Osteogenic differentiation potential and quantification of fresh and cryopreserved dental follicular stem cells-an in vitro analysis

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Abstract

Purpose: To isolate and characterize mesenchymal stem cells of dental follicle from fresh and cryopreserved samples and to test any significant difference in their osteogenic differentiation potential by using digital imaging software. We also investigated whether the cryoprotectant used and its concentration is able to maintain cell count and viability.

Methods: Mesenchymal stem cells (MSCs) were isolated from dental follicle of impacted third molars. The osteogenic differentiation potential of dental follicle stem cells was assessed using alizarin red and alkaline phosphatase staining followed by digital imaging quantification of the stains.

Results: Dental follicle cells have shown typical characterisation by exhibiting the stem cell stromal markers and hematopoietic markers, but there was variance in the percentage of expression in fresh and cryopreserved samples. There was considerable osteogenic differentiation potential in the fresh sample compared to cryopreserved sample. The cell count and viability were preserved in both samples.

Conclusions: The results in the study have shown wide variation of osteogenic differentiation potential in fresh and cryopreserved samples. Also, the cryoprotectant was found to be effective in its purpose at the specified concentration.

Key Words: Dental follicle; Mesenchymal stem cells; Cryopreservant; Osteogenic differentiation; Digital imaging

Introduction

Mesenchymal stem cells (MSCs) have proved to be invaluable in regenerative medicine because of their two remarkable characteristics of self-renewal and multi-lineage differentiation. Stem cells can broadly be classified as Embryonic Stem Cells (ESCs) and Adult Stem Cells (ASCs), based on their origin and differentiation potential[1,2,3]. The ASCs isolated from various dental tissues are commonly referred to as Dental Stem Cells (DSCs). MSCs were first isolated by Gronthos et al. from dental pulp (DPSCs)[4] and subsequently from the dental pulp of human exfoliated deciduous teeth (SHEDs)[5]. The dental follicle is a loose connective tissue that surrounds the developing tooth and is separated from dentin by an epithelial layer (Hertwig’s sheath) with three distinct stem cell populations (hDF1, hDF2, hDF3), distinct morphologies, gene expression and differentiation potential[6,7]. Dental follicle stem cells (DFSCs) are known as the progenitor cells for the formation of all periodontium related tissues, including periodontal ligament, cementum and alveolar bone[8]. DFSCs can be isolated from developing tissues, compared to other stem cells of dental tissue origin[9]. DFSCs also express surface antigen markers similar to the other dental stem cells (positive for CD44, CD105, CD29, negative for CD34, CD117, the putative stem cell markers Notch-1 and Nestin)[10]. DFSCs are obtained easily after tooth extraction. DFSCs are also available from inflamed or diseased dental tissues and exhibit properties similar to those of DFSCs obtained from healthy tissues[11]. Interestingly, the DFSCs manifest characteristics of both mesoderm and ectoderm, which contributes to the union of mesenchymal stem cells (required for the formation of odontoblasts, cementoblasts, osteoblasts, fibroblasts) and epithelial stem cells (involved in forming ameloblasts), which is imperative for regenerating a new tooth[12,13]. DFSCs exhibit fibroblastic morphology and excellent proliferative capacity, with selective adherence to solid surfaces. They can be cryopreserved for either research or future medical therapy. Cryopreservation is done to prevent contamination by microorganisms and other cell lines and used for long term maintenance. Freezing media is a serum-free media with dimethyl sulphoxide (DMSO) in 10% concentration as cryoprotectant. There is controversy regarding the optimal cryoprotective agent and the concentration required to minimise cytotoxicity of the banked cells, the minimum number of cells required for cryopreservation to obtain viable cell lines after thawing, the storage time and the storage temperature.

The objectives of our research were to isolate and characterise mesenchymal stem cells from the dental follicle of fresh and cryopreserved samples harvested from third molar sites and to test any significant difference in their in vitro osteogenic differentiation potential by quantifying the expression of alkaline phosphatase and alizarin red stains using digital imaging software. We have come across many methods for cryopreservation of dental stem cells to maintain their pluripotency after cryopreservation. But literature is still lacking related to the concentration and method of cryopreservation adequate to maintain cell viability and count without causing cytotoxicity. Here, we assessed the efficacy of the
cryopreservant in a predetermined concentration in maintaining cell count and viability, as well as the scope for preserving fresh samples for osteogenic differentiation compared to cryopreserved ones.

**Methods**

**DFSC culturing and cryopreservation**

Discarded dental follicles from extracted third molar sites from patients in the age range of 14 to 28 years who underwent removal of immature impacted third molars for orthodontic reasons were taken for the isolation and culture of DFSCs. Nine samples were obtained from healthy subjects and processed on the same day. No associated pathology was noted in any of these samples. Tooth gerns were removed under approved guidelines and dental follicle separated and placed in Dulbecco's phosphate buffered saline (D-PBS). Under aseptic conditions, the tissues were minced into 1–3 mm explants, transferred to sterile bottles, washed with PBS. To this, 1 ml collagenase 3mg/ml was added for tissue digestion and placed in agitation in a shaker incubator at 37°C for 40 min to separate cells. When taken out after 40 min, cells were resuspended using pipettes, and the solution was checked for cells. If found positive for cells, the solution was centrifuged at 1500 rpm for 5 min to get the cell pellet. After adding 4 ml DMEM to the test tube, the cells were resuspended and the solution pipetted into 25 cm² tissue culture vented flasks and placed in an incubator in a humidified atmosphere at 37°C with 5% CO₂ for the culture of DFSC. DFSCs were cultured in alpha minimum essential medium (GIBCO); total media was 500ml, DMEM-440 ml, 10% FBS-50ml, Pen-strep-5ml, NEAA-5ml. Following trypsinisation, cells were counted, centrifuged with PBS, the supernatant removed, 4 ml DMEM was added and the mixture subcultured similarly in 75 cm² tissue culture flasks for 2 to 3 passages, with six samples being cryopreserved for later use. For cryopreservation, the cells were resuspended in 1ml freezing media and aliquoted to two cryogenic storage vials. Freezing was carried out using the gradual cooling method, initially at temperatures of -80°C in a freezer and later moving the mixture into a liquid nitrogen tank with liquid nitrogen in two phases, a liquid phase at -80°C in a freezer and later moving the mixture into a liquid nitrogen tank with liquid nitrogen in two phases, a liquid phase at -80°C in a freezer and later moving the mixture into a liquid nitrogen tank with liquid nitrogen in two phases, a liquid phase and a solid phase of liquid nitrogen at -196°C for 40 min. Photographs were taken in 5X and 10X magnification of each well, and the images analysed quantitatively by using Image J software according to the standardised protocol. Software setup was done initially by going to menu-analyse-set scale-distance in pixels=637.6, known distance 1, unit of length in mm=OK-type of image=RGB <split image image=adjust threshold, and colour thresholding used. The pixels in the threshold range are displayed in red. All samples were evaluated using the same threshold — select analyse-measurement-select area, fraction, limit to threshold, display label=OK=analyse=measure. We used area percentage as our read out and calculated the fold change in the staining area.

**Results**

**Rate of cell growth and morphology**

Cells were found positive for cells in 25 ml tissue culture flasks (TCF) under an inverted microscope. Cells were noticed positive at 5 to 7 days in all samples, showing spindle-shaped fibroblast morphology. Two samples were discarded due to contamination. Six samples underwent 2 passages before cryopreservation, and the last subculture was done in 75 ml TCF. One sample which did not undergo cryopreservation became positive for stem cells in 7 days and underwent 3 passages and survived long term culture for 44 days before being taken for characterisation and osteogenic differentiation. Cell growth was calculated by taking the cell count before freezing the samples for an average of 21 days, and the average cell count was 1.607 X 10⁶ cells/ml. Samples were cryopreserved for a period ranging from 3 months to one year before being taken for characterisation. Although cells were positive and viable in cryopreserved samples, cell count had decreased to an average of 0.58 X 10⁶ cells/ml, as they were stored in 2 separate vials while being frozen, so they were subjected to subculture before undertaking characterisation and differentiation.

**Characterisation of MSC markers in fresh and cryopreserved samples**

All hematopoietic stem cell markers were found to be negative or weakly positive, and HLA-DR was found to be negative in all samples, regardless of whether they were cryopreserved or fresh. CD 34 and CD 14 showed variable expression of sometimes positive and sometimes negative, as they also represent primitive stromal cells other than hematopoietic cells. Fresh sample without cryopreservation showed 95% fluorescence for all stromal cell-associated MSC markers, whereas cryopreserved samples showed an average immunofluorescence of 92.3% for CD44APC, 90.6% for CD73PE, 85.6% for CD 29PE, 97% for CD13APC, 90.5% for CD90FITC, 15% for CD14APC and 43.2% for CD 105PE. MSC phenotypic analysis by FACS is shown in Figure 1 and Table 1.
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**Figure 1:** MSC Phenotypical analysis

**Table 1:** Distribution of MSC and Hematopoetic marker expression

| Sample name | CD45 FITC | CD34 FITC | CD31 APC | CD14 APC | HLADR PE | CD73 PE | CD29 PE | CD44 APC | CD13 APC | CD90 FITC | CD105 PE | CD146 PE |
|-------------|-----------|-----------|----------|----------|----------|--------|--------|----------|----------|----------|---------|---------|
| 22 MPMF     | 0         | 5         | 0        | 5        | 0        | 76     | 62     | 85       | 95       | 76       | 21      | 30      |
| 220486      | 0         | 2         | 0        | 2        | 0        | 98     | 99     | 99       | 85       | 99       | 90      | 90      |
| 2134MPMF    | 0         | 0         | 0        | 0        | 3        | 99     | 99     | 99       | 99       | 99       | 90      | 0       |
| DFSCP2      | ND        | ND        | 0        | 2        | 0        | 98     | 99     | ND       | ND       | ND       | ND      | 35      |
| DF22212     | ND        | ND        | ND       | ND       | ND       | 85     | 70     | ND       | ND       | 95       | 0       | ND      |
| DF311dp     | 0         | ND        | 14       | 0        | 95       | 98     | 93     | ND       | 92       | 70       | ND      | ND      |
| ND-not determined | | | | | | | | | | | | |

**Figure 2:** MSC osteodifferentiation of fresh sample
Osteogenic differentiation of fresh and cryopreserved samples

Alizarin red staining and digital imaging quantification of stains

Two samples were selected for osteogenic differentiation for 15 days, one fresh and one cryopreserved. Staining with alizarin red S allows a demonstration of extracellular calcium deposits in a bright orange-red colour. Staining revealed that although DFPCs cultured in a control medium (CM) were able to form mineralised nodules, they were markedly less than from DFPCs cultured in test samples in osteogenic medium (Figures 2 and 3). As alizarin red staining is somewhat nonspecific, quantification of the calcium content of the deposited matrix was also done by digitally quantifying the stain image at a fixed threshold in both samples. In the cryopreserved sample, the fold change observed for alizarin red stain was 5.513, while in the fresh sample the change was 43.369 (Table 2).

Alkaline phosphatase ALP staining and digital imaging quantification of stains

When cultured for 15 days in an osteogenic medium, DFPCs showed a dense mineralised plexus in test samples (Figures 2 and 3).

Table 2. Fold change distribution of fresh and cryopreserved samples for the ALP and AzR stains showing osteogenic differentiation potential.

| Samples          | Test % | Control % | Fold change |
|------------------|--------|-----------|-------------|
|                  | ALP    | AzR       | ALP         | AzR       | ALP         | AzR         |
| Cryopreserved dp  | 6.607  | 4.416     | 0.460       | 0.678     | 13.363      | 5.513       |
| Fresh dp         | 4.391  | 18.369    | 0.021       | 0.414     | 208.09      | 43.369      |

ALP-alkaline phosphatase, AzR-Alizarin red

Figure 3: MSC osteodifferentiation of cryopreserved sample

Discussion

Comparison of fresh and cryopreserved samples

Recent literature has reported limited but significant differences in the expression of physiological properties between fresh and thawed samples. However, prospective randomised clinical studies to assess their therapeutic value are still lacking. The possibly compromised immunomodulatory activity directly after thawing may be a reason for failure in clinical trials, as the recovery time for MSCs from the cryo-stun effect is still unknown. A few studies have revealed altered physiological properties such as membrane asymmetry which lead to cell injury and disintegration, while some studies reported no differences between fresh and cryopreserved DFPCs.
we assessed the efficacy of the cryopreservant in 10% concentration as well as the scope of preserving fresh samples for osteogenic differentiation compared to cryopreserved ones. The easy accessibility to dental MSCs makes them an excellent alternative to BMSCs for use in clinical trials. However, the cryopreservation procedure employed must follow standardised protocols to safeguard the biological product, as they are intended for clinical use. Factors like type, concentration, dilution of the cryoprotective agent (CPA), the freezing rate and thawing conditions should be taken into account. DMSO as a CPA used in research is a cytotoxic chemical that has been reported to cause epigenetic changes and cytotoxicity at levels routinely used for MSC cryopreservation. A review of the literature showed that 80% of stem cell banks most commonly employ 10% DMSO. The survival and number of colonies formed by MSCs were significantly decreased by reducing the concentration of DMSO, and the magnitude of this decrease was inversely proportional to DMSO concentration. The recommendation to cryopreserve stem cell populations of at least 1 million cells in order to compensate is followed in this study. Controlling the freezing rate is essential to minimise the damage caused by cell dehydration and ice crystal formation. A slow and controlled freezing rate of 1–2°C per minute is generally considered optimal for maintaining MSC viability during cryopreservation. The ability of DFSCs to maintain their characteristics for years after cryopreservation is critical if they are intended for clinical use. Although, there was a difference noticed in the average cell count before and after cryopreservation, which can be attributed to the separation of cells into two vials before cryopreservation, this study did not find any significant change in viability or cell count in cryopreserved samples. The average cell count was 1.607X10^6 cells/ml before cryopreservation and 0.58 X 10^6 cells/ml after cryopreservation in each vial. The major drawbacks of the study were the limited number of fresh samples employed to check differentiation potential. Moreover, we were unable to check the recovery time for thawed samples for optimal osteogenic differentiation, and also could not test for pre osteoblast markers like RUNX 2 or osterix. However, we were successful in gaining insight into using fresh samples whenever possible for better osteogenic results, emphasising that preservation of fresh samples is a technique-sensitive procedure.

Conclusions

A fresh sample could ensure long term survival during cultures, undergoing multiple cell passaging with good proliferation rates, and retained more than 95% fluorescence in the expression of all mesenchymal stem cell markers. It also exhibited marked osteogenic differentiation potential compared to the cryopreserved one. Further, this study proves that DFSCs can be cryopreserved and stored for long periods without losing cell count and viability, by using a cryoprotectant in the specified concentration.

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Abbreviations

MSC : Mesenchymal Stem Cell
ASC : Adult Stem Cell
ESC : Embryonic Stem Cell
DSC : Dental Stem Cell
DPSC : Dental Pulp Stem Cell
DFSC : Dental Follicle Stem Cell
SHED : Stem cells from Human Exfoliated Deciduous teeth
SCAP : Stem Cells from Apical Papilla
PDLSC : Periodontal Ligament Stem Cell
GMSC : Gingival Mesenchymal Stem Cell
NDP-SC : Natal Dental Pulp Stem Cell
DMSO : Dimethylsulfoxide
D-PBS : Dulbecco’s Phosphate Buffered Saline
DMEM : Dulbecco’s Modified Eagle Medium

Potential Conflicts of Interests

None

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