

The effects of cryopreservation on mesenchymal stem cell (MSC) phenotype are not well documented; however this process is of increasing importance for regenerative therapies. This study examined the effect of cryopreservation (10% dimethyl-sulfoxide) on the morphology, viability, gene-expression and relative proportion of MSC surface-markers on cells derived from rat adipose, bone marrow and dental pulp. Cryopreservation significantly reduced the number of viable cells in bone marrow and dental pulp cell populations but had no observable effect on adipose cells. Flow cytometry analysis demonstrated significant increases in the relative expression of MSC surface-markers, CD90 and CD29/CD90 following cryopreservation. sqRT-PCR analysis of MSC gene-expression demonstrated increases in pluripotent markers for adipose and dental pulp, together with significant tissue-specific increases in CD44, CD73–CD105 following cryopreservation. Cells isolated from different tissue sources did not respond equally to cryopreservation with adipose tissue representing a more robust source of MSCs.

Introduction

Non-hematopoietic stem cells have been described in many tissues and were originally termed fibroblastic colony-forming-units because they readily adhered to culture dishes and formed colonies of fibroblast-like cells [2,20–23]. These cells are currently referred to as mesenchymal stem cells (MSCs) [1] because of their ability to differentiate into a variety of mesenchymal cell types such as adipose tissue, cartilage and bone [20–23]. MSCs were first identified in the bone marrow but have since been isolated from almost every tissue in the body [1,8,28]. Dental stem cells isolated from the gelatinous pulp region of the tooth were identified by Gronthos et al. [8], and have been employed for a wide range of regenerative therapies, most prominently neuronal and orthopaedic therapies [8,13,26]. Of the large number of tissues from which MSCs have been isolated adipose tissue is perhaps the most clinically useful, since this tissue contains relatively large numbers of stem cells (⩽10%) when compared with the bone marrow (0.001–0.1%) that can be isolated in large volumes, relatively non-invasively with minimal patient discomfort [25,28].

The capacity of mesenchymal stem cells (MSCs) to survive long-term storage and maintain their phenotype upon thawing is critical if they are to be banked and used for future therapeutic purposes. Reductions in cell viability and alterations in the expression of gene and phenotypic cell surface markers may have implications for the therapeutic application of MSC, including reduced functional and differentiation capacity. Cryostorage represents a physical insult to cells resulting in structural and molecular changes within cells. To protect cells from damage during the cryopreservation process and to maximise cell recovery cryoprotectants are incorporated within the freezing medium. The concentration of cryoprotectants added to the medium is one of the primary factors governing the survival of frozen cells. The majority of published cryopreservation protocols incorporate 10% DMSO in order to prevent the formation of intra- and extra-cellular crystals during the freezing process [4,24]. However, recent studies have reported that the survival and number of colonies formed by MSCs is significantly decreased following cryostorage and that the magnitude of this decrease is inversely proportional to DMSO concentration [19]. Moreover, the use of DMSO as a cryoprotectant has been shown to be ineffective at protecting some cell types from cold shock (0 to +8 °C) during the freezing process [11], highlighting the need for further investigation into the effects of cryostorage using 10% DMSO on MSC viability and self-renewal.

It is presently unknown whether post-thaw MSCs retain the same potential for regenerative therapeutic applications as their non-cryopreserved counterparts. The response of stem cells to

Abbreviations: DMSO, dimethyl sulfoxide; ADSC, adipose-derived stem cell; BMSC, bone marrow stem cell; DPSC, dental pulp stem cell; MSC, mesenchymal stem cell; FACS, fluorescence-activated cell sorting; sqRT-PCT, semi-quantitative reverse transcriptase polymerase chain reaction; PDT, population doubling time.

* Corresponding author. Address: School of Dentistry, St Chads Queensway, Birmingham, West Midlands B4 6NN, UK.
E-mail address: owendavies777@outlook.com (O.G. Davies).

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cryopreservation can include a reduction in cell viability due to cold-shock and/or the toxic effects of DMSO, and changes in the expression of stem cell-related markers, cytoskeletal disassembly, delayed apoptosis, and osmotic and oxidative stresses [27]. These factors may have an influence on the functionality of MSCs and reduce their applicability for regenerative therapies. It has subsequently been suggested that the effects of cryopreservation may be responsible for the failure of a randomised phase III clinical trial using random donor MSCs in the treatment of steroid resistant graft-versus-host disease (NCT00366145) [7]. At present, much of the information concerning the effects of cryopreservation on MSCs is difficult to interpret as studies frequently isolate MSCs from different tissue sources and store them for variable periods of time [17,22].

In the present study, we examined the effect of 14 days cryostorage on the viability, morphology, gene expression and immunophenotype of adipose-derived cells (ADCs), bone marrow cells (BMCs) and dental pulp cells (DPCs) derived from rats. The rat was used as a model since MSCs isolated from this species have a very similar cell-surface marker profile and differentiation potential when compared with human MSCs [9]. The use of a rat model also enabled the comparison of cells isolated from several tissues within the same animal, thereby limiting potential error caused by intra-species variation. A traditional graded freezing protocol (4 °C, 1 h; −20 °C, 2 h; −80 °C, overnight; −136 °C, 14 days) was adopted throughout this study since previous work has demonstrated that this method is no less effective in maintaining post-thaw viability of MSCs compared with controlled freezing, with consistent nucleation observed [18]. This work was undertaken to determine if cryopreservation with 10% DMSO affected the viability and the capacity of ADCs, BMCs and DPCs to survive fluorescence-activated cell sorting (FAC), which is arguably the most routinely used procedure for MSC isolation. The effect of cryopreservation on the cell-surface marker profile of MSCs was analysed using FACS to assess whether cryopreservation influenced the proportion of MSCs within heterogeneous cultures. Additionally, the expression profiles of pluripotent/multipotent genes between cryopreserved and non-cryopreserved cells were compared, as changes in these transcripts may alter their functionality and differentiation potential, and may therefore limit the clinical potential of these cells.

Methods

Cell culture

Adipose, bone marrow and dental pulp tissues were isolated from six week old male Wister Hann rats (weight ~120 g) (Aston University, Pharmaceutical Sciences Animal House, Birmingham, UK; ethical approval reference: BCHDent286.1471.TB). Cells were isolated from each tissue using a standard protocol [6].

Cryopreservation of cell isolates

To prepare cells for cryostorage, ~80% confluent passage 1 cultures containing approximately 2.5×10^6 cells per 75 cm^2 culture flask (Nunc, UK) were detached using 0.25% trypsin, 1 mM EDTA·4-Na (2.5 g/L trypsin in 0.38 g/L EDTA) (Gibco, UK), and centrifuged at 900 g for a period of 5 min. The supernatant was aspirated and an equal volume of cells re-suspended in 0.4% Trypan blue solution to provide a cell viability count. 1×10^6 cells were re-suspended in cryogenic medium [90% FBS containing 10% dimethyl sulfoxide (DMSO)] [15] and the cell suspensions transferred to cryogenic vials that were prepared for liquid nitrogen storage by incubation at 4 °C for 1 h, then at −20 °C for 2 h and subsequently −80 °C overnight. Frozen cell suspensions were then transferred to liquid nitrogen storage. 10 vials of cryostored cells were recovered from storage by thawing in a 37 °C RS Galaxy S + incubator (RS Biotech, UK) for ~5 min. To remove residual cryogenic medium prior to culture, the contents of each vial were transferred to 15 mL Falcon® tubes containing 5 mL α-MEM + 10% FBS and centrifuged at 900 g for 5 min. Cell viability was measured immediately after thawing using the Trypan blue exclusion assay and approximately 2×10^5 cells seeded in 25 cm^2 culture flasks (Nunc, UK). Cells were cultured to approximately 80% confluence, at which point Trypan blue cell counts were performed and population doubling times (PDT) calculated using the following equation:

$$PDT = T \ln 2 / \ln(Xe/Xb)$$

T = incubation time, Xb = cell number at the beginning of incubation, Xe = cell number at the end of incubation.

Cell viability during flow cytometry

To examine the capacity of cryostored cells to undergo flow cytometric cell sorting, cryostored and non-cryostored cells established at passage 2 were cultured until ~80% confluent (80% confluency was reached after 5 days for ADCs and BMCs, and after 7 days for DPCs). Cells were detached using 0.25% trypsin, 1 mM EDTA·4Na (2.5 g/L trypsin in 0.38 g/L EDTA) (Gibco, UK), centrifuged at 900 g for 5 min, neutralised with α-MEM + 10% FBS and the resulting suspensions transferred to 15 mL Falcon® tubes. Cell suspensions were incubated in FACS buffer (sterile PBS + 1% FBS) and maintained under constant agitation using an orbital shaker, mimicking conditions experienced during flow cytometry. The number of viable cells were determined every 30 min during this incubation period at 4 °C for a total period of 5 h by adding 0.4%

Table 1
DNA sequences, annealing temperatures, and cycle numbers for primers used in the sqRT-PCR reaction. All primers were designed using Primer Blast software (<http://ncbi.nlm.nih.gov/tools/primer-blast/>) and manufactured by Invitrogen, UK.

Gene name	Sequence (5′ → 3′)	Annealing temperature (°C)	Cycle number	Accession number
Normalisation				
GAPDH	F-CCCATCACCATCTTCCAGGAGC;R-CCAGTGAGCTTCCCGTTCAGC	60.5	21–27	NM_017008
Pluripotent markers				
Klf4	F-ATCATGGTCAAGTTCCCAGC; R-ACCAAGGACCATCGTTTAGG	60.5	35	NM_052713
C-myc	F-CTTACTGAGGAAACGGCGAG; R-GCCCTATGTACACCGGAAGA	60.5	35	BC091699
Nanog	F-TATCGTTTTGAGGGGTGAGG; R-CAGCTGGCACTGGTTTATCA	60.5	35	NM_001100781
Lin28	F-TTTCTTGTTTCCCCCAAATG; R-AGAGGGGCTGGTTGTAAGGT	60.5	35	NM_001109269
SOX-2	F-ATACAAGGGAATTGGGAGGG; R-AAACCCAGCAAGAACCCTTT	60.5	25	NM_001109181
Multipotent markers				
CD44	F-TGGGTTTACCCAGCTGAATC; R-CTTGCGAAAGCATCAACAAA	60.5	33–37	NM_012924.02
CD105	F-TTCAGCTTTCTCCTCCGTGT; R-TGTGGTTGGTACTGCTGCTC	60.5	41–45	NM_001010968
CD73	F-GGACTGATTGATCCCCTCCT; R-TTGTCCCTGGATTTGAGAGG	60.5	25	NM_002526
CD29	F-AATGGAGTGAATGGGACAGG; R-TCTGTGAAGCCCAGAGGTTT	60.5	25	NM_017022.2
CD90	F-AGCTCTTTGATCTGCCGTGT; R-CTGCAGGCAATCCAATTTTT	60.5	26	NM_012673

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