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Research Article

ISOLATION, CULTURE AND CRYOPRESERVATION OF HUMAN BONE MARROW – DERIVED MESENCHYMAL STEM CELLS

Chung C Doan, Nhung H Truong, Ngoc B Vu, Tam T Nguyen, Hoang M Nguyen, Khue G Nguyen, Si M Do, Ngoc K Phan, Phuc V Pham*

Laboratory of Stem Cell Research and Application, University of Science, Vietnam National University, HCM City, Vietnam

Email: pvphuc@hcmuns.edu.vn

ABSTRACT: Human bone marrow derived mesenchymal stem cells (BMMSCs), a source of important autologous stem cells without ethical debate, have been used in both experimental and clinical treatment. Therefore, this research aims to isolate, culture and cryopreserve BMMSCs. Mesenchymal stem cells (MSCs) were preserved in each cryovial by slow freezing – rapid thawing method. Post-cryopreserved MSCs was analyzed the viability and the stemness by their expression of some specific markers, differentiation potential and proliferation ability. The results of this research showed it is easy to isolate the BMMSCs by culturing the mononuclear cells derived from human bone marrow in DMEM/F12 plus 15% FBS, 10 ng/ml FGF and 1% antibiotic–mycotic; the viability rates of thawed MSCs were 72.95+/-6.14%. Cryopreserved MSCs maintained the stemness after 1 year storage in nitrogen liquid. They expressed CD13, CD44, CD90, CD166 markers and were negative for CD14, CD34, CD45 and HLA-DR markers in similar to fresh MSCs. In addition, they also owned and maintained adipogenic differentiation potential. So that BMMSCs can be in vitro cultured, proliferated and preserved and used for long periods of time in regenerative medicine.

Keywords: Bone marrow, Bone marrow derived mesenchymal stem cells, Mesenchymal stem cells, Stem cell cryopreservation.

Abbreviations: BM-Bone marrow, BMMSCs-Bone marrow derived mesenchymal stem cells; HSCs-Hematopoietic stem cells; MSCs-Mesenchymal stem cells.

INTRODUCTION

Mesenchymal stem cells (MSCs) were isolated from various sources such as bone marrow, umbilical cord blood, adipose tissue 0 and identified by the minimal criteria of The Mesenchymal and Tissue Stem Cell Committee of the International Society for Cellular Therapy [7]. One notable characteristic is plastic – adherent ability of MSCs when they are cultured in appropriate media. Another property, MSCs must have differentiation potential into osteoblasts, adipocytes and chondroblasts in *in vitro* culture with known stimulators. Lastly, MSCs were confirmed with the phenotype that were positive for CD73, CD90, CD105 and negative for CD45, CD34, CD14 or CD11b, CD79a or CD19 and HLA-DR. Bone marrow, a source of hematopoietic stem cells (HSCs) and MSCs, is useful for basic research and clinical applications. While HSCs can create circulating blood cells, including red blood cells, monocytes, platelets, granulocytes and lymphocytes, MSCs which possess two important characteristics of long – term self renewal, can be induced to differentiate into various functional cell types of mesodermal tissues [11]. In addition to generating other mesenchymal tissues, many studies have also revealed that MSCs could differentiate into non – mesodermal tissue – specific cells under appropriate conditions such as hepatocytes, cardiomyocytes, neuron like cells [23]. BMMSCs could be *in vitro* isolated because of their adherent and rapidly proliferative characteristics. They also retained their multipotency after some passages [4]. Furthermore, they were a source of autologous stem cells, without causing an immune response with the host and associating with any ethical debate. These results indicated the benefit of multipotential MSCs for both cell therapy, tissue engineering purposes in regenerative medicine [17] and clinical applications in order to treat a diverse variety of diseases such as lung fibrosis, spinal cord injuries, stroke and cardiovascular diseases [2, 8].

Bone marrow – derived cells have been utilized as a source of MSCs. However, the characteristics of MSCs from fresh bone marrow may change during long-term culture, like genotypic change, phenotypic instability, and transformation, loss of differentiation capacity and decrease of proliferation ability or senescence. The limitations in BMMSC culture will result in one of the notable difficulties that are the lack of MSC sources for both research and clinical applications in the future. Therefore, the maintenance of MSC initial characteristics is crucial, and it may be possible to preserve them by satisfactory cryopreservation technologies [27]. As the technology to preserve cultured cells by freezing methods that have been developed recently, stem cell cryopreservation, especially BMMSCs, has become more favorable [3]. This is especially significant because the supply of stem cells that are limited could be happed any time. Furthermore, efficient cryopreservation may be more practical in order to save time, money [10] and may be essential for the establishment of a bone marrow – derived MSC bank. In fact, if long term cryopreserved MSCs still retained differentiation ability and high viability, they would be expected to become a useful cell source for regenerative medical progression, especially autologous transplantation.

Two methods are used to cryopreserve almost cell lines. They are slow freezing–rapid thawing method and vitrification method [13]. The slow freezing–rapid thawing method using dimethylsulfoxide (DMSO) as a cryoprotectant can be easily applied to the cryopreservation of MSC lines such as BMMSCs [27], human dental pulp – derived mesenchymal stem cells [21], and umbilical cord blood – derived mesenchymal sten cells [15]. In most research, cells are suspended in freezing medium containing 5 – 10% DMSO, transferred into cryovials and then frozen by steps with slowly decreasing temperatures: 4°C for 10 min, – 20°C for 1 hour, – 80°C for 1 – 2 days and – 196^{o}C for 3 – 6 months. Using this method can obtain some advantages, like a large cell volume which might be frozen in one vial are required for applications such as drug screening or clinical applications. In addition to keeping balance to protect cryopreservated cells, it also prevent the damage caused by various factors, including crystal formation in the cytoplasm of cells, fracture, toxic and osmotic damage by decreasing temperature slowly with low concentration of cryoprotectants [24]. However, it is difficult to eliminate injuries by intracellular ice formation which is the main source of fracture and damage to the cytoplasm [9]. It is also a time – consuming procedure and requires a programmable freezer. Alternately, vitrification, a rapid freezing method using a high concentration of cryoprotectants, could also be used. Vitrification can totally eliminate damages caused by intracellular crystal formation of cells [17]. It is also advantageous because of controlling penetration of cryoprotectants and dehydration rate, preventing prolonged temperature shock and damage from intracellular ice formation. Besides, it is a relatively short time and nonprogrammable temperature decreasing procedure [13]. However, the vitrification method requires a high concentration of permeable or nonpermeable cryoprotectants exposed to cells before plunging into liquid nitrogen. It results in extreme osmotic stresses and chemical toxicity for cryopreservated cells [26]. The vitrification method has been commonly used to cryopreserve many different cell types such as embryonic stem cells (ESCs) [17] and umbilical cord – derived mesenchymal stem cells (UCMSCs) [10]. Thus, the goals of this research are isolation, culture and cryopreservation of human bone marrow – derived mesenchymal stem cells in order to supply stem cell resource for research and experimental treatment in mouse models at the laboratory of stem cell research and application.

MATERIALS AND METHODS

BMMSC isolation and culture

Bone marrow (BM) sample was obtained from a patient, being negative for HBV, HIV, HCV and some other sexually transmitted diseases, experienced surgery with the written consent of the patient and the authorities of Orthopedic Hospital in Ho Chi Minh City, Vietnam. Under local anesthesia, about 15 – 20 ml of bone marrow suspension was harvested from posterior iliac crest of donor and captured in a 50 ml tube (Falcon, BD Bioscience) containing the same volume of heparinized (10 U/mL) phosphate – buffered saline (PBS) to prevent clotting. The mixture of bone marrow and heparinized/PBS was kept at 4^{o}C prior to further processing in the laboratory. Mononuclear cells were isolated as a previously described protocol with some modifications. Briefly, bone marrow was loaded on the top with an equal volume of Ficoll – Paque (Sigma – Aldrich, Co, USA) preloaded in a centrifuge tube and centrifuged at 3000 rpm for 20 minutes in room temperature.

The top layer of mononuclear cells was collected and washed with PBS two times and DMEM-F12 medium one time. The isolated cells were suspended in DMEM-F12, supplemented with 15% FBS, 10 ng/ml FGF and 1% antibiotic – mycotic (Sigma – Aldrich, Co, USA) and seeded into 25 cm^2 flasks at a density of 5.10^5 cells/cm^2. The cells were cultured at 37°C, 5% CO_2. After 3 days, non – adherent cells were removed by washing twice with PBS and replacing the medium. The cell density and morphology was monitored under an inverted microscope. When these primary cultured cells reached 70 – 80% confluence, they were harvested using 0.25% Trypsin/EDTA (Sigma – Aldrich, Co, USA) and sub cultured at a ratio of 1:3. Then, the third passage cells were used for subsequent studies [11, 12].

Cell cryopreservation

BMMSCs at third passage were trypsinized by 0.25% trypsin/EDTA (Sigma – Aldrich, Co, USA) and centrifuged at 3,000 rpm for 5 minutes at room temperature to remove extra trypsin. Approximately 10^6 cells/ml re-suspended cryopreseved medium DMEM/F12 plus 10% DMSO. Cryovials were frozen and sequentially stored with slowly decreasing temperatures, incubated at 4°C for 10 minutes, cooled to – 20°C for 1 hour, and then cooled to –80°C for 1 days. Immediately afterwards, the cryovials were immersed in liquid nitrogen.

Cryopreserved cell recovery

Following storage, cryopreserved cells were thawed by rapidly immersing the cryovials in a water bath at 37°C. After warming for about 40–60 seconds, all cell suspension were resuspended in culture medium plus 30% FBS and centrifuged at 3,000 rpm for 5 minutes to collect cells at the bottom of the centrifuge tube. After thawing, cell number and the viability of post – cryopreserved MSCs was determined by Cell Viability Analyzer (Beckman Counter, USA).

Flow cytometry

To confirm that cryopreserved MSCs and fresh MSCs can maintain their immunophenotypic characteristics, MSCs were subjected to flow cytometry analysis. Briefly, fresh and cryopreserved MSCs were harvested using 0.25% Trypsin/EDTA and suspended at a concentration of 10^6 cells/ml by PBS. Then, they were with each FITC or PE – conjugated monoclonal antibody for 20 minutes at 4^oC in the dark. After the washing step, the labeled cells were analyzed by FACSCalibur cytometter (BD Biosciences, San Jose, CA) using Cell Quest Pro software (BD Biosciences). Immunophenotyping of MSCs was performed with antibodies, including anti – CD13 – PE, anti – CD14 – FITC, anti – CD34 –FITC, anti – CD44 – PE, anti – CD45 – FITC, anti – CD90 – PE, anti – CD166 – PE and anti –HLA-DR – FITC. They were all purchased from BD BioSciences.

In vitro differentiation

Differentiation potential of pre and post–cryopreserved MSCs can be assessed by differentiation into adipocytes. To induce adipogenic differentiation, MSCs were seeded on 6-well plates cells with 10^4 cells/well, and cultured in DMEM/F12 plus 10% FBS. Cells with nearly 70 – 80% confluence were exposed to DMEM (Sigma–Aldrich, Co, USA), supplemented with 0.5 mM 3-isobutyl-1-methylxanthine, 10 µM Dexamethazone, 100 µM Indomethacine, 10 µg/ml Insulin, 10% FBS (Sigma – Aldrich, Co, USA) and 1% antibiotic – mycotic for 2 – 3 weeks. The medium was changed every third or fourth day. Intracellular lipid droplets indicating adipogenic differentiation were observed under a light microscope and confirmed by Oil Red solution (Sigma–Aldrich, Co, USA), as an indicator of intracellular lipid accumulation.

RESULTS AND DISCUSSION

Morphological characteristics of MSCs

BM – derived mononuclear cells were seeded with a density of 5.10^5 cells/cm^2 and formed adherent heterogeneous cell populations after 3 days in culture, consisting of round and spindle – shaped cells (Fig. 1A). BM derived stem cell candidates have been shown to be morphologically distinguishable from adherent hematopoietic cells and monocytes. 7 days after the initial seeding, MSC candidates proliferated slowly and developed into small colonies (Fig. 1B).

Purification of MSC candidates was achieved by removing nonadherent cells during subsequent changes of medium. In the first passage of culture, fibroblast – like cells reached 70 – 80% confluence at day 12. When subcultured, heterogeneous cell populations had the same fibroblast – like shape after the third passage (Fig. 1C). Post – cryopreserved BMMSCs closely resembled pre – cryopreserved BMMSCs in morphology (Fig. 1D). Cryopreserved MSCs after thawing maintained morphology, proliferation. These results suggested that freezing process did not affect the morphology as well as the proliferation of MSCs [27].

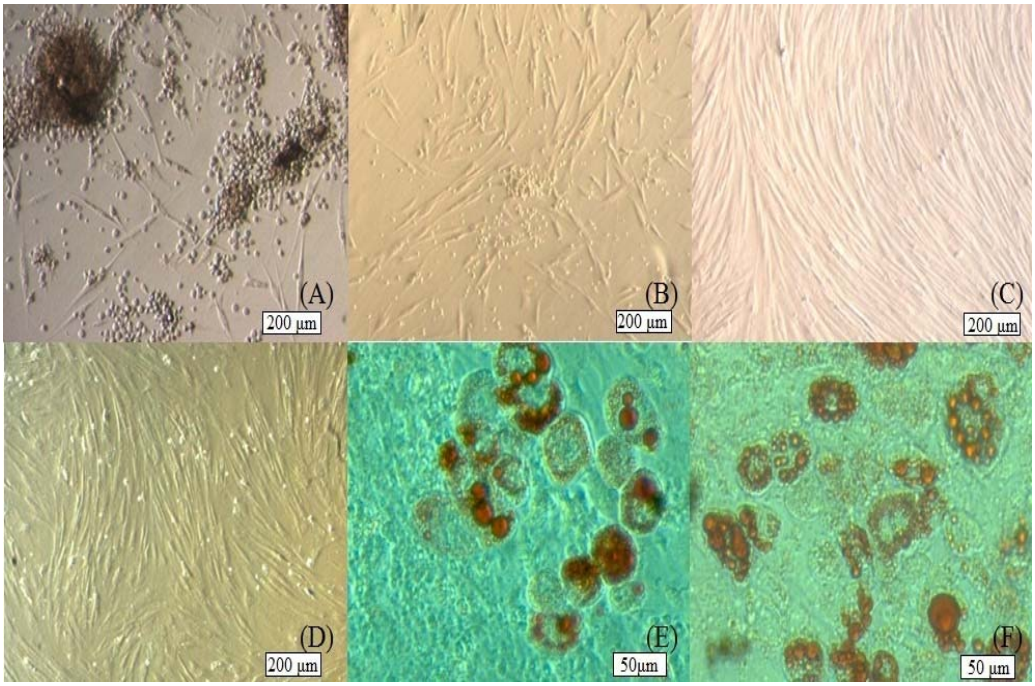

Figure 1. Isolation and differentiation of human BMMSCs. The population of MSC candidates were cultured after 3 days (A), after 7 days (B), after the third passage (C). The population of post – cryopreserved MSCs were cultured after 7 days (D). Adipocytes, differentiated from fresh MSCs (E) and post – cryopreserved MSCs (F), exhibited red color with Oil red staining.

In previous reports, the population of MSCs was known to be difficult to isolate because they were usually contaminated by hematopoietic progenitors and stem cells [5]. In addition, other ideals were suggested that the cells were heterogeneous in morphology and surface molecules 0. It was emphasized the presence of differently shaped and sized cells. The small ones, referred to stem cells, proliferated more rapidly than the large ones, seem to as mature MSCs [6]. In this study, however, we indicated that a relatively homogeneous population of human bone marrow – derived MSCs can be easily isolated by a normal method involving in the biological characteristics of MSCs, including their shape and size, their tendency to adhere, and their ability to help form colonies in culture. MSCs obtained by this method were relatively homogeneous and had significant proliferation, selfrenewal. In addition, seemly, MSCs might not proliferate if they were not separated from non – MSCs or dead cells. It could be explained by some soluble growth factors generated by non – MSCs or debris of dead cells, might inhibit the proliferation of MSCs. This speculation was supported by the fact that MSC candidates could lose their proliferation capacity if culture medium was not frequently changed. The presence of two differently shaped and sized cell populations also was recognized in initial passage and the cultured cell population became relatively homogeneous after passage three. In addition to adherent property, MSCs can be obtained by sorting cells on the basis of their differences in size and cytoplasmic granularity in morphology characteristics 0 or specific surface markers in immunophenotypic characteristics [1, 12].

Expression of surface markers

Flow cytometry analyses indicated that fresh MSCs were negative for CD14 (1.70%), CD34 (0.32%), CD45 (0.19%), HLA-DR (0.52%) and dimly positive for CD166 (78.25%). The cells were also strongly positive CD13 (96.32%), CD44 (91.72%), CD90 (96.58%) (Fig. 2). All markers are commonly used in the analyses of MSCs. These data coincide well with the reported data of MSC characteristics [11, 25].

To assess the stemness of the cryopreserved MSCs after thawing and culturing, these cells were also checked for surface markers expression. Analysis results also showed that MSCs were positive with markers CD13 (97.01%), CD44 (94.07%), CD90 (96.07%), CD166 (82.05%) and the rate of negative markers are CD14 (3.53%), CD34 (0.51%), CD45 (2.92%), HLA-DR (0.70%), respectively. This result is similar to the immunophenotypic characteristics of fresh MSCs (Fig. 2) ($p<0.5$). It was concluded that cryopreservation did not affect the immune-phenotype of human BMMSC populations.

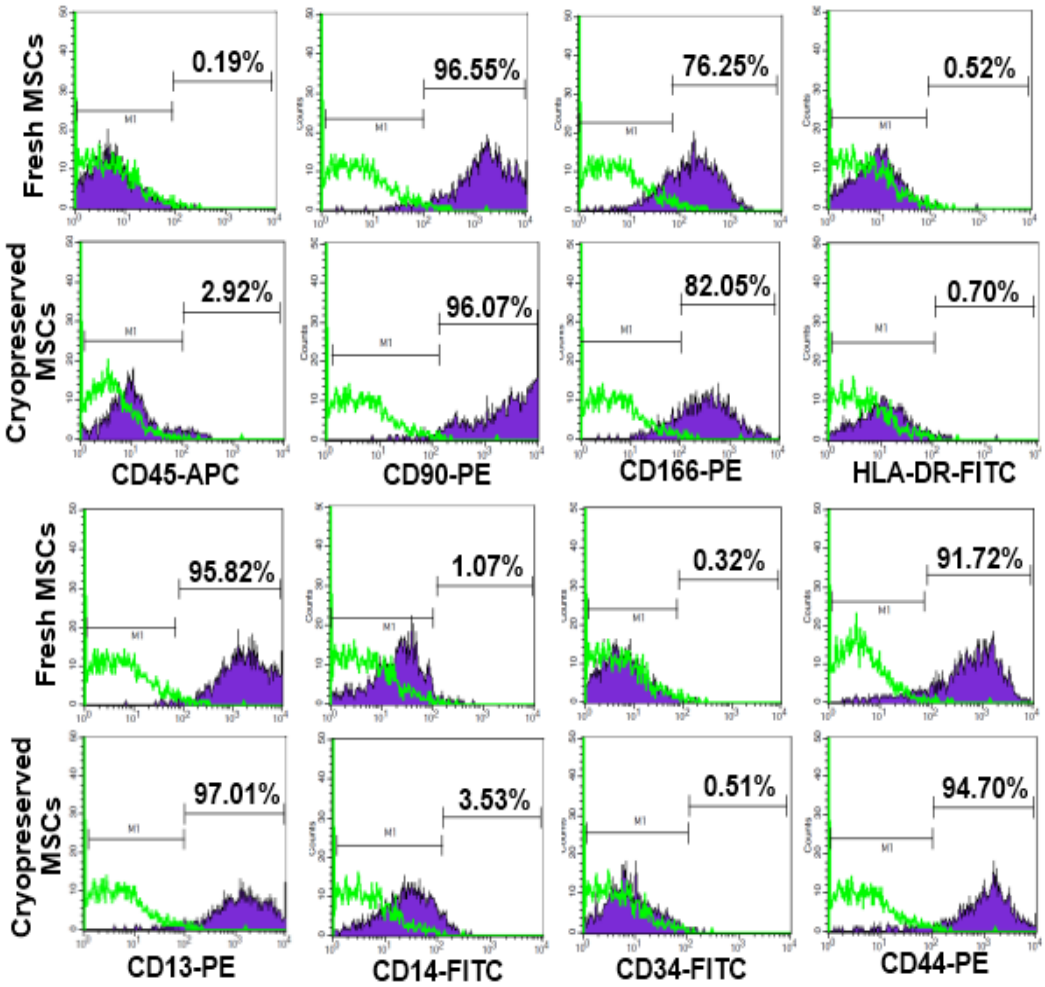

Figure 2. FACS analyses of fresh and cryopreserved BMMSCs. Fresh cells (A) and cryopreserved cells (B) were labeled with FITC – conjugated antibodies against CD13, CD14, CD34, CD44, CD45, CD90, CD166 and HLA-DR. Cells were analyzed with a FACSCalibur. The x axis indicates fluorescence intensity (FI). The y axis indicates cell counts.

Characterization of BMMSCs is also based on the expression of specific markers. Mesenchymal stem cells, both cryopreserved cells and fresh cells strongly expressed CD13, CD44, CD90 and dimly with CD166. They also were negative for CD14, CD34, CD45 and HLA-DR. These results demonstrated that BMMSCs, in this study, is relatively satisfactory to recommended minimal criteria of The Mesenchymal and Tissue Stem Cell Committee of the International Society for Cellular Therapy. CD14, CD34, CD45 and HLA-DR are common markers of circulating blood cells. Among of them, marker CD14, lipo-polysaccharide receptor, is a surface antigen of monocytes, macrophages and endothelial progenitor cells. The CD34 protein, a surface glycoprotein, expressed on developmentally early hematopoietic stem cells and progenitor cells as well as endothelial cells [14]. It has been suggested that CD34 negative stem cells may generate not only hematopoietic progenitors but also specific mesenchymal precursors, such as osteoclasts, chondrocytes, myocytes, adipocytes and others.

The CD45 molecule, known as the leukocyte marker, typically expressed on all hematopoietic cells at high levels. HLA-DR, known as HLA molecule Class II, is expressed in monocytes, macrophages, and lymphocytes, and its function is the presentation of exogen antigens to T_h lymphocytes (CD4) [20]. Since BMMSC population might be easily contaminated with blood cells, marker expression analysis involved in negative marker, including CD14, CD34, CD45 and HLA-DR is essential. Consequently, this proves that analyzed fresh MSCs do not contaminate to hematopoietic cell lines and some other cell lines.

Besides, analyzed cell population is also strongly positive for the markers CD13, CD44, CD90 and dimly positive for CD166. CD13, Aminopeptidase N (APN), a surface adherent protein, exhibit on various cell lines. Thy-1 or CD90 is a glycoprotein on the surface of cells. This protein was first detected in thymocytes. Currently, CD90 is considered a serious protein marker to identify mesenchymal stem cells from bone marrow, umbilical cord, umbilical cord blood, and some other sources [19]. The CD44 expression has also been demonstrated on human MSCs. CD44, hyaluronate receptor, is involved in leukocyte attachment to and rolling on endothelial cells. Recently, two markers CD44 and CD166, another adherent molecule, are used extensively to identify MSC from umbilical cord blood, adipose tissue and bone marrow [11, 16]. The positive and negative marker percentage of resuscitated hMSCs was similar to fresh MSCs. It showed that recovered MSCs possessed not only a homogenous morphology but also cell surface antigen profile with fresh cells.

Differentiation of MSCs

Both fresh and cryopreserved MSCs remained their differentiation potential into adipocytes. After cultured with adipogenic medium, MSCs began changing into ovoid morphology and accumulating intracellular lipid droplets which could be detectable in induced cells for 7 days. At the beginning of the two weeks, the lipid droplets enlarged and invaded the entire cytoplasm. The control cells showed no detectable lipid droplets. After 3 weeks of induction, Oil red staining positive adipocytes from fresh and cryopreserved MSCs were red color (Fig. 1E and Fig. 1F), which demonstrated the committed differentiation of MSCs into adipocytes when cultured in induced medium with known adipogenic factors. This result also revealed that the cryopreservation did not influence the differentiation potential of MSCs [27].

Viability of thawed MSCs

After thawing, the viability rates of cryopreserved groups were 72.95+/-6.14%. It could be explained by extrinsic factors in a culture that may affect the viability and proliferation of cells, including medium composition, serum, pH, or trypsinization effectiveness, etc. However, there were no significant differences in viability between them.

Cryopreservation, a method to preserve cells, is as important as cell culture method. The application of cryopreserved MSCs in cell therapy also requires preservation of their differentiation and proliferation ability. Thus, it is essential to investigate whether the freezing process influenced not only their proliferation ability but also their differentiation potential. The study of Bruder [3] revealed that resuscitated MSCs could be sub-cultured for many passages without noticeable loss of differentiation capacity. Furthermore, these cells did not lose their differentiation capacity after cryopreservation. After storage for 1 year, cryopreserved cells had a fairly high degree of viability, about 73% indicating that long-term cryopreservation for several months is not a critical problem. Most of the thawed cells can attach surface of culture flasks, similar to primary cultured cells. In addition to characteristics described above, another defining feature that thawed MSCs still kept was their differentiation potential or their ability into adipocytes when compared with that of primary cultured cells. These observations clearly showed cryopreserved MSCs could be stored and maintained high degrees of viability and differentiation potential. Indeed, our present study demonstrated that the thawed MSCs exhibited three essential stem cell characteristics, including the extensive self-renewal capacity, the multilineage potential and the specific marker expression [21]. With these results, we believed that cryopreserved MSCs might become a promising candidate cell source for cell – based therapy and autologous transplantation without a number of fresh stem cells. It also helps avoid the risk and possible damage caused by multiple bone marrow harvests in some cases, requiring duplicate treatments with cells or fresh bone marrow to expand a large number of cells. Furthermore, it is possible to establish an individual cell bank from bone marrow of donors or patients, used for treatment of the same individual in the future. Consequently, if cryopreserved MSCs were managed as an individual cell bank, it would be easy to supply numerous MSCs in indispensable cases.

CONCLUSION

BMMSCs are relative homogeneity and capacity for renewal and multipotentiality to differentiate. These cells are positive for the markers CD13, CD44, CD90, CD166, negative for the hematopoietic cell marker CD34, CD45 and several other markers CD14, HLA-DR. The results also showed that the freezing process does not significantly affect to the viability, proliferation, differentiation, differentiation capacity and immune-phenotypic characteristics of cryopreserved MSCs. The cryopreserved MSCs might be a good cell source for therapeutic or diagnostic purposes in regenerative medicine.

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