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RESEARCH ARTICLE

DIFFERENTIATION POTENTIAL OF OVINE BONE MARROW DERIVED MESENCHYMAL STEM CELLS

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Abstract

Mesenchymal stem cells from bone marrow were harvested from femur of adult sheep and tripotential property of bone marrow derived mesenchymal stem cells (BM-MSCs) was accessed. Mesenchymal stem cells (MSCs) are multipotent adult stem cells present in all post-natal organs, which can differentiate into different tissues originating from mesoderm ranging from bone and cartilage to cardiac muscle. BM-MSCs have been identified as a multipotent stem cells that have been shown to differentiate *in-vitro* into osteocytes, chondrocytes and adipocytes. In the present study BM-MSCs were isolated by Percoll density gradient centrifugation, cultured to a confluent monolayer. BM-MSCs were subcultured for further passages. Cells harvested at passage 4 were maintained in differentiation medium for an optimal time period of 21 days and their differentiation into oteocytes, chondrocytes and adipocytes was observed by histochemical staining. Differentiated cells in osteogenic, chondrogenic and adipogenic medium were positive for Alizarin Red S, Toluidine blue and Oil Red 'O' respectively with a distinct cell morphology. This findings suggested that ovine bone marrow as a reliable source of adult stem cells which can differentiate into mesodermal lineages.

INTRODUCTION

Adult stem cells were present throughout the lifetime of the individual and were involved in continued development of tissues, replacement and repair in certain disease states (Young *et al.,* 1995). Friedenstein *et al.* (1968) first described the mesenchymal stem cells (MSCs) from bone marrow. MSCs are morphologically fibroblast-like cells and characterized by their ability to both self-renew and differentiate into tissues of mesodermal origin (osteoblasts, adipocytes, chondrocytes and myocytes) which has a tremendous medical potential in relation to regeneration of bone and cartilage (Pittenger *et al.,* 1999 and Robey and Bianco, 2006). Presently, the therapy based on the adult multipotent stem cells is becoming, consequently, an important scientific subject where usual contemporary medicines fail to treat the disease. It is possible due to high proliferation capacity and multilineage differentiation ability of MSCs. It also has already been proved that most promising stem cell source in clinical practice is represented by bone marrow with more focus on candidate stem cells especially haematopoietic stem cells and mesenchymal stem cells (Serban *et al.,* 2008).

MATERIALS AND METODS

Bone tissue from femur of adult sheep was collected in a ziplock cover containing normal saline with Betadine (5%), antimycotic and antibiotics, (100 units/ml of pencillin, 100g/ml of streptomycin) from Corporation Slaughter House, Chennai. It was processed within 60 minutes of collection of sample. Isolation of mono nuclear cell fraction was done by Percoll density gradient centrifugation in the Centre for Stem Cell Research and Regenerative Medicine, Madras Veterinary College (Ramakrishnan *et al.,* 2013). Isolated mononuclear cells were seeded at a density of 1×10^6 cells/ml in T-25culture flasks with 5 ml of Dulbecco's modified Eagle's medium-high glucose (DMEM-HG) (GIBCO®). The spent medium was changed at 2-3 days interval until the confluency was attained. After 70-80% confluency the cells were detached from the culture flask by using 0.25% trypsin/EDTA (Cat. No.-T4049, Sigma®) for 2 minutes at 37°C for further passaging. On reaching the confluency cells were passaged subsequently and maintained upto P4 level. The subcultured cells of passage 4 (P4) were plated at a density of 3×104 cells/ well in a 12 well plate with 1.5 ml of HG-DMEM and was allowed to get confluent (Plate 1). Then the medium was replaced by same volume of differentiating medium composing of basal medium (Osteocyte/Chondrocyte differentiation Basal Medium (GIBCO®, Cat. No. A10069-01 and HiAdipoXL Basal Medium Cat No. AL521, HiMedia® (Part-A) supplemented with osteocytic growth factors (Cat. No. A10066-01, GIBCO®), chondrocytic growth factors (Cat. No. A10064-01, GIBCO®) and adipocytic growth factor (Cat No. AL521, HiMedia® (Part-B)) growth factors in the ratio of 10:1 and cells were incubated at 37°C and 5% CO_2 with change of medium in every three days.

After 21 days of induction, osteogenic differentiation was confirmed by Alizarin Red S staining. For staining, the cultures were fixed with 4% formaldehyde for 20 minutes at room temperature and then subjected to 2% Alizarin Red S solution for 5 minutes to detect the presence of mineralized matrix in the culture.

Similarly at the end of 21 days of induction period of chondrogenic differentiation, the same way cells were fixed with 4% formaldehyde and stained with 0.04% Toluidine blue for 10 minutes to detect the presence of proteoglycans in the culture.

Likewise the cells subjected to adipogenic differentiation medium was maintained for 14 days. At end of the induction period, cells were fixed with 4% formaldehyde and stained with 0.3% Oil Red 'O' for 50 minutes which was confirmed by accumulation of lipid rich vacuoles.

RESULT

In the present study, trilineage differentiation capacity of adult ovine BM-MSCs into osteocyte, chondrocyte and adipocyte was confirmed by the histochemical staining technique as demonstrated by Baghaban Eslaminejad *et al.* (2009) and Heidari et al. (2013).

When cultured in osteogenic medium for a period of 21 days, BM-MSCs proliferated extensively to become over confluent and showed a progressive change in cell morphology and followed a complex pattern in cell arrangement and formation of extracellular matrix. The differentiated cells showed a positive reaction to Alizarin Red S in which the cells and extracellular matrix was stained red (Fig-1). Chondrogenic differentiation was evident by the presence of proteoglycan rich soft collagen matrix component, glycosaminoglycans which stained basophilic by Toluidine blue staining (Fig-2). Cells in chondrogenic differentiation medium became enlarged and elongated gradually. Differentiated cells in the adipogenic medium were more or less flat in morphology and larger in size as compared to osteocytes in osteogenic and chondrocytes in chondrogenic medium. Adipogenic induction was apparent by the accumulation of lipid rich vacuoles within the differentiated cells stained by Oil Red 'O' stain (Fig-3).

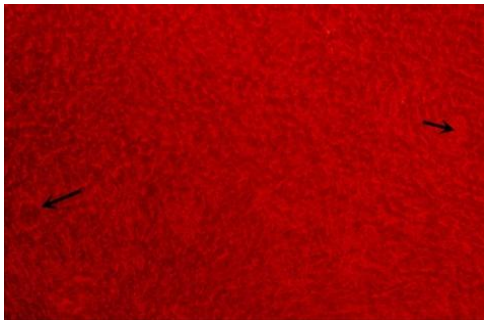

Figure-1 Photomicrograph of adult Ovine BM-MSCs (arrow) showing osteogenic differentiation at P4 level (arrow) Alizarin Red S x 200

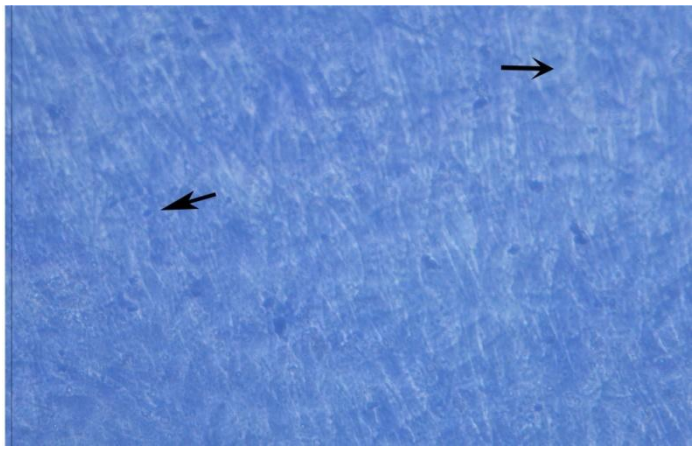

Figure-2 Photomicrograph of adult Ovine BM-MSCs (arrow) showing chondrogenic differentiation at P4 level (arrow) Toluidine Blue X200

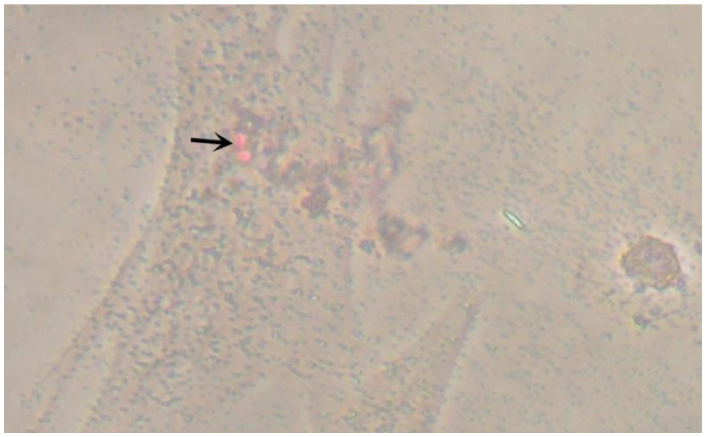

Figure-3 Photomicrograph of adult Ovine BM-MSCs showing adipogenic differentiation at P4 level (arrow) Oil Red 'O' x 400

DISCUSSION

A novel way to confirm the presence of MSCs in the adult tissues is, by their ability to differentiate into bone, cartilage and fat *in-vitro* in addition to its morphologic or phenotypic characteristics (Chamberlain *et al.*, 2007). Differentiation is a process which changes the cell in size, shape, membrane potential, proportion of its constituents and metabolic activity as a result of modification in gene expression as reviewd by De Schauwer *et al.*, 2011. This study was focused on multilineage differentiation potential of MSCs under specific culture conditions. Over time deposits of calcium was demonstrated by Alizarin Red S in osteogenic induction and Toluidine blue staining verified chondrogenic induction by staining acid mucosubstances within the extracellular matrix. Since Oil Red 'O' stains neutral glyceride droplets, it was used to identify adipogenic induction. Accumulation of lipid droplets was seen within the flat differentiated cell (Chamberlain et al., 2007; De Schauwer *et al.*, 2011 and Heidari *et al.*, 2013). Efficacy of MSCs to differentiate into various lineages varies with source of the tissue selected. BM-MSCs showed good osteogenic and chondrogenic differentiation as reported by Bochev *et al.*, 2008 and Vishnubalaji *et al.*, 2012, where as in the present study differentiation of BM-MSCs into adipogenesis was upto little extent as also reported by De Schauwer *et al.* (2011), they found the necessity of rabbit serum to induce adipogenesis in equine MSCs. Since Rentsch *et al.* (2010) demonstrated that ovine bone marrow derived mesenchymal stem cells share the similar morphologic, immunophenotypic and functional characteristics as their human counterpart and characteristic of ovine BM-MSCs is known to a little extent, further studies in this regard has yet to be explored.

CONCLUSION

Along with mesodermal lineage differentiation potential, MSCs have the capacity to transdifferentiate into ecto and endodermal lineages which made it to stand as an important aspect in regenerative medicine. An emerging research in the field of BM-MSCs focused that it can be engrafted in multiple tissues and underwent site specific differentiation into osteocytes, chondrocytes, adipocytes, myocytes, cardiomyocytes, hepatocytes, astrocytes, bone marrow stromal cells and thymic stroma. Our observation here also support that adult bone marrow derived mesenchymal stem cells can differentiate into osteocytes, chondrocytes and adipocytes as evident by Alizarin Red S, Toluidine blue and Oil red 'O' staining technique.

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