A STUDY OF THE BONE MARROW DERIVED MESENCHYMAL STROMAL CELLS IN RATS – PROLIFERATION AND IMMUNOPHYNOTYPIC MARKERS

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ABSTRACT. Mesenchymal stem cells derived from bone marrow (BM-MSCs) have the ability to differentiate into multiple cell lineages. Although the cultivation of these cells has led to a number of characterisation studies, some significant morphological and immunohistochemical properties are still lacking. In this study, isolation of BM-MSCs, morphological features, cell viability, immunophenotypic properties and cryopreservation of BM-MSCs were examined in detail. The results demonstrate that the cells isolated from BM-MSCs were plastic adherent and had fibroblastic spindle shape after three passages and get confluent monolayer cells 70-80% after 4-7 days post-subculture. Based on the cell viability analysis, the BM-MSCs showed an increase in cell viability starting from passage 1 until passage 10. Immunophenotypic analysis demonstrated that BM-MSCs were positive for CD44 and CD105 and negative for CD34. Functional analysis of cryopreservation of BM-MSCs from P6 after 6 months expressed good proliferation rate and cell viability.

Keywords: rat bone marrow, stem cells, CD markers, indirect immunoperoxidase

INTRODUCTION

Medullary bone cavities of the organisms after birth contains a variety of cell types, including haematopoietic lineage cells and mesenchymal stem cells (MSCs), making up the marrow stroma a heterogeneous cell population. MSCs are characterised *in vitro* by their fibroblast-like morphology. In 2006, ISCT (International Society for Cellular Therapy) defined three main criteria describing human MSCs in order to more accurately identify the cells. This means, cells should simultaneously meet these criteria in order to be defined as mesenchymal stem or stromal cells: (1) adherence to plastic surface, (2) positive for CD105, CD73, CD90 but negative for haematopoietic and endothelial marker molecules CD45, CD34, CD14, CD11b, CD79, CD19 and negative for HLA-DR (3) have the potential to differentiate into adipocyte, osteoblast, chondrocyte lineages, in response to appropriate stimuli *in vitro*.

Bone-marrow-derived MSCs have been isolated from a diversity of species, including mouse (Peister *et al*., 2004), rat (Javazon *et al*., 2001), rabbit (Johnstone *et al*., 1998), and human (Colter *et al*., 2001). Rodent MSCs were confirmed more difficult (Aubin, 1999), although this is not without argument (Javazon *et al*., 2001). The practical difficulties

in preparing MSCs from rodent bone marrow have limited the number of possible experiments, because animal transplantation models are required for preclinical studies. The selection of appropriate cell populations is apparently crucial for the outcome of *in vivo* experiments with MSCs. (Friedenstein *et al*., 1976).

Rat bone marrow-derived MSCs isolated from different laboratories all over the world share at least two characteristics: (1) can be cultivated for a long period of time, (2) stimulation and differentiation into mesodermal lineage cells including osteoblasts (Lorenzi *et al.* 2008), chondrocytes (Ahmed *et al.,* 2006; Peng *et al.,* 2008), and adipocytes (Lorenzi *et al.,* 2008; Peng *et al.,* 2008).

There are numerous features for the BM-MSCs but the most common are immunohistochemical characterisations, proliferation rates and cell viability, and morphological changes. Therefore, in this study, morphological and immunophenotypic properties of BM-MSCs were examined in detail.

MATERIALS AND METHODS

Isolation and culturing of RBM-MSCs

To establish the BM-MSCs culture, rats 2 to 3 weeks old, were sacrificed by cervical dislocation and their femurs and tibiae were carefully cleaned from adherent soft tissue. The tip of each bone was removed, and the bone marrow (BM) was harvested by inserting a syringe needle (27-gauge) into one end of the bone and flushing with MEM-Earle medium (Biochrom, Berlin, Germany). The BM cells were filtered through a 70-mm nylon mesh filter (Falcon, USA). Cells were plated into 6-well plastic cell culture plate at a density of 25×10^6 cells per well in MEM containing 15% foetal bovine serum (FBS, Sigma), 2 mm L-glutamine (Gibco, USA), 100 µ/ml penicillin (Sigma) and 100 µ/ml streptomycin (Sigma). Cultures were kept at 37 °C in a humidified atmosphere containing 95% air and 5% CO_2 (Nadri and Soleimani, 2007).

Passaging and subculture MSCs

Passaging of the cells were in suspension culture at cell density (>10^6 cells/ml). Typically, the first passage was done on day 6 post-initial culture. The MSCs are sub-cultured at approximately 80-90% confluence to prevent contact inhibition of growth and spontaneous differentiation (Solchaga *et al*., 2004). The culture medium was aspirated and the cells were washed three times with PBS and detached by incubation with 1 ml of trypsin for 1 minute at 37 °C. In order to dislodge the cells, the flask was gently rocked, then 5 ml of culture media containing 15% FBS was added to stop the trypsin action. Cells were counted using a haemocytometer chamber (Yang *et al.,* 2006)

Growth curve

The cells grown with cultivation usually has characteristic patterns in which three phases including lag, log and plateau phases can be recognised. In this research, the growth curve was plotted for each MSC derived from bone marrow. For this purpose, the

passage-3 cells derived from each tissue were plated at 5×10^4 cells/well in 12-well culture plates and allowed to become confluent. On a regular daily basis, some cells were trypsinised and cell number was determined by a haemocytometer count. Growth curves were drawn from the data.

Determination of cell viability

Counting of viable cells by using a haemocytometer chamber was according to the method described by Yang *et al.* (2006). Confluent monolayer cells of 25 cm^2 flasks were trypsinised and centrifuged at 1500 rpm for 10 minutes at 4 °C. At this juncture, the cell pellet was re-suspended with MEM complete growth media. For viable cell counting, 0.5 ml of trypan blue stain was added to 0.5 ml of cell suspension (v/v). After a gentle mix, 20 µl of the stained cells were dropped on a haemocytometer and promptly examined to count and calculate viable cell numbers by using the following formulae recommended by Phelan (1998).

$$\text{Cells/ml} = \begin{matrix}\text{average}\\ \text{count}\\ \text{per four}\\ \text{corners}\end{matrix} \times \begin{matrix}\text{dilution}\\ \text{factor}\\ (2)\end{matrix} \times \begin{matrix}10^4\\ (\text{correction}\\ \text{factor})\end{matrix}$$

Total number of cells and total number of viable (unstained) cells in original suspension were counted to calculate percent viable cells as follows:

$$\begin{matrix}\text{Total cells}\\ \text{in original}\\ \text{suspension}\end{matrix} = \text{cells/ml} \times \begin{matrix}\text{total original volume}\\ \text{of the whole cell}\\ \text{suspension}\end{matrix}$$

$$\begin{matrix}\%\\ \text{viable}\\ \text{cells}\end{matrix} = \frac{\text{Number of unstained cells}}{\text{Total number of cells}} \times 100$$

The protocol for immunostaining technique

The indirect immunoperoxidase test (IIP) was done by using the method followed by (Yang *et al.,* 2011). The mesenchymal stem cells were fixed with cold methanol: acetone (50:50 v/v) for 5 minutes. The glass slides were then immersed in 1% H_2O_2 in absolute methanol for 30 minutes. The PBS was then added to the glass slide for 15 minutes. The glass slides were then air dried. The primary antibody of the following CD markers (CD44, CD105 and CD34) were diluted 1:1000 with PBS and added to the glass slide, incubated for 1 hour at room temperature. The glass slides were then washed 3 times with PBS for 5 minutes each. The rabbit anti-rat IgG-HRP conjugated secondary antibody was then added to the glass slides (1: 1000) and incubated for 1 hour at room temperature. DAB substrate solution (DAB reagent set, Invitrogen, USA) was then added to the glass slides and incubated for 10 minutes in a dark room. The slides were mounted with buffer glycerol and examined under a light microscope.

Cryopreservation

Following the method recommended by Freshney *et al. (*1987), one-day-old subcultures of BM-MSCs at 6th passages were harvested and re-suspended to a concentration of 105 cell/ml in MEM media supplemented with 10% FBS, 1% antibiotic of penecillin-streptomycin, 10% dimethyl sulphoxide DMSO (DMSO; MERCK®, Germany). The cell suspension was dispensed into 1 mL cryopreservation

ampoules and kept initially at –20 °C for 1 hr, and then at –75 °C overnight and finally transferred into liquid nitrogen (–196 °C). The frozen cells were recovered from storage 6 months post-storage by thawing under running water at 37 °C. The freezing medium was removed by centrifugation, the cells were then suspended in MEM (15% FBS) and examined for viability, counted using a haemocytometer after staining with trypan blue. The viable cells were seeded into 25 cm^2 cell culture flask and monitored.

RESULTS

Isolation of Bone Marrow Derived-Mesenchymal stem cells

Mesenchymal stem cells were isolated according to their adherence to a tissue culture flask. After 1 day of cultivation, most of the cells were still mononuclear cells (Figure 1A). Three days later the cells extended and became elongated shape (Figure 1B). Five days later the cells became confluent, spindle-shaped (Figure 1C). After 7 days the cells grew and form 70-80% confluent monolayer (Figure 1D).

Cell viability

The cell proliferation and viability were analysed. A growth curve was determined to ensure that cells used in the following experiments were within the exponential growth phase. As shown in Figure 2, cells proliferated rapidly in day 3 to 5 and cell viability remained close to 95% and the number of cells began to decline on day 10 and the viability 75%.

Cryopreservation

The cryopreservation results showed that cells of BM-MSCs recovered after 6 months of storage in liquid nitrogen at P6, grew well to confluence within five days. The average viability for recovered cells was estimated about 55%.

Immunophenotyping analysis of MSCs

To determine the phenotypic nature of MSCs, the surface antigens CD44, CD105 and CD34 were examined by immunophenotypic staining technique. The results of immunophenotypic analysis showed that the adherent cells were strongly stained with CD44+ and CD105+ and the cells appeared in brown colour (DAB stain**)** (Figures 3A and 3B) while no colour stain appeared in the CD34 (Figure 3C).

DISCUSSION

Up till now, the bone marrow (BM) is still an invaluable source of adult pluripotent stem cells, including haematopoietic stem cells and MSCs. The therapeutic potential of BM MSCs for applications such as tissue engineering and gene therapy is enormous. Investigators demonstrated that these cells could be differentiated *in vitro* into cells with mesenchymal lineages such as adipocytes, chondrocytes, myoblasts and osteoblasts.

The first attempt to isolate MSCs were carried out by Friedenstein *et al*. (1987) to establish and develop a simple method to isolate and culture MSCs *in vitro*. It has been widely used in the actual practice in the laboratories. While there are still

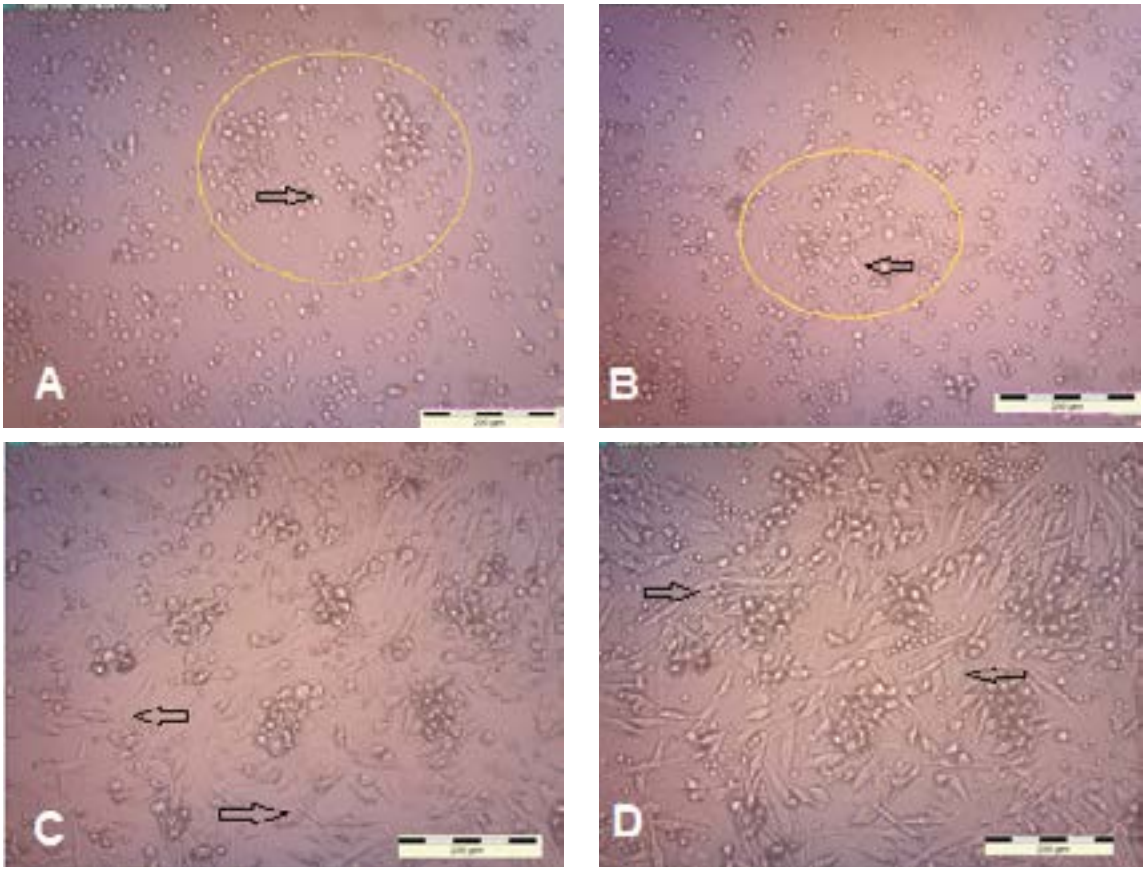

Figure 1. Morphologi of Rat MSCs primary culture. **A:** The cells after 1 day in culture, the MSCs were appeared oval-shaped (×10). **B:** The cells after 3 days in culture, the MSCs were appeared elongate-shaped (×10). **C:** The cells after 5 days of culture, the cells formed a monolayer of adherent cells (spindle shape) (×10). **D:** The cells after 7 days in culture, the MSCs were appeared a (80-90% confluency) (×10).

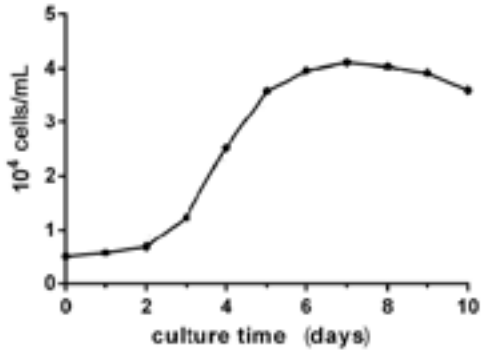

Figure 2. Growth curve of bone marrow-derived MSCs.

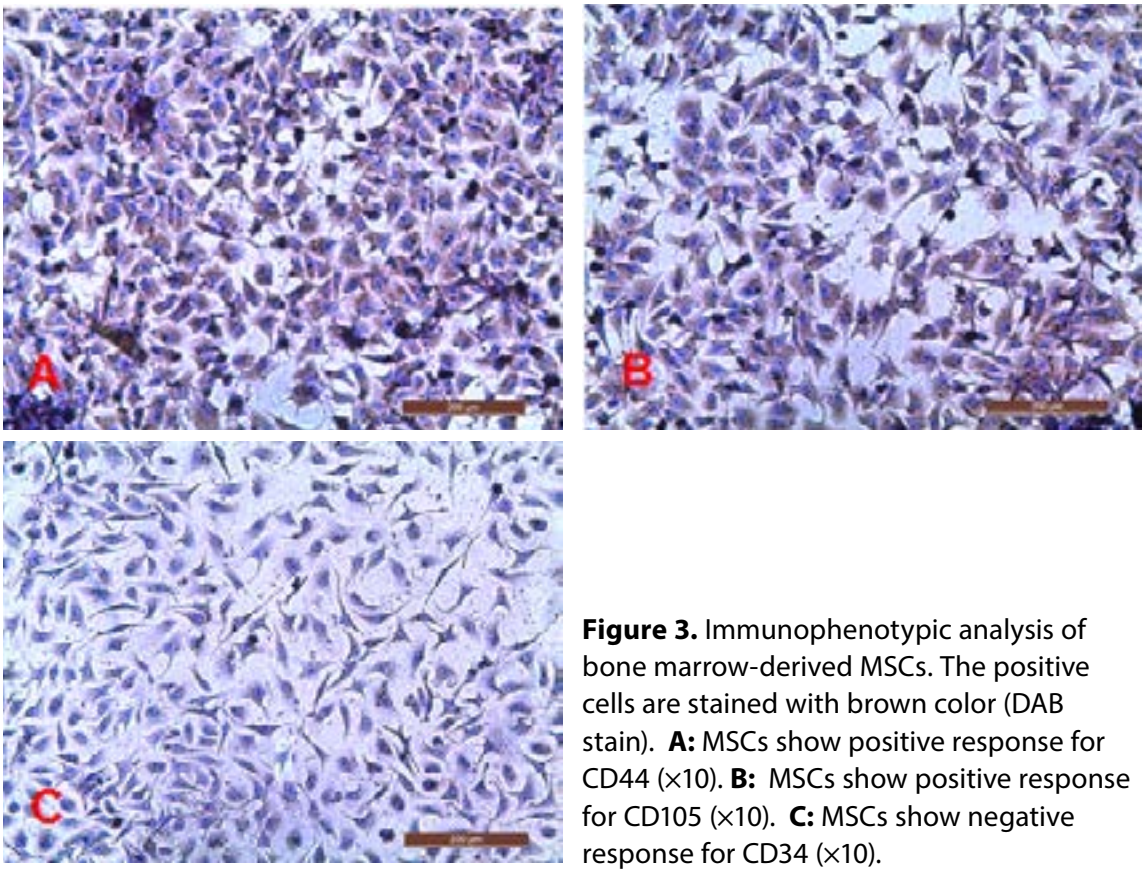

Figure 3. Immunophenotypic analysis of bone marrow-derived MSCs. The positive cells are stained with brown color (DAB stain). **A:** MSCs show positive response for CD44 (×10). **B:** MSCs show positive response for CD105 (×10). **C:** MSCs show negative response for CD34 (×10).

difficulties in harvesting a large number of MSCs for basic and clinical research. Common methods for isolating MSCs include whole marrow direct adherence, density gradient centrifugation, flow cytometry and immunobead methods. Flow cytometry and immunobead methods are considered high in cost and technically difficult.

The comprehensive implementation of these methods is unconscionable for animal test investigation. Common methods for isolating MSCs include whole marrow direct adherence and density gradient centrifugation. Density gradient centrifugation refers to the extraction of mononuclear cells for adherent culture according to the proportion of mononuclear bone marrow cells. Whole marrow direct adherence refers to regular culture medium changing to remove non-adherent cells based on the stem cell adherence characteristic to achieve MSC purification. Direct adherence is simple and convenient, obtains more MSCs than density gradient centrifugation and has an appropriate cell density for growth in culture flasks. In addition, MSCs purity increases after medium changes.

The present study showed that the morphological features of rat-derived MSCs were heterogeneous consisting of round cells in the beginning and converted to spindle-shaped, and finally became confluent monolayer (Clark and Keating *et al.,* 1995). These data are consistent with previous reports from rat bone-marrow-derived cells (Colter *et al.,* 2000).

Growth curve analysis was performed to measure proliferation of cells and phases of their growth throughout 8 days. The BM-MSCs had no lag phase and started proliferating immediately at day 2 of cultivation and reached the peak at day 7 and then it began to decline until day 10 of cultivation and this result in line with (Ahmed *et al.,* 2014).

In this study, the cell viability had shown a decline after 10 passages, because MSCs undergo replication senescence during the *in vitro* expansion, after a certain number of cell divisions. This phenomenon was first described in the 1960s by Leonard Hayflick (Hayflick *et al*., 1965), where he found that the course of long-term cultivation finally stopped after a specific number of cell proliferation.

The results of the indirect immunoperoxidase test showed that all MSCs were positive for CD44 and CD105 and negative for CD34. These results were also observed by several researchers (Colter *et al*., 2000; Xu *et al.,* 2004; Kern *et al.,* 2006), they demonstrated that the BM, derived MSCs showed a high percentage of CD105 and CD44 positive cells by immune staining and negative for CD34.

In conclusion, in the present study, the rat BMSCs isolated in a simple principle method of adhesion, it is possible to establish an effective method of harvesting and homogenous population of bone marrow. The BMSCs showed phenotypic characteristics of which point towards the stem cell-like features.

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