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Cells isolated from cryopreserved dental follicle display similar characteristics to cryopreserved dental follicle cells

Hefeng Yang [a, b, 1], Jie Li [c, 1], Jingjing Sun [b, d], Weihua Guo [b, d], Hui Li [b, d], Jinlong Chen [b, d], Yu Hu [e], Weidong Tian [b, d, **], Song Li [a, *]

[a] Department of Dental Research, The Affiliated Stomatological Hospital of Kunming Medical University, Kunming, Yunnan 650500, PR China
[b] National Engineering Laboratory for Oral Regenerative Medicine, Sichuan University, Chengdu 610041, PR China
[c] Chongqing Key Laboratory of Oral Diseases and Biomedical Sciences, College of Stomatology, Chongqing Medical University, Chongqing, 401147, PR China
[d] Department of Oral and Maxillofacial Surgery, West China Hospital of Stomatology, Sichuan University, Chengdu 610041, PR China
[e] Department of Orthodontics, The Affiliated Stomatological Hospital of Kunming Medical University, Kunming, Yunnan 650031, PR China

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A B S T R A C T

Dental follicle tissue is a promising resource of mesenchymal stem cells for cytotherapeutic approaches and tissue engineering applications. There are two procedures for banking of human dental follicle stem cells have been reported. Conventional method requires cell isolation, expansion and immediate cryopreservation. Whereas dental follicle stem cells can be isolated from cryopreserved dental follicle fragments. The aim of this study was to compare the characteristics of dental follicle cells isolated from cryopreserved fragments (DFCs-CF) with dental follicle cells recovered from cryopreserved cells (DFCs-CC). Dental follicle fragments obtained after mechanical disaggregation were divided into two parts, with one part maintained in culture, while another part underwent cryopreservation. Dental follicle fragments and dental follicle cells from fresh tissue were stored in liquid nitrogen for 3 months. After thawing, the isolation, morphology, proliferation, cell cycle, colony-forming-unit ability, stemness-related marker expression, apoptosis, and multi-lineage differentiation potential of DFCs-CF were tested compared with DFCs-CC. DFCs-CF expressed mesenchymal stem cells marker, proliferated well, showed similar levels of mRNA for stemness- and apoptosis-related genes and exhibited the capacity of multi-lineage differentiation similar to those of DFCs-CC. These results imply that cryopreservation of dental follicle fragments is an effective banking method for isolation of dental follicle cells.

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1. Introduction

Since mesenchymal stem cells (MSCs) are capable of self-renewal, multi-lineage differentiation, regeneration and immunosuppression, they are emerging as one of the most important cell types with great therapeutic promise [15,20,25]. MSCs, first isolated from bone marrow, have now been isolated from various tissues, including adipose tissue, umbilical cord, epidermis and dental tissue [2,27]. Recently, precursor cells were isolated and characterized from dental pulp, periodontal ligament, exfoliated deciduous teeth, immature wisdom teeth and dental follicle, these cells display the characteristics of MSCs [12,18]. Dental tissues are easy to get, less traumatic but abundant of mesenchymal stem cell, so it becomes an important source of mesenchymal stem cells and has great significance for stem cell treatment and dental tissue engineering [6,7,29].

One of the ultimate goals of cell therapy and tissue engineering research is to establish an industry based on building tissue engineering products. In order to maintain the stability of the product supply and meet the irregular market needs, long-term preservation of cells will be necessary to realize the potential commercial value of regenerative medicine. Therefore, the storage of stem cell and the construction of stem cells bank becomes the essential foundation of regeneration treatments based on stem cells. Cryopreservation is the best choice for long-term storage of cells at

* Corresponding author. Department of Dental Research, The Affiliated Stomatological Hospital of Kunming Medical University, No.1168, Chunrongxi Road, Chenggong District, Kunming 650500, PR China.
** Corresponding author. Department of Oral and Maxillofacial Surgery, West China School of Stomatology, Sichuan University, No.14, 3rd Section, Renmin South Road, Chengdu 610041, PR China.
E-mail addresses: drtwd@sina.com (W. Tian), lisong59@sohu.com (S. Li).
[1] These authors contributed equally to this work.

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present. However, how to reduce effects on the biological characteristics of cryopreserved cells is one of the important challenges for cryopreservation [10,32].

There are a variety of methods used in deep cryopreserved storage of cells and tissues, including cryoprotective agent (CPA) addition, programmed slow freezing, and rapid freezing. Among which, the most common approach to prevent the risk of ice crystal damage is adding cryoprotective agent (CPA) such as Me2SO during cell cryopreservation [8,18,22]. The most widely used method for banking of bone marrow stromal cells (BMSCs) consists of isolation, in vitro expansion and subsequent storage in liquid nitrogen. However, the economics of cell isolation and cultivation especially when clinical application of cells have to satisfy good manufacturing practice (GMP) standards need to be take into consideration [1]. Some scholars concluded that cryopreservation of clinical samples, like bone tissue sample, whole teeth sample, and dental follicle tissue could be an effective way for cell isolation and cultivation [3,27,30]. To the best of our knowledge, there is lack of comparison report in these two cryopreservation ways: cryopreservation before or after cell isolation.

2. Materials and methods

2.1. Cryopreservation of human dental follicles

Dental follicles were obtained from 5 patients (average age 19 years; 2 females and 3 males) after immature impacted wisdom teeth extraction during clinical orthodontic treatment at West China Hospital of Stomatology, Sichuan University with informed consent from patients. Tissues were transferred to the laboratory in cold PBS containing 100 unit/mL penicillin and 100 μg/ml streptomycin (Hyclone, Logan, UT, USA) within 2 h.

Under aseptic condition, the dental follicles were washed with PBS, minced into approximately 1–3 mm^2 explants using fine scissors and divided into two experimental groups, each group composed of tissues from five donors. Prior to freezing, minced dental follicle tissues in 1.8 ml cryovials containing 1 ml cryoprotective medium (10% Me2SO, 90% FBS) were kept in a refrigerator at 4 °C for 30 min to attempt full equilibration, then cooled at approximately −1 °C/min from 4 °C to −80 °C in a freezing container (Nalgene, Rochester, NY, USA). The cryovials were subsequently transferred to liquid nitrogen (LN2). The rest of the fragments were used for further cell isolation.

The cryopreserved dental follicle tissues were stored in liquid nitrogen for at least 3 months. The cryovials were then thawed by immersing them in a circulating water bath at 37 °C until thawed and the tissues were then washed twice by centrifugation with culture medium (α-MEM supplemented with 10% fetal bovine serum and 1% penicillin-streptomycin solution) at 1000 rpm for 2 min for further cell isolation.

2.2. Isolation and culture of dental follicle cell

Dental follicle cells were isolated from fresh and cryopreserved dental follicle tissues, the method has been described previously [27]. Briefly, the tissue blocks were washed by PBS, digested in collagenase type I (0.1 U/ml; Sigma-Aldrich, St. Louis, MO, USA) and dispase (1 U/ml; Sigma-Aldrich, St. Louis, MO, USA) for 1 h at 37 °C, and then incubated in α-MEM supplemented with 10% fetal bovine serum and 1% penicillin-streptomycin solution (Sigma-Aldrich, St. Louis, MO, USA) in a humidified atmosphere at 37 °C and 5% CO_2. Cell culture medium was changed every 3 days.

Cells (passages 0), derived from fresh dental follicles, were cryopreserved at 1×10^6/ml in 1.8 ml cryovials containing 1 ml cryoprotective medium (10% Me2SO, 90% FBS) were kept in a refrigerator at 4 °C for 30 min to attempt full equilibration, then cooled at approximately −1 °C/min from 4 °C to −80 °C in a freezing container. The cryovials were subsequently transferred to liquid nitrogen (LN2) for at least 3 months storage and thawed in a circulating water bath at 37 °C. Cells isolated from cryopreserved dental follicle were not further cryopreserved. Cells from passages 3 were used for the experiments.

2.3. Immunofluorescence staining

Cells isolated from cryopreserved dental follicle (DFCs-CF) and cryopreserved dental follicle cells (DFCs-CC) were seeded in 6-well plates (Corning, NY, USA) for further culture. At 80% confluence, the cells were washed three times with PBS after being fixed in 4% paraformaldehyde for 30 min, and stained with the following antibodies: anti-Stro-1, anti-Vimentin, and anti-CK-14 (R&D Systems, Minneapolis, MN, USA) according to the manufactures' protocols. Then the cells were visualized with relevant Alexa Fluor® 488 or 555 conjugated secondary antibodies (Invitrogen, Carlsbad, USA). The stained cells were observed under a fluorescent microscope (Olympus, Tokyo, Japan).

2.4. Flow-cytometric analysis

To characterize the immunophenotype of DFCs-CF and DFCs-CC, flow-cytometric analysis was used to detect the expression of mesenchymal stem cell associated surface markers at passages 3. Cells were trypsinized, re-suspended, stained with the following monoclonal antibodies: anti-human-CD3-FITC, -CD33-FITC, -CD34-FITC, -CD45-FITC, -CD29-PE, -CD44-FITC, -CD90-FITC, -CD105-PE, -CD166-PE (all from BD Biosciences, San Jose, CA, USA) according to the manufactures' protocols. The flow cytometry was performed using BD Accuri® C6 and analyzed using FACSDiva software (BD Biosciences, San Jose, CA, USA).

2.5. Cell proliferation and colony-forming unit assay

To evaluate the viability of DFCs-CF and DFCs-CC, Cell Counting Kit-8 (CCK-8, Dojindo, Japan) assay was performed according to the manufacturer's protocol. Briefly, 2×10^3 DFCs-CF and DFCs-CC were seeded into 96-well plates (Corning, NY, USA) and kept in standard culture conditions from day 1 to day 8. Each sample was added with 110 μl culture medium containing 10 μl CCK-8. After incubation at 37 °C for 2 h, six parallel replicates were prepared and the absorbance in each well was measured at 450 nm using a spectrophotometer (Thermo Fisher Scientific, Pittsburgh, PA, USA).

To assess the self-renewal capacity of the cells, a total of 100 cells were seed into a 100-mm plate (Corning, NY, USA). Following cultivation for 14 days, the medium was removed, and the cells were fixed with methanol/acetic acid (3:1) for 5 min, and stained with Giemsa for 2 min at room temperature. The stained cells were observed and photographed under a phase-contrast inverted microscope (Olympus, Tokyo, Japan). Stained colonies with >50 cells were counted.

2.6. Cell cycle analysis

At 80% confluence, single-cell suspensions of DFCs-CF and DFCs-CC were harvested and washed twice with PBS. Subsequently, the cells were fixed in cold 70% dehydrated alcohol at 4 °C for 2 h. After further washing, cell suspensions were treated with RNase A at 37 °C for 30 min stained with propidium iodide (PI; Sigma-Aldrich, St. Louis, MO, USA) at 4 °C for 30 min, and then subjected to cell cycle analysis by flow cytometry [28]. The amounts of cells residing in G1 phase, S phase, and G2/M phase were determined.

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