



Original Article

Evaluation the Ability to Isolate and Culture Stem Cells from Frozen Human Umbilical Cord Tissue

Nguyen Thi Nhung^{1,2}, Do Trung Kien^{2,3}, Nguyen Thi Hiep¹,
Tran Thi Huong Giang¹, Nguyen Van Hanh^{1,2,*}

¹*Institute of Biotechnology, Vietnam Academy of Science and Technology,
18 Hoang Quoc Viet, Hanoi, Vietnam*

²*Graduate University of Science and Technology, Vietnam Academy of Science and Technology,
18 Hoang Quoc Viet, Hanoi, Vietnam*

³*Fisheries and Technical Economic College, Tu Son, Bac Ninh, Vietnam*

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Abstract: Umbilical cord-derived mesenchymal stem cells have been determined to be effective in cell therapy and regenerative medicine. Cell cryopreservation is widely used for long term preservation. However, cell cryopreservation is costly and labor consume. Therefore, cryopreservation of human umbilical cord (HUC) tissue has become an alternative solution to reduce the risks of disease and open up opportunities for later treatment for families in recent years. In this study, we evaluated the ability to isolate and the characteristic of mesenchymal stem cells (MSCs) derived from fresh and frozen human umbilical cord tissue. The results showed that the HUCMSCs were isolated successfully from both fresh and frozen HUC tissues. The expression of MSCs cell surface marker CD73, CD90, CD105 in group 1 were higher than those in group 2, CD73 (99.42 vs 95.2%); CD90 (99.74 vs 96.6%); CD105 (99.64 vs 96.15%), respectively. However, they are similar in morphology and osteogenic and adipose differentiated ability. The results of the study would be useful for stem cell research, cell therapy and regenerative medicine.

Keywords: Cell therapy, cryopreservation, mesenchymal stem cell, human umbilical cord tissue.

1. Introduction

Mesenchymal stem cells (MSCs) has been considered as one of the most effective tool for cell therapy since they have capacities for self-renewal; proliferation, multilineage

differentiation, and immunomodulatory properties [1-3]. These cells were first identified and isolated from bone marrow [4]. However, the isolation of MSCs from bone marrow is painful and complex [1]. Therefore, alternative sources of MSCs have been suggested such as umbilical cord tissue, adipose tissue, peripheral and umbilical cord blood [2]. The criteria to define human MSCs by International Society for Cellular Therapy are:

* Corresponding author.

E-mail address: nvhanh@ibt.ac.vn

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i) MSCs must be plastic-adherent when maintained in standard culture conditions; ii) MSCs must express CD105, CD73, and CD90 and lack expression of CD45, CD34, CD14 or CD11b, CD79a or CD19, and HLA-DR surface molecule; iii) MSCs must differentiate into osteoblasts, adipocytes, and chondroblasts *in vitro* [5].

Human umbilical cord (HUC) has been recognized as a promising source for MSCs with their advantages of noninvasive and insignificant ethical concerns [6]. The MSCs from HUC have painless collection procedure and faster self-renewal properties than those in bone marrow stem cells [1]. Moreover, they can differentiate into the three germ layers that promote tissue repair, modulate immune responses and anticancer properties [1, 2]. In our previous study, MSCs derived from fresh HUC tissues could differentiated into Hepatocyte-like cells [7]. However, the disadvantage of fresh HUC tissues that it could only be collected at the day of childbirth.

Cryopreservation have important roles in maintenance of MSCs function and avoiding adverse effects caused by long term culture [8, 9]. Several types of cells derived from human umbilical cord including endothelial and epithelial cells have been isolated from frozen tissue [10, 11]. Recently, an effective method for cryopreservation of human umbilical cord vein endothelial cells has been reported [12]. The slow freezing with low level of DMSO is commonly used for MSCs preservation [13]. Nevertheless, cell cryopreservation is costly and time consume. Therefore, cryopreservation of HUC tissue is considered as one of alternative methods for MSCs preservation. However, the influence of cryopreservation post-thaw and long term culture on HUCMSCs remains little known. Therefore, this present study examined the effect of HUC tissue cryopreservation on the isolation, proliferation, surface marker expression, differentiation ability of HUCMSCs. The study would provide more information for the ability isolation HMSCs from frozen-thawed tissue and potential of them.

2. Materials and Methods

2.1. Isolation of the Human Umbilical Cord Mesenchymal Stem Cells

The HUC samples were obtained from healthy donors at the Hanoi Obstetrics and Gynecology Hospital. These donors were given full information about the purpose of using the specimen and the samples were taken with the agreements of them. The HUCs were transferred to the laboratory and washed in sterile phosphate buffered saline (PBS) at least 3 times to remove red blood cells. A middle HUC piece of approximately 2-3 cm was cut from the whole cord for processing. Subsequently, the HUC piece was cut into explants of small pieces (1 -2 mm³) with a sterile scalpel and placed into 4 well plates (Nunc, UK) prefilled with culture Dulbecco's Modified Eagle's Medium (DMEM/F12) supplemented with 15% FCS), 10 ng/mL bFGF, 10 ng/mL EGF, 1X ITS, 2 mM L-glutamine and 1% antibiotic (penicillin/ streptomycin, all material from Sigma-Aldrich, USA) and cultured in an incubator with humidified atmosphere, 5% CO₂ at 37 °C. The medium was changed every 2-3 days and cells were passaged when they reached 70% confluence (group 1).

2.1. Freezing and Thawing of Human Umbilical Cord Tissue

The HUC tissues were frozen by slow freezing method utilizing 10% DMSO. Briefly, the medium for freezing is DMEM/F12 supplemented with 10% DMSO,... The HUC tissues in small pieces (1 -2 mm³) were put in the freezing medium and then divided into cryotubes. The procedure was addressing under sterile bench and on ice. The cryotubes contained HUC tissues were then reduced temperature gradually by keeping at 4 °C in 30 min, 0 °C in 60 min, -20 °C in 20-30 min and then in liquid nitrogen [8]. The HUC tissues were kept in liquid nitrogen bank in one week before thawing. For thawing, the cryovials were transferred into water bath at 37 °C until all melting. the HUC tissues were washed in DMEM/F12 at least 3 times to get free DMSO.

The tissues were then cultured in 4 well dishes as described above (group 2).

2.2. Measurement of Cell Growth and Proliferation

The cell number was obtained at each time point by staining with 0.4% Trypan blue and an improved Neubauer hemocytometer (Germany). For detection of the doubling time of the human umbilical cord mesenchymal cell (HUCMSCs), they were calculated using the obtained cell counts at every passage after each 36 h. The procedure was repeated with cells from three separate cords, aliquots of 1×10^5 HUCMSCs were plated into 35-mm Petri dishes to obtain the doubling time [3].

The cell growth assays were performed in 7 days. The HUCMSCs were cultured at a density of 5×10^4 HUCMSCs cells per 35-mm Petri dish in culture medium. The total cell number was recorded each day, from day 1 to day 7 of culture. The cells were trypsinized in 3-5 min, neutralized by DMEM and centrifuged at 3000 round per minute (rpm) in 3 min. The cells were suspended and counted [3].

2.3. FACS Determination of Human Umbilical Cord Mesenchymal Stem Cells Phenotype

Flow cytometry was performed for the cells from fresh and frozen HUC at passage 3. To determine cell surface marker expression, cell suspensions (1×10^6 cells) were incubated with 10 μ l of each antibody of CD90-FITC, CD73-APC, and CD105-PE antibodies (Miltenyi Biotec Inc. USA) for 10 min at 2-8 °C in the dark. The cells were then washed three times by buffer (PBS+ 0.5% BSA+ 2 mM EDTA). Samples were sorted on a FACS BD flow cytometer. The resulting data was analyzed using CellQuest software (BD Bioscience).

2.4. Adipogenic and Osteogenic Differentiation

In *in vitro* differentiation assays, the cells were performed as follows [7]. For inducing the adipogenic differentiation, the HUCMSCs from passages 3 were plated at a density of 1×10^4 cells/well of 4 well plates (Nunc, UK) in medium for expansion which supplemented with 10 μ g/L insulin, 1 μ M Dexamethasone and

0.5 μ M IBMX (all from Sigma). The medium was changed every 3 days. After 3 weeks, cells were stained with Oil Red O (Bio Basic Canada Inc.) to make lipid vesicles visible.

For inducing the osteogenic differentiation, the procedure was similar with adipogenic differentiation. However, the medium was DMEM/F12 containing 10% FBS, 50 μ g ascorbic acid, 10 nM dexamethasone, and 10 mM β -glycerophosphate [3]. The medium was replaced by fresh one every three days for 21 days. After 21 days, the cells were fixed with paraformaldehyde 4% at room temperature for 10 minutes and then stained with Alizarin red (Bio Basic Canada Inc.) for illustration of calcium deposition for 20 minutes.

3. Results and Discussion

3.1. Isolation of Mesenchymal Stem Cells Derived from Fresh and Frozen-thawed Human Umbilical Cord Tissue

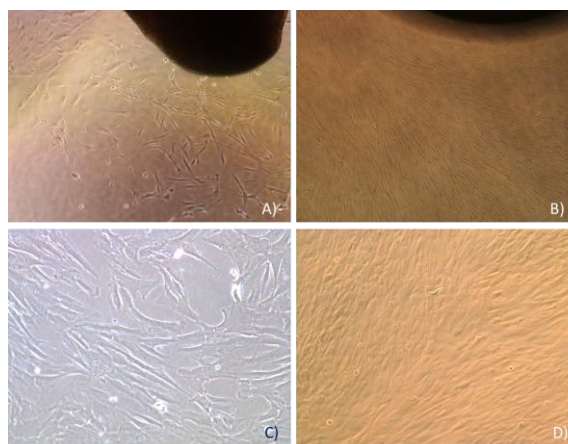


Figure 1. The cells expanded from the fresh tissue (A) and the frozen tissue (C), the morphology of HUCMSCs confluence at passage 2 from fresh tissue (B) and frozen tissue (D), (the photo in A, B were under objective 10x and the photo in C, D were under objective 20x).

The MSCs derived from fresh HUC tissues (group 1) were expanded after 7 to 10 days (Figure 1A). The cells reached 50-60% confluence after 14-18 days. Meanwhile, the MSCs derived from frozen HUC tissues were

started expanding after 15 days and reached 50-60% confluence after 20-25 days (Figure 1 C) (group 2). The morphological observation of HUMSCs was performed using an inverted microscope following culture for passage 2, they exhibited a typical fibroblast-like and there were similar between two groups (Figure 1 B and 1 D).

In present research, there were no differences in morphology of MSCs derived from fresh and frozen HUC tissues. The cells have characteristic of MSCs such as plastic-adherent and fibroblast morphology (cells are long and thin, tapered at both ends, with large round nuclei), and they were the same in many reported [3, 6, 10].

3.2. Proliferation Rate of Human Umbilical Cord Mesenchymal Stem Cells

To evaluate changes in proliferation rate according to cells source, we calculated population-doubling time (Table 1) and increased cells number (Figure 2). The proliferation of MSCs derived from fresh and frozen HUC tissue was evaluated from passage 2 to 5. The results were similar to report in embryonic stem cells [15]. Park et al., [15] showed that, in the early passage the population-doubling time of ESCs was 37.6 h, whereas population-doubling time of ESCs in middle or late passage was about 30.0 h.

Table 1. The proliferation of fresh and frozen HUCMSCs expressed by total cell number in each group from passage 2 to 5. Values are expressed the mean ± SEM (n=3 in each group)

Group	The time for population-doubling (h)			
	P2	P3	P4	P5
Group 1	28.42 ± 0.60	26.34 ± 0.55	27.69 ± 0.20	25.71 ± 0.55
Group 2	36.00 ± 0.93	33.75 ± 0.55	40.00 ± 0.39	40.00 ± 0.63

In the group 2, doubling time was tended to be longer. At the P2, the population-doubling time was 36 h, but it was increased upto 40 h at passage 4 and passage 5. Although it was less

than 60 ± 3 hours on reported of Chen et al., [14].

The cell concentration increasing through 7 days culture in almost cells when cells grew exponentially (Figure 1). The number of MSCs derived from frozen tissue were similar in three days, after that they had a bit delay in expansion and proliferation compared to those derived from fresh ones. Cai et al., [12] reported that the level of ICAM-1 in cells was significantly decreased in the post-cryopreserved at 24 weeks and the tube-like structure-forming potential was weakened with increasing cryopreservation duration yet the morphological and the proliferation were not significant differential.

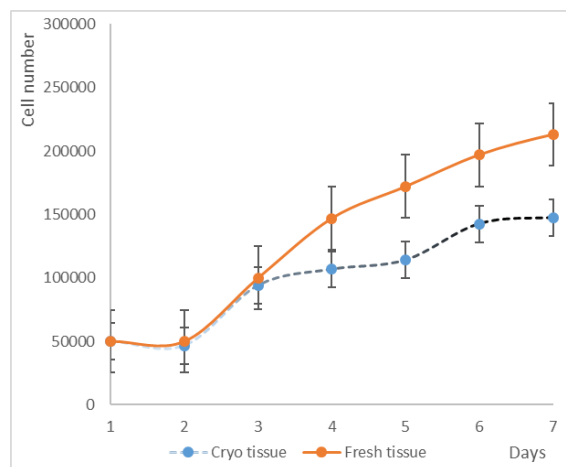


Figure 2. Total cell number was recorded every day for a total of 7 days. Values are expressed the mean ± SEM (n=3 in each group).

3.3. Effect of Human Umbilical Cord Tissue Cryopreservation on the Surface Marker Expression of Mesenchymal Stem Cells

The flow cytometry was performed for fresh and frozen HUCMSCs at passage 2 to determine cell surface marker expression of CD73, CD90 and CD105. The results showed high level expression of the mesenchymal stem cell markers in both group 1 and 2. In group 1, the expression of CD73 (99,42%), CD90 (99,74%), CD105 (99,64%) in MSCs derived from fresh HUC tissue (Fig. 3). Similarly, the expression of CD 73 (95,2%), CD90 (96,6%)

and CD105 (96,15%) in MSCs derived from frozen HUC tissue in group 2 (Figure 4). In this study, the phenotypic markers specific for mesenchymal stem cells in group 2 were lower than those in group 1, but they were still higher than those in several previous publications [3, 12, 14].

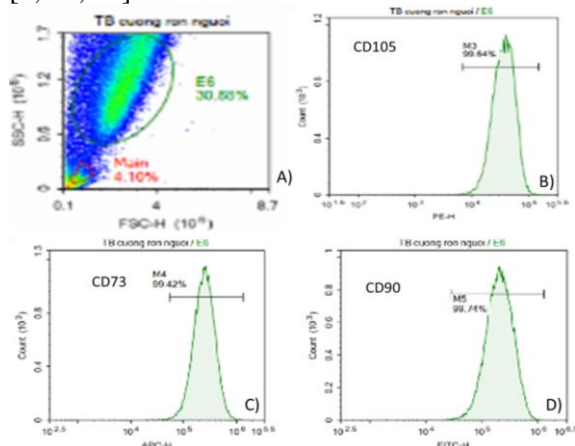


Figure 3. Flow cytometry of HUCMSCs from fresh tissue. Cells positive to CD105 (A) CD73 (B), CD90 (C).

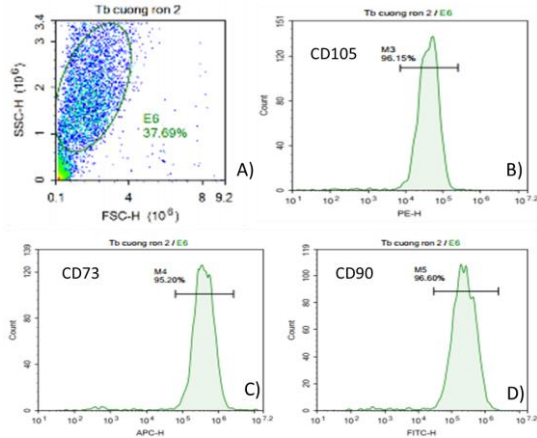


Figure 4. Flow cytometry of HUCMSCs from frozen tissue. Cells positive to CD105 (A) CD73 (B), CD90 (C).

3.4. Differentiation Potential of Human Umbilical Cord from frozen-tharwing Tissue

The HUCMSC could be differentiated to adipogenic cells. The Lipid droplet appeared after three weeks and became oversized little by little at the end of the differentiation period

(Figure 4 A and 4 B). After three weeks of osteogenic differentiation, the HUCMSCs derived from fresh and frozen tissues expressed in colored crystals after staining with Alizarin red (Figure 4 C and 4 D).

The HUCMSC possess stem cell properties, such as proliferation capacity, self-renewal potential, and multiple differentiation capacity. Therefore, these cells may be a potential source of cell therapy for a variety of diseases [10]. However, while their potential allows for the development of several MSC-based therapies, assessments of cell longevity, loss of differentiation related to culture, preservation by specific markers, etc. can help with quality control and stem cells for commercial and clinical applications [14].

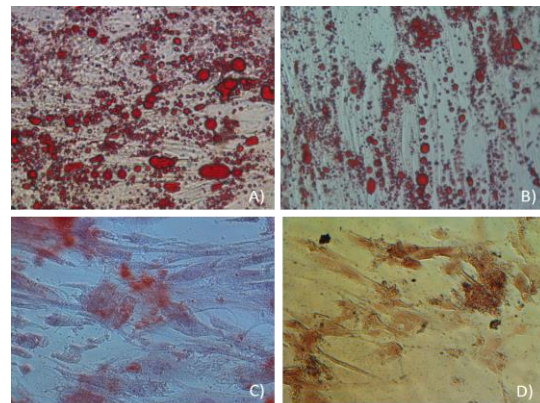


Figure 5. Oil Red and Alkaline phosphatase activity in HUCMSCs. The cell positive of Oil Red staining from fresh tissue (A) and frozen tissue (B), the HUCMSCs from fresh tissue (C) and frozen tissue (D) became Alkaline phosphatase positive (the photo under objective 20x).

4. Conclusion

In conclusion, the HUCMSCs were isolated from frozen HUC tissue successfully. The growth rate and percentage of marker positive with HUCMSCs derived from frozen tissue were lower than those from fresh tissue. However, HUCMSCs obtained from both fresh and frozen tissues were similar in morphology, osteogenic and adipose differentiated ability.

Further studies should be addressed to evaluate the characteristic of MSCs in

long term storage and potential of them in application in cell therapy and regenerative medicine.

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