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An Approach to Derive Bone Marrow Mesenchymal Stem Cells with Therapeutic Significance

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ABSTRACT

"Mesenchymal Stem cells (MSCs)" are distinct type of cells naturally present in the body that has the ability to separate into any cell type like bone, cartilage, muscle, nerve, tooth etc. There are numerous studies available to efficiently isolate culture, characterize and expand the bone marrow mesenchymal stem cells. Similar such method was utilized to isolate the MSCs from femoral and tibial bone of female wistar rats and the standardized method was used to culture and expand MSCs. The cultured cells were determined to be MSCs with their hallmark properties of spindle shape morphometry, cellular plasticity & cell adherence property. The cells after expansion were subjected to flow cytometry analysis for characterization. The BMSCs showed positive expression for CD90 & negative expression for CD34. In the present investigation, we obtained the BMSCs that would have potential clinical significance as it was performed in a GMP environment with all necessary requirements that could enable its therapeutic value.

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INTRODUCTION

Stem cells have the significant prospective for developing differentiated cell types like bone (Saraswathi *et al.*, 2015), cartilage (Goldberg *et al.*, 2017), muscle (Cai *et al.*, 2018), fat (Jumabay, 2015), nerve (Kumar and Perumal, 2015), tooth (Saraswathi and Saravanakumar, 2010), endothelial cells (Wang *et al.*, 2017) in the body during early life and growth. There are various reasons for dedicated research activities to develop the best suitable stem cells that would have therapeutic value. There are certain limitations with the progress of "stem cell research" due to ethical reasons. "Embryonic stem cells (ESCs)" though

possess remarkable therapeutic importance, the utilization of ESCs have got ethical considerations that hasn't progressed further for its application.

Other sources of stem cells are already identified in the body that can be easily extracted and cultured. The sources include, peripheral blood, bone marrow, umbilical cord, etc. Each type of the sources has advantages as well as demerits. It requires using number of growth factors to use peripheral blood as a source for deriving the required mesenchymal cell population as there is a limited availability of MSCs in peripheral blood. Umbilical cord though is the richest source of MSCs, the access is quite limited with respect to the rat models. Bone marrow is also one of the richest sources of MSCs which is easy to access and just requires a genuine protocol and approval from animal ethics committee to utilize bone marrow for research use.

"Bone marrow derived MSCs (BMSCs)" are vastly elaborated and there are standardized methods available for efficient isolation, culture and expansion. Rat bone marrow is obtained by cutting the epiphyseal ends of femoral and tibial long bone of wistar rat. Under sterile conditions bone was collected in a petri dish by flushing it through "phosphate buffered saline (PBS)" comprising antibiotic solution. The bone marrow collected on a petri dish is further culture and expanded. BMSCs are multifaceted and once a stem cell started multiplying, every novel cell has the ability either to stay as stem cell or develop into another cell type with specialized function such as cartilage, bone, muscle, nerve and tooth. BMSCs reach into the target site during injury to repair and replace tissue loss. During stem cell non-availability from the basal layer or delayed healing, ex-vivo cultured stem cells can be used an alternate to treat and repair dermal injuries. Mesenchymal treated chronic and acute wounds accelerate closing of wound with augmented epithelisation, formation of granulation tissue and angiogenesis. An alternate to surgery, recent approaches for treating cutaneous wound is incomplete without stem cells. This differentiation stem cell potential, according to their niche has enumerable therapeutic application. So that, it can be benefited by common people- in trauma & burns, old age patients – in delayed wound healing and defence personals – during military operations.

MATERIALS AND METHODS

Animals

The test facility is registered with CPCSEA Guidelines of India. (No.1182/PO/Rc/S/08/CPCSEA). Animals were housed individually with stainless steel grill cages and provided with standard rodent pellet feed and daily UV purified RO filtered water ad libitum. Sterilized clean paddy husk was taken as the material for bedding. Room Temperature and relative humidity were maintained. The test room facility was provided with 12hrs light and 12hrs dark conditions throughout the experiment. The IAEC approval number of this study is MTR/IAEC/PRU/013-15.

Bone marrow mononuclear cells isolation

Two female rats weighing 80gms was used for isolation of BMSCs. The wistar rat was sacrificed using diethyl ether; femoral and tibial bones were dissected and collected under fume hood in GMP environment. Muscles, connective tissues attached to the bone were cleaned and rinsed PBS comprising antibiotic solution. Under laminar flow, Marrow from bones was collected by cutting the epiphyseal ends and flushing it with PBS having antibiotic solution and was carefully laid over gradient solution (FICOL). The cells were centrifuged at 400 rpm for 30min and the clear white buffy coat (mononuclear cells) was collected into fresh sterile 15ml falcon tubes. The obtained mononuclear cells were rinsed two times in PBS to obtain cell pellet.

Cell culture and expansion

The cell pellet was suspended to Dulbeccco's minimal essential minimum medium (DMEM) (Gibco®, Grand Island, NY, USA) supplemented with Fetal bovine serum (FBS) (10%) containing antibiotic solution. The cell suspension was then seeded to T25 flask and incubated at 37°C and 5% carbon dioxide. Culture was maintained up to 7 days with media replacement every two days for the appearance of spindle shape morphology. The spindle shaped cells (BMSCs) were passed with trypsin (0.25%) and reseeded for obtaining 70-80% confluence. Cell viability was determined by trypan blue exclusion test.

FACS Analysis

Cells on reaching 70-80% confluence were subjected to flow cytometer analysis using Floid imaging cell station (Applied Bio system). BMSCs were labelled with antibody that is particular to cell surface molecule and tagged to fluorescence. Cells passes via laser beam in the device sort's only BMSCs labelled with cell surface antibody by emitting fluorochrome. The amount of fluorescence represents individual BMSCs cell component. BMSCs were positive for CD90 and negative for CD34 antibody (Ramos *et al.*, 2016). BMSCs labelled cell surface antibody tagged to fluorescence is subjected to flow analysis were separated based on their charges.

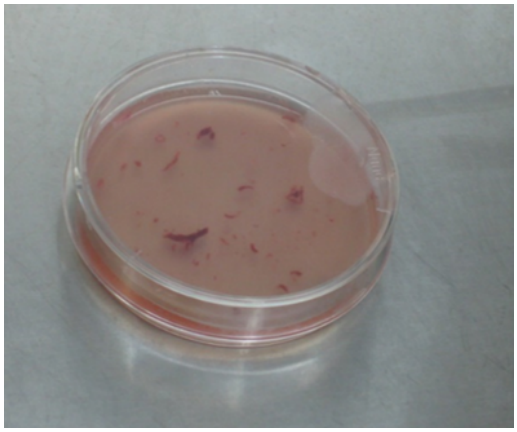

Figure 1: Rat Bone marrow cells

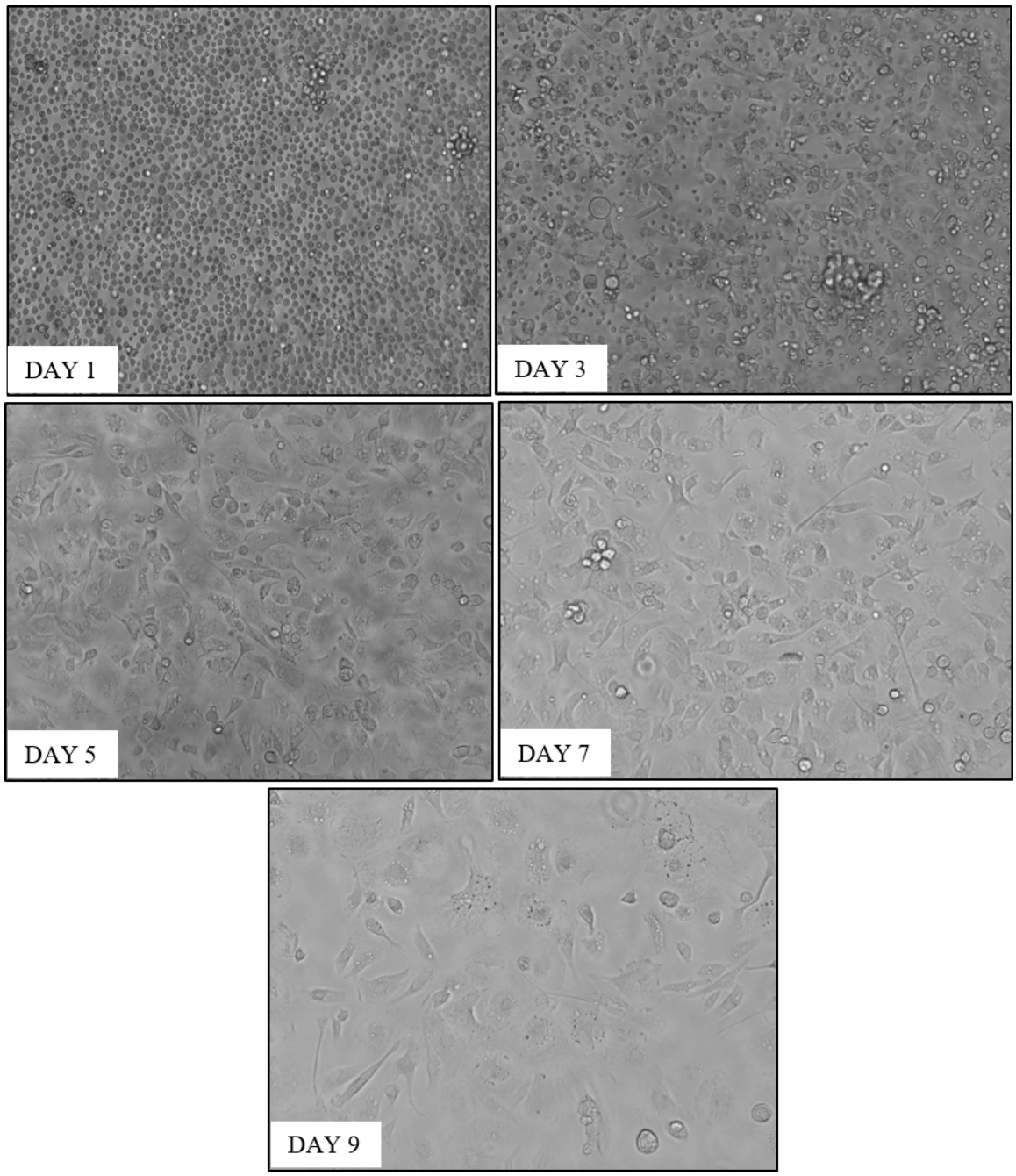

Figure 2: Observation of cell culture

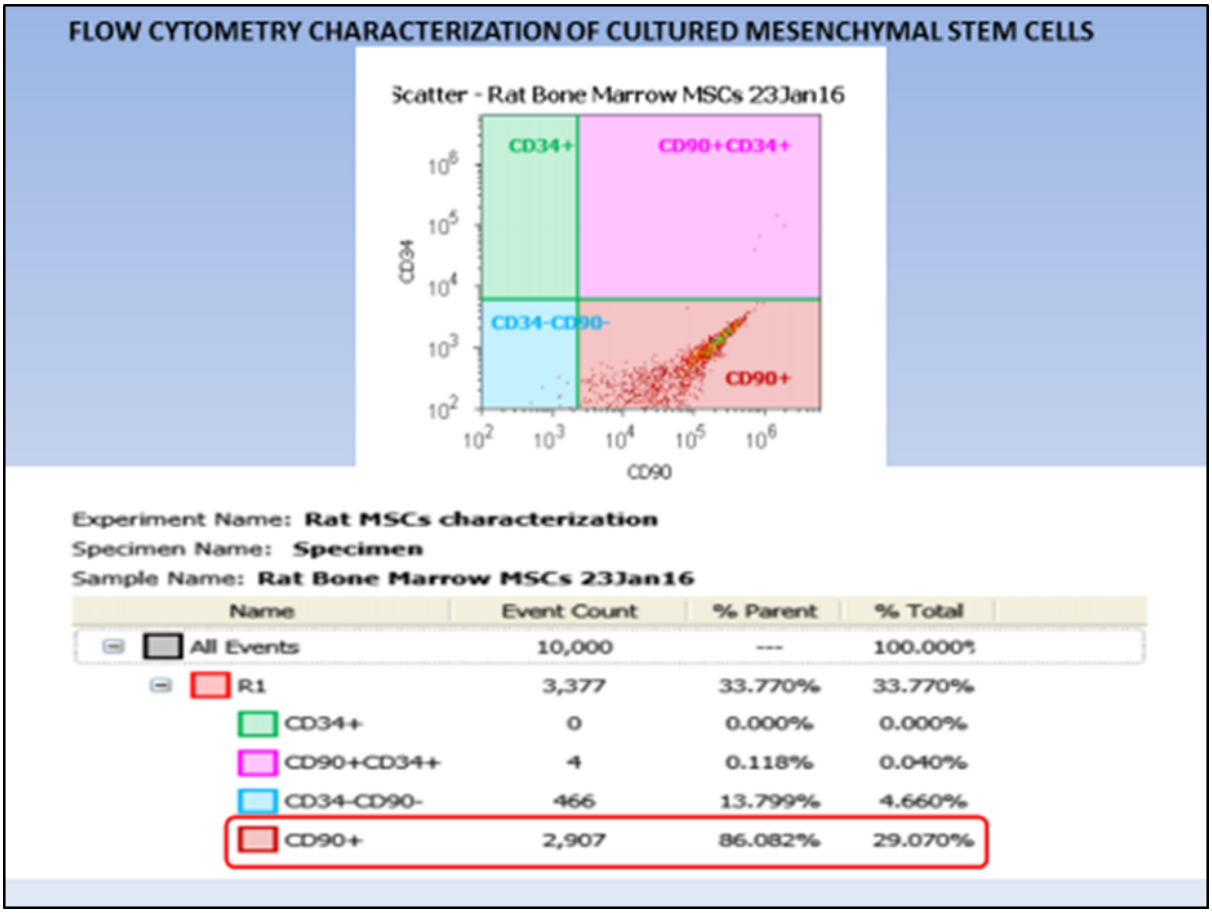

Figure 3: Flow cytometry analysis

An electric field deflects the charged cell which was collected to different containers. Cells were further cultured and expanded.

RESULTS AND DISCUSSION

Bone marrow cells collected from epiphyseal ends by flushing with PBS containing antibiotic solution (Figure 1). Sample centrifuged at 400 rpm for 30min to separate cells from plasma. Cell suspension seeded to T25 flask and incubated at 37°C and 5%carbon dioxide (Figure 2). Rat Bone marrow cell culture and expansion. Day: 1, cells appear to be in rounded in shape. Day: 3, spindle shape cells continue attached to the bottom of the flask where the round cells kept suspended inside medium and were eliminated with subsequent change in media. Day: 5, Cells began to multiply and make small colonies. Day: 7, cells appear to be spindle in shape with increased number of cell colonies. Day: 9, spindle and flattened cells were appreciated. Flow cytometry analysis of rat bone marrow mononuclear cells in which BMSCs express antibodies CD90 for positive expression and CD34 for negative expression-Figure 3.

Bone marrow cells were actively present in the body circulation and reach into the target site during injury to repair and replace tissue loss. During stem cell unavailability from the basal layer or delayed healing, ex-vivo cultured stem cells can be used an alternate to treat and repair dermal injuries. Isolation of stromal cells form bone marrow has earlier been reported by (Dexter *et al.*, 1981) but it was difficult to get pure stromal cells. In this study BMSCs were isolated from bone marrow extracted by gradient density centrifugation method using FICOL (1.077 g/ml). White buffy coat Mononuclear were obtained after centrifugation. This could be due to density of cells, when changed slightly in DMEM. This method is simple and can be taken for obtaining pure BMSCs. BMSCs when young appear round in shape. On Day 1, all the cells in cultured flask appear to be rounded in shape. This could be because of the unavailability of cellular nutrition in initial phase of cell culture. In our study, during passaging of cell, few cells appeared to be flattened in shape while other appeared spindle. Day 3, spindle shape cells remained attached to the bottom of the flask. Bone marrow mesenchyme stem cells exhibited plastic adherence characteristics. While the rounded cells continued to stay inside medium and were eliminated with subsequent change in media.

BMSCs resemble fibroblast in morphology and form colonies. This characteristic nature of BMSCs has been reported by (Bing *et al.*, 2016) in rabbit, rat, human, mouse and monkey. Day 5 cells began to proliferate and form small colonies. BMSCs perpetuate and form small to large colonies. This could be due to paracrine cell signalling. This mechanism of paracrine signalling favours angiogenesis and tissue remodelling such as fibroblast proliferation and epithelisation in all phase of wound repair. BMSCs undergo similar healing process to retain tissue homeostasis (Hocking and Gibran, 2010).

Fibroblasts are mesenchymal cells derived from the embryonic mesoderm. In our study BMSCs adhered to plate and exhibited fibroblastic spindle shape morphology. Day 7 cells seemed to be shaped like spindle with increased number of cell colonies. This could be probably due to higher integrin level in cultured stem cells which increases the proliferation potential of BMSCs as stated by (Jones *et al.*, 1995). Ex-vivo differentiated BMSCs may possess cytokines, growth factors and integrin's. This may provide an organised microenvironment that constitute mini-niche for ex-vivo differentiated cells.

On flow cytometry analysis, Bone marrow mono nucleated cells exhibited positive expression for CD90 and negative expression for CD34 antibody. This indicates Bone marrow mono nucleated is haematopoietic lineages, while Stromal cells are non-hematopoietic in lineage (Dexter *et al.*, 1981; Chen *et al.*, 1991; Mafi, 2011). BMSCs acquired an indefinite shape from round to flat due to property of cellular plasticity (Grove *et al.*, 2004). BMSCs on Day 9 appreciated being flattened. This confirms growth of cells due to DMEM nutritive medium. It appears flatter due to rapid expansion capacity of BMSCs inside nutritive medium of T25 flask. BMSCs appeared spindle in shape on day 9 and this could be due to elongated cytoplasmic processes. BMSCs are homologous to fibroblast in morphology.

Fibroblast is connective tissue cells that secrete type I & type III collagen (Shieh *et al.*, 2019). Fibroblast adheres to collagen and elastic fibres which they lay down. Fibroblasts are particularly active during wound repair, multiplying and laying down fibrous matrix which becomes invaded by numerous blood vessels (Noble *et al.*, 2019). BMSCs could lead to wound healing as they are principle source of fibroblasts cells (Dehkordi *et al.*, 2019). Fibroblast moves to the injured target tissue to secrete collagen matrix Collagen provides strength and stability to the underlying tissue in order to retain normal structural and functional anatomy.

CONCLUSION

There are various occurrences, wherein the ultimately derived MSCs lose the native potential and doesn't withstand the environment or gets contaminated due to mishandling. The recovered MSCs would be of less value and doesn't propagate to the level of expected outcome with respect to the therapeutic applications. Therefore, there is a necessity for high-quality stem cells that will withstand the in vitro as well as the ex vivo conditions to effectively involve in regeneration of the desired cell type. Our method has thus provided such a type of MSCs that will have better therapeutic value and will generate successful regeneration after transplantation.

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