

A NOVEL METHOD FOR ISOLATION AND TRILINEAGE DIFFERENTIATION OF RAT BONE MARROW DERIVED MESENCHYMAL STEM CELLS

SANKARI D^{1*}, THANGARAJU P², ALWIN D³, THAMARAI KANNAN P⁴

¹Department of Biotechnology, Faculty of Science and Humanities, SRM University, Kattankulathur, Chennai, Tamil Nadu, India.

²Department of Animal Biotechnology, SRM Medical College Hospital and Research centre, SRM University, Kattankulathur, Chennai, Tamil Nadu, India. ³Department of Animal House, SRM Medical college Hospital and Research centre, SRM University, Kattankulathur, Chennai, Tamil Nadu, India. ⁴Department of Animal Biotechnology, Translational Research Platforms for Veterinary Biological, Tamil Nadu Veterinary and Animal Sciences University, Madavaram, Chennai, Tamil Nadu, India. Email: sankari.biotech09@gmail.com

Received: 20 May 2015, Revised and Accepted: 11 June 2015

ABSTRACT

Objective: Goal of this study is to find a simple method for isolation, colony formation and Trilineage differentiation of Mesenchymal stem cells from bone marrow of Wistar Rat. Adherent capacity, morphology, trilineage differentiation and colony formation of bone marrow mesenchymal stem cells were studied in low glucose, high glucose Dulbecco modified eagle medium with various concentration of fetal bovine serum.

Methods: Mesenchymal stem cells were isolated from bone marrow of Wistar rat by Ficoll (sigma) density gradient centrifugation with plastic adherence method. Bone marrow was collected from femur and Tibia of 6-weeks-old Wistar rat; Bone marrow was cultured in Dulbecco's modified Eagles's medium (DMEM) with low glucose, high glucose supplement (Invitrogen) and 10%, 15% of fetal bovine serum (FBS) at the density of 1×10^6 , incubated at 37°C in 5% of CO₂; adherent capacity, colony formation were studied. Differentiation of bone marrow mesenchymal stem cells (BMMSCs) was monitored under suitable differentiation medium. The cells were refeed every 3-4 days and passaged when the cells reached 80-90% confluences.

Result: BMMSCs were adhered in tissue culture flask at 24 hrs, 48 hrs in high glucose and low glucose DMEM with 10% of FBS, respectively. Colony formation was faster in high glucose and low glucose DMEM with 15% of FBS compared to high glucose and low glucose DMEM with 10% FBS. Morphological changes were observed in BMMSCs high glucose, low glucose DMEM with 10% FBS, but no changes were found in the differentiation of BMMSCs in high glucose, low glucose DMEM. Differentiation of BMMSCs was nourishing in third passage cells.

Conclusion: High glucose DMEM with 10% FBS is a good supplement for adherence of cells whereas high glucose, low glucose DMEM with 15% FBS can be utilized for rapid Colony formation. Third passage BMMSCs is fruitful for differentiation of BMMSCs.

Keywords: Wistar rat, Bone marrow, Mesenchymal stem cells, Adherent capacity, Colony formation, Differentiation, Osteoblast, Chondrocytes, Adipocytes.

INTRODUCTION

Organ transplantation is replaced by stem cell therapy. Stem cell therapy is an interdisciplinary research area. Stem cells are biologically active molecules compared with other cell types, found in all organisms; stem cells are two types, embryonic stem cells, and adult stem cells. Cells which are isolated from blastocyst is called embryonic stem cells, adult stem cells can be isolated from head to toe of human being and animals, they are unspecialized cells, capable of long-term self-renewing, repairing and replacing damaged cells tissues. During cell division, each new cell has potential either to remain a stem cell or become a more specialized function, such as a nerve cell, muscle cell, red blood cell, brain cell, etc. Due to their multilineage potency, stem cell plays a vital role in cell therapies and regenerative medicine. Due to ethical issues on embryonic stem cells, the adult stem cells can be utilized for cell therapy and regenerative medicine. Mesenchymal stem cells are multipotent stromal cells and can be isolated from various sources, but bone marrow is the most abundant source. Rat bone marrow contains two different types of stem cells, hematopoietic stem cells which are involved in the formation of blood cells and nonhematopoietic stem cells which are responsible for multilineage capacity. Bone marrow mesenchymal stem cells (BMMSCs) are a promising source for future clinical use due to their self-renewing, proliferation, multilineage differentiation, mineralizing cells, and immune modulation. Mesenchymal stem cells provide support and maintenance of Hematopoietic stem cells

which are present in bone marrow [4,10] Mesenchymal stem cells (MSCs) were first discovered 130 years ago by German pathologist Cohnheim, [3] After several decades, the Multipotent capacity of MSCs were recognized by Fridenstein *et.al.* [1,3] in 1976. MSCs are present very low in number at bone marrow (1:100000) but proliferation and expansion capacities are higher in proper culture medium [2] MSCs can differentiate into various cell types, like bone, cartilage, adipose, muscle, hepatocytes [6,11], and endothelium [7] ISCT recommend three minimal criteria to define MSCs. First MSCs must be plastic adherence in standard culture conditions. Second must express CD105, CD73, and CD90. Third MSCs must differentiate to osteoblasts, adipocytes and chondrocytes *in vitro* [5,11]. Karyotype analysis also recommend to characterize MSCs [13]. BMMSCs can be grown efficiently in suitable culture condition compared with MSCs from other sources [11]. Rat MSCs from bone marrow also contains neuroprotective properties [14].

METHODS

Experimental animal

Six weeks age young male albino rats of Wistar strain, weighing approximately 60-70 g, were acclimatized and housed in the central animal house, SRM MCH, and RC. All animals were kept in 12:12 hr light: Dark cycle, at a room temperature of 24±2°C. Rats were fed with standard rat pellet supplied by Provimi animal nutrition India Ltd, Bangalore, India, and water ad libitum provided. The experiments were

conducted according to the ethical norms approved by Institutional Animal Ethics Committee Guidelines of SRM University (IAEC No. 073/IAEC/2013).

Collection of sample

Rats were euthanized with thiosol (150 mg/kg b.wt). According to the principle and procedure, the femur and Tibia were removed, under the supervision of on duty veterinary medical officer in accordance to the ethical norms of Institutional Animal Ethics Committee.

Isolation and expansion of MSCs

BMMSCs were isolated by Ficoll (sigma) density gradient centrifugation with plastic adherence method (Combination method). Bone marrow sample was diluted with equal volume of DPBS, centrifuged at 400 g for 30 minutes. Ficoll hypaque was added to separate the Mononuclear cells and the cells were transferred aseptically in to high glucose DMEM and low glucose DMEM with 10%, 15% fetal bovine serum (FBS) (Invitrogen), 1% penicillin, and 1% streptomycin. No additional growth factors required for this protocol.

Culture of MSC in two different medium with various concentration of FBS

Isolated cells were seeded into two different medium low glucose and high glucose DMEM with 10%, 15% FBS, incubated at 37°C with 5% CO₂. Non-adherent cells were removed after 24 hrs (high glucose DMEM) and 48 hrs (low glucose DMEM). The culture medium was replaced every 24 hrs to avoid possible differentiation effects of various cytokines originating from MSCs. Cells were Passaged using the standard trypsinization method, and cells were counted by trypan blue staining method (Sigma).

Subculture of BMMSCs

Adherent cells were subcultured at 80-90% of confluence (7th day) [9,10]. Medium was discarded, cells were rinsed twice with DPBS and 1-2 ml of Trypsin ethylenediaminetetraacetic acid was added to lift the adherent cells followed by addition of 5-7 ml of DMEM to stop further enzymatic action on the BMMSCs. Cells were centrifuged at 1200 rpm for 10 minutes at 37°C; Pellet was collected and suspended in DMEM. Subcultured cells were seeded at the density of 1×10^6 .

Colony forming unit

Third passage cells which were grown in low glucose and high glucose DMEM were detached and grown for 14 days in the new culture plates. After 14 days, cells were fixed with 4% para formaldehyde and stained with Giemsa stain.

Trilineage differentiation potential of BMMSCs

Differentiation potential of BMMSCs was carried out on third passage cells.

Adipogenic differentiation of MSCs

Third passage cells were used to induce the differentiation in BMMSC (20,000cells/cm²) MSC growth medium was replaced by adipogenic differentiation medium and kept in humidified atmosphere with 4-6% CO₂. Furthermore, refeeded the cells with adipogenic differentiation medium every 3-4 days and after 7 days media was removed and washed with DPBS, cells were fixed with 4% paraformaldehyde, HSC LipidTOX, and Oil O Red were added to visualize the adipogenic differentiation.

Chondrogenic differentiation of MSCs

To induce chondrogenic differentiation, third passage cells (4000/cm²) were used. Mesenchymal stem cells growth medium was replaced by the chondrogenic differentiation medium. Humidified atmosphere with 4-6% CO₂ was maintained, and the medium was replaced every 3-4 days. After 14 days, media was removed and washed in DPBS. Alcian blue was utilized to identify the chondrogenic differentiation.

Osteogenic differentiation of MSCs

Osteogenic differentiation was induced by culturing third passage BM-MSCs (4000 cells per cm²), up to 21 days in Osteogenic induction medium (Invitrogen) and kept in humidified atmosphere with 4-6% CO₂, with changing the medium every 3-4 days. After 21 days, cells were rinsed with DPBS, fixed with 4% formaldehyde for 30 minutes; Osteocytes and calcium deposition were identified with Alizarin Red stain.

RESULTS

Isolation and culturing of MSCs in low glucose DMEM

Ficoll hypaque and density gradient method

The Rat bone marrow cell suspension was layered on Ficoll and isolated by density gradient method (combination method), and the cells were cultured in tissue culture flask with low glucose DMEM. MSC were easily identified by a plastic adherent, grown in monolayer and glistening was identified. BMMSCs were attached in the tissue flask at day 2, and the round cells were removed by repeated media changes. Adherent cells showed spindle shape and started to proliferate in the first passage and gradually grew to colonies. Homogenous and Elongated cells were observed at passage 3. Colonies were observed with Giemsa stain (Fig. 1).

Isolation and culturing of MSCs in high glucose DMEM

Ficoll hypaque and density gradient method

The Rat bone marrow cell suspension was layered on Ficoll and isolated by density gradient method (combination method) and cultured in the tissue culture flask with High glucose DMEM. MSC were easily identified by a plastic adherent, grown in monolayer and glistening was identified BMMSCs were attached in the tissue flask at day 1; round cells were removed by repeated media changes. Adherent cells showed spindle shape and started to proliferate at day 2 and gradually grew to colonies. Cells were showed spindle morphology in the second passage. Homogenous and Elongated cells were observed in the third passage. After 14 days, the third passage cells were stained with Giemsa stain (Fig. 2).

Trilineage differentiation of BMMSCs

BMMSCs were differentiated *in vitro* using adipogenic, chondrogenic and osteogenic induction medium. Following 7 days of Adipogenic induction, the cells stained with HCS LipidTOX and Oil Red O showed adipocytes. MSCs were differentiated into chondrocytes in chondrogenic induction medium. After 14 days, cells were stained with Alcian Blue. Osteocytes were observed when cells grown for 21 days in the osteogenic induction medium, osteocytes, and calcium deposition were identified in Alizarin Red stain (Fig 3).

DISCUSSION

Bone marrow is the most abundant source of MSCs, many methods are available to isolate Mesenchymal stem cells from bone marrow, such as plastic adherence, erythrocyte lysis buffer, Ficoll separation, density gradient centrifuge, immunomagnetic beads selection, adding growth factors, etc [8]. In this study, we utilized Ficoll separation with density gradient centrifuge (combination method). BMMSCs were easily adhered on the plastic culture plate, but hematopoietic contamination, irregular morphologies of BMMSCs were found till 1st passage, after the first passage HSC contamination was removed by repeated culturing and 90% pure homogenous population of MSCs were found on the tissue culture plates. The homogenous population was observed at third passage. Colony formation was faster in DMEM (LG and HG) with 15% FBS; expansion of BMMSCs under suitable culture condition is still laborious. BM niche is a powerful environment for MSCs that encompass osteoblasts, adipogenic cells, and chondrogenic cells, stromal cells, extracellular proteins and signaling molecules [11,12]. BMMSCs were cultured on three different culture medium at 37°C

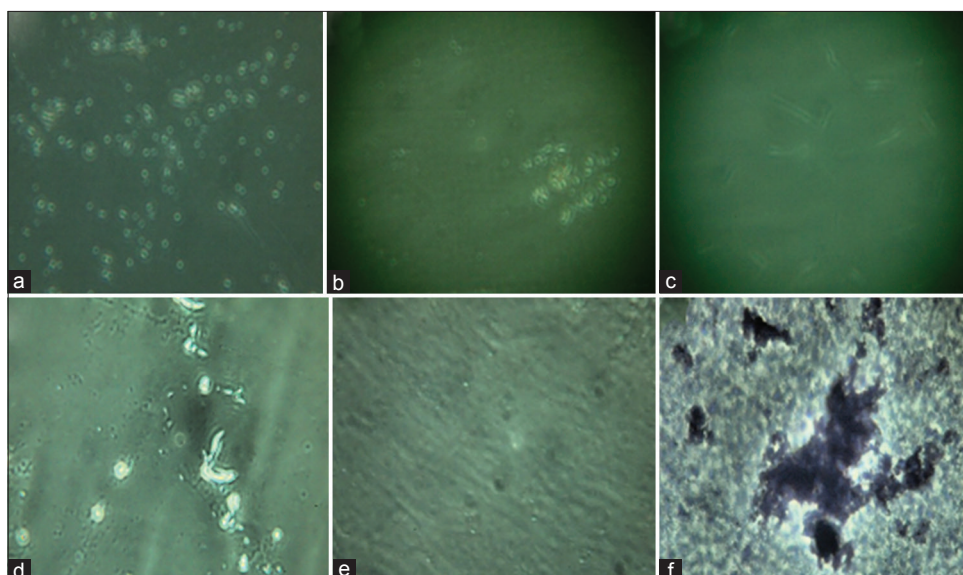


Fig. 1: Morphologies of bone marrow mesenchymal stem cells in low glucose DMEM: (a) Passage 0 at day 2, (b) at day 3, (c) passage 1 at day 2, (d) passage 2 at day 2, (e) passage 3 at day 2, (f) passage 3 at 14 day stained with giemsa stain

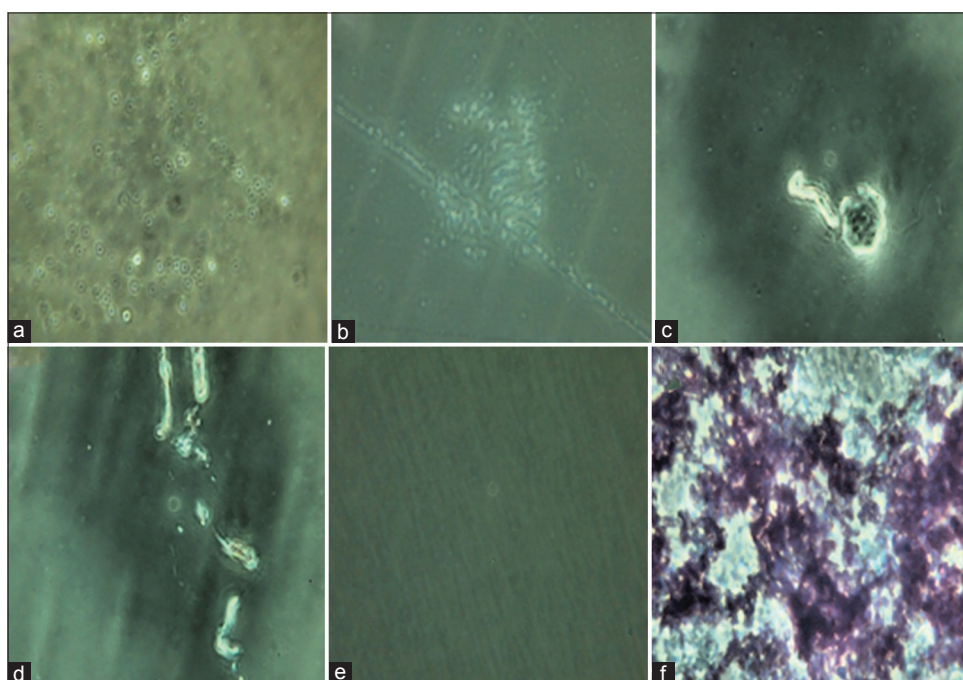


Fig. 2: Morphologies of bone marrow mesenchymal stem cells in high glucose DMEM: (a) passage 0 at day 1, (b) at day 2, (c) passage 1 at day 2, (d) passage 2 at day 2, (e) passage 3 at day 2, (f) passage 3 at 14 day stained with Giemsa stain

in 5% CO₂, after 7 days chondrogenesis was analyzed, adipogenic cells were found on the adipogenic induction medium on 14th day, osteogenic differentiation was found after 21 days on the tissue culture plates, plastic adherence, multilineage differentiation are important biological characters to identify the bone marrow-derived MSCs. Due to the inherent heterogeneity of MSCs, there are no unique surface markers to characterize the BMMSCs. Thus, we focused two biological characters of BMMSCs.

CONCLUSION

Ficoll density gradient centrifugation with the plastic adherence method is felicitous for the isolation, expansion. High glucose DMEM with 10% FBS is a good supplement for adherence of cells whereas

high glucose, low glucose DMEM with 15% FBS feasible for the rapid colony formation. BMMSCs have showed homogenous population and spindle elongated morphology at third passage. Third passage cells were successfully differentiated into adipocytes, chondrocytes and osteocytes Based on these important characteristics, third passage cells can be utilized for cell therapy and regenerative medicine.

ACKNOWLEDGMENTS

We would like to thank Department of Biotechnology, Faculty of science and Humanities, SRM University, Kattankulathur, SRMMCH&RC SRM University, Kattankulathur, ISISM, SRM University, Kattankulathur and Southern India Scientific Corporation, Chennai.

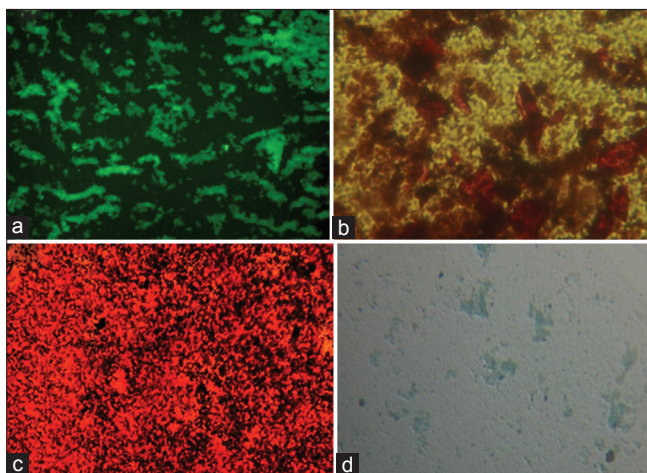


Fig. 3 *In vitro* characterization of Third passage bone marrow mesenchymal stem cells (BMMSCs). (a,b) Adipogenic differentiation of BMMSCs was confirmed by HSC LipidTOX and Oil O Red, (c) Osteogenic differentiation of BMMSCs was confirmed by Alizarin Red, (d) chondrogenic differentiation of BMMSCs was confirmed by Alcian Blue.

REFERENCES

- Friedenstein AJ, Gorskaja JF, Kulagina NN. Fibroblast precursors in normal and irradiated mouse hematopoietic organs. *Exp Hematol* 1976;4(5):267-74.
- Beyer Nardi N, da Silva Meirelles L. Mesenchymal stem cells: Isolation, *in vitro* expansion and characterization. *Handb Exp Pharmacol* 2006:249-82.
- Prockop DJ. Marrow stromal cells as stem cells for nonhematopoietic tissues. *Science* 1997;276(5309):71-4.
- Pittenger MF, Marshak DR. In: Marshak DR, Gardner RL, Gottlieb D, editors. *Stem Cell Biology*. New York: Cold Spring Harbor Laboratory Press, Cold Spring Harbor; 2001.
- Dominici M, Le Blanc K, Mueller I, Slaper-Cortenbach I, Marini F, Krause D, *et al*. Minimal criteria for defining multipotent mesenchymal stromal cells. The international society for cellular therapy position statement. *Cytotherapy* 2006;8(4):315-7.
- Kang XQ, Zang WJ, Song TS, Xu XL, Yu XJ, Li DL, *et al*. Rat bone marrow mesenchymal stem cells differentiate into hepatocytes *in vitro*. *World J Gastroenterol* 2005;11(22):3479-84.
- Oswald J, Boxberger S, Jørgensen B, Feldmann S, Ehninger G, Bornhäuser M, *et al*. Mesenchymal stem cells can be differentiated into endothelial cells *in vitro*. *Stem Cells* 2004;22(3):377-84.
- Polisetti N, Chaitanya VG, Babu PP, Vemuganti GK. Isolation, characterization and differentiation potential of rat bone marrow stromal cells. *Neurol India* 2010;58(2):201-8.
- Ayatollahi M, Salmani MK, Geramizadeh B, Tabei SZ, Soleimani M, Sanati MH. Conditions to improve expansion of human mesenchymal stem cells based on rat samples. *World J Stem Cells* 2012;4(1):1-8.
- Meirelles Lda S, Nardi NB. Murine marrow-derived mesenchymal stem cell: Isolation, *in vitro* expansion, and characterization. *Br J Haematol* 2003;123(4):702-11.
- Yameen Z, Leavesley Z, Upton Z, Xiao Y. Multilineage differentiation potential of bone and cartilage cells derived from explant culture. *Open Stem Cell J* 2009;1:10-9.
- Wilson A, Trumpp A. Bone-marrow haematopoietic-stem-cell niches. *Nat Rev Immunol* 2006;6(2):93-106.
- Fu WL, Zhang JY, Fu X, Duan XN, Leung KK, Jia ZQ, *et al*. Comparative study of the biological characteristics of mesenchymal stem cells from bone marrow and peripheral blood of rats. *Tissue Eng Part A* 2012;18(17-18):1793-803.
- Taran R, Mamidi MK, Singh G, Dutta S, Parhar IS, John JP, *et al*. *In vitro* and *in vivo* neurogenic potential of mesenchymal stem cells isolated from different sources. *J Biosci* 2014;39(1):157-69.