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Hypoxia enhances the viability, growth and chondrogenic potential of cryopreserved human adipose-derived stem cells

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ABSTRACT

Cryopreservation is the only existing method of storage of human adipose-derived stem cells (ASCs) for clinical use. However, cryopreservation has been shown to be detrimental to ASCs, particularly in term of cell viability. To restore the viability of cryopreserved ASCs, it is proposed to culture the cells in a hypoxic condition. To this end, we aim to investigate the effect of hypoxia on the cryopreserved human ASCs in terms of not only cell viability, but also their growth and stemness properties, which have not been explored yet. In this study, human ASCs were cultured under four different conditions: fresh (non-cryopreserved) cells cultured in 1) normoxia (21% O_2) and 2) hypoxia (2% O_2) and cryopreserved cells cultured in 3) normoxia and 4) hypoxia. ASCs at passage 3 were subjected to assessment of viability, proliferation, differentiation, and expression of stemness markers and hypoxia-inducible factor-1 alpha (HIF-1α). We found that hypoxia enhances the viability and the proliferation rate of cryopreserved ASCs. Further, hypoxia upregulates HIF-1α in cryopreserved ASCs, which in turn activates chondrogenic genes to promote chondrogenic differentiation. In conclusion, hypoxic-preconditioned cryopreserved ASCs could be an ideal cell source for cartilage repair and regeneration.

1. Introduction

Stem cell therapy is becoming a promising option for the treatment of degenerative diseases such as diabetes, spinal cord injury, stroke, myocardial infarction, Parkinson's and Alzheimer's diseases [24]. Among all types of stem cells, mesenchymal stem cells (MSCs) have been found to be a potential candidate in those therapies due to their unique properties, including multilineage differentiation ability, immunosuppressive and paracrine functions [1]. In recent years, adipose tissue was found to be a better source of MSCs due to its abundance and ease of harvest with minimally invasive procedures [13]. Given that long-term cultured human adipose-derived stem cells (ASCs) have raised biosafety concerns such as chromosomal aberration, spontaneous malignant transformation [11,28], and senescence [29], therefore it is necessary to preserve and store the ASCs at the early culture for clinical use.

Cryopreservation is the existing common method for preservation or storage of cells below freezing point between −80 °C and −196 °C, in order to halt all cellular function and metabolism [2]. However, cryopreservation processes (freezing and thawing) involve abrupt changes in thermal, chemical and physical environment surrounding the cells and thus may cause cellular damages including cell death [17]. Such damages can be reduced with the use of a cryoprotective agent (CPA) to reduce cell shrinkage during dehydration and to prevent the formation of intracellular ice which causes the cell rupture during cryopreservation [36]. The most common CPAs used are permeating CPAs, *e.g.*, glycerol and dimethylsulfoxide (DMSO), with DMSO has been demonstrated to be superior to glycerol due to its relatively higher potential in penetrating cell membrane [16]. Although cryopreservation has been shown to maintain phenotype and functional properties (*e.g.*, stemness and differentiation potential) of ASCs, but it may still induce cryo-injury to cells as indicated by their reduced post-thaw viability (75–85%) [31,34]. Studies have shown that cryopreservation is detrimental to cell viability, which could be due to the cryopreservation processes (*e.g.*, freezing and thawing) and the

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toxic effects of a high concentration of DMSO [12,27], leading to the activation of cell apoptosis. This mechanism has been reported to be the main cause of the low survival rate of cryopreserved cells [14]. To restore the viability of cryopreserved ASCs, it is hypothesized that one of the ways to do this is by incubating the cryopreserved ASCs in a hypoxic condition since this condition has been shown to support or enhance cellular growth and viability [4].

In general, inspired oxygen tension drops from atmospheric level (approximately 21%) to 2%–9% when oxygen reaches the organs and tissues [9,30]. However, most *in vitro* cell cultures are maintained at approximately 21% O_2, which is in contradicted to the oxygen concentration (<4%) at adipose tissues where ASCs usually reside [23,26]. Several studies have clearly shown the negative impact of atmospheric oxygen tension on ASCs and also the positive impact of low oxygen tension on ASCs. These include a relatively lower cell viability and proliferation rate, and higher degree of DNA damage in ASCs cultured in high oxygen tension, as compared to those cultured in low oxygen tension [6,10,33]. Although evidences have shown that hypoxia is able to contribute to the increase of viability and proliferation of non-cryopreserved or fresh ASCs, but the effect of hypoxia on cryopreserved ASCs is still unknown. Herein, the aim of this study is to investigate the effect of hypoxia on viability, proliferation and stemness properties of cryopreserved human ASCs.

2. Materials and methods

2.1. Isolation and culture of human ASCs

This study was approved by the Medical Ethics Committee of University Malaya Medical Centre (Reference no: 996.46). Human adipose tissues were harvested from 6 healthy female donors aged 25–35 years who were undergoing Caesarean section with prior informed written consent. Isolation of ASCs was performed conform the declaration of Helsinki using protocols as described elsewhere [34,35]. ASCs were divided into 2 groups: 1) **FN**: a normoxic group in which ASCs were cultured at 21% O_2 and 5% CO_2 at 37 °C and 2) **FH**: a hypoxic group in which ASCs were placed in an oxygen controlled incubator (Galaxy 170 R, New Brunswick Scientific, USA) supplied with nitrogen to maintain 2% O_2 (low oxygen tension) at 5% CO_2 and 37 °C. Both oxygen levels were confirmed with a Jenway 970 portable dissolved oxygen meter (Bibby Scientific Limited, Staffordshire, UK). The cells were sub-cultured up to passage 3 (P3) prior to be used for all the tests.

2.2. Cryopreservation of human ASCs

Cryopreservation of ASCs was performed using a slow freezing method. Approximately 1×10^6 ASCs at the end of passage 2 were suspended in a cryomedium containing 10% (v/v) DMSO (Sigma-Aldrich) and 90% (v/v) fetal bovine serum (FBS), and loaded into a cryogenic vial (Nalgene, Thermo Fisher Scientific Inc, USA). The cryovial was moved into "Mr. Frosty" (Nalgene), a freezing container which offers a freezing rate of 1 °C/min, before being kept in −80 °C overnight. The cryovial were then kept in liquid nitrogen at −196 °C for 1 month. After 1 month, the frozen cells were thawed rapidly in a waterbath at 37 °C. The cells were then resuspended in culture medium and centrifuged to remove the CPAs. The cryopreserved ASCs were divided into 2 groups: 1) **CN**: a normoxic group in which ASCs were cultured at 21% O_2 and 5% CO_2 at 37 °C, and 2) **CH**: a hypoxic group in which ASCs were placed in an oxygen controlled incubator with 2% O_2 and 5% CO_2 at 37 °C. The cells were sub-cultured to P3 before being harvested for testing and analysis.

2.3. Morphological examination, cell viability and proliferation assay

Morphology of fresh (non-cryopreserved) and cryopreserved ASCs at P3 cultured in both normoxia and hypoxia were observed through microscopic examination. To assess their cell viability, a trypan blue exclusion assay (Gibco) was conducted. Total dead cells and live cells were counted, and percentage of cell viability was determined. On the other hand, proliferation potential of ASCs was performed using a Resazurin reduction assay (Sigma-Aldrich). In brief, the cells were seeded in a 24-well plate with 3×10^4 cells per well and incubated overnight for cell attachment. The assay was conducted after 24 h (day 1), on days 3 and 7. The absorbance of Resazurin at wavelengths of 570 nm and 595 nm was measured using a FLUOstar Optima microplate reader (BMG Labtech, Offenburg, Germany). Percentage of Resazurin reduction was then determined using a formula recommended by manufacturer. Finally, the cell number of ASCs corresponding to the percentage of Resazurin reduction on each day of culture was determined.

2.4. Immunophenotyping

The expression of cell surface markers in ASCs at P3 cultured on day 7 was determined using immunophenotyping. In brief, ASCs were trypsinized using Accutase cell detachment solution (Innovative Cell Technologies, Inc., San Diego, USA). About 5×10^5 cells were subjected to staining with specific antibodies conjugated with fluorochrome as follows: FITC-conjugated CD105, CD90, CD45, CD34 and HLA DRDPDQ and PE-conjugated CD73, CD14 and CD19 (Becton Dickinson, San Jose, USA), each for 30 min in the dark on ice. Cells stained with FITC-conjugated mouse IgG_1 & IgG_2 isotypes, and PE-conjugated mouse IgG1 & IgG2 isotypes (Becton Dickinson) were used as negative controls. The data were acquired with a minimum of 10,000 events per sample using a flow cytometry system (BD FACSCanto II, Becton Dickinson) followed by analysis using a FlowJo software (Treestar, OR, USA).

2.5. Cell differentiation assay

ASCs at P3 cultured on day 7 from each group were evaluated for their adipogenic, osteogenic and chondrogenic differentiation potential. For adipogenic differentiation, ASCs were cultured in an adipogenic induction medium containing DMEM/F12 with 10% FBS, 200 μM indomethacin (Sigma-Aldrich), 0.5 μM isobutyl-1-methyl xanthine (Sigma-Aldrich), 1 μM dexamethasone (Sigma-Aldrich) and 10 μM insulin (Sigma-Aldrich) for 21 days. The cells were stained using Oil red O (Sigma-Aldrich) to observe the presence of lipid droplets.

For osteogenic differentiation, ASCs were cultured in an osteogenic induction medium containing DMEM/F12 with 10% FBS supplemented with 100 nM dexamethasone, 10 mM b-glycerophosphate (Sigma-Aldrich), and 0.05 mM ascorbic acid-2-phosphate (Sigma-Aldrich) for 21 days. The cells were stained using Alizarin red (Sigma-Aldrich) to observe the presence of calcium deposits.

For chondrogenic differentiation, ASCs were cultured in a chondrogenic induction medium composed of DMEM/F12, 1% FBS, 1% antibiotic-antimycotic, 1% vitamin C, 1% glutamax, 1% ITS premix (Becton Dickinson), 50 μg/ml ascorbic acid-2-phophate, 100 nM dexamethasone, 40 μg/ml L-proline (Sigma-Aldrich), 10 ng/ml TGF-β1 and 50 ng/ml IGF-1 (Peprotech, Rocky Hill, USA) for 21 days. The cells were stained using Alcian blue (Sigma-Aldrich) to observe the presence of proteoglycan. The cells were also counterstained with 0.1% Nuclear Fast Red Solution (Sigma-Aldrich). Further, the gene expression of specific markers for each differentiation was