

Original Research Article

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Assessment of *In vitro* Differentiation Potential of Canine Bone Marrow Derived Mesenchymal Stem Cells to Skeletal Myocytes

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ABSTRACT

Mesenchymal stem cells are multipotent/pluripotent stromal cells that can differentiate into a diversity of cell categories depending upon the micro-environment to that they are exposed. The multipotent differentiation potential of MSCs is generally towards a constrained sum of cell types familiar to the tissue of origin and maintains the capacity for self-renewal. To assess the differentiation potential of MSCs to skeletal myocytes a male mongrel dog of 3 years was used to carry out the research. The marrow derived mesenchymal stem cells were harvested with mononuclear cells and cultivated in monolayer culture in T-25 tissue culture flasks till they attained of 80-90 % confluency. At 4th passage the cells were driven towards skeletal myogenesis by addition of skeletal myocytes differentiation medium containing 5-azacytidine, foetal horse serum, basic fibroblast growth factor, dexamethasone and hydrocortisone. The cells which differentiated showed positive results of RT-PCR for the early myogenic differentiation factors Myf5 and Myogenin and Hoechst 33342 staining. The Hoechst 33342 stainin, RT-PCR analysis and scanning electron microscopy leads to the positive result for differentiated skeletal myocytes *in vitro*. The differentiated skeletal myocytes can be used in tissue engineering and veterinary therapeutics for repairing the damaged skeletal myocytes.

Keywords

MSCs, Skeletal myocytes, Myf5, Myogenin, Hoechst 33342.

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Introduction

Mesenchymal stem cells (MSCs) or stromal progenitor cells is a population of stem cells. Mesenchymal stem cells regenerate tissues of mesenchymal lineages by differentiation, and trans-differentiate into cells derived from other embryonic layers, including neurons and epithelia in skin, lung, liver, intestine, kidney, and spleen. These cells form an elemental part of haemopoietic

micro environment which supports blood cell development. These cells are multipotent in nature as they can differentiate into some mesodermal lineages including adipocytes, osteocytes and chondrocytes . Additionally MSCs have a distinct artifice to suppress immuno mediated inflammatory responses (Griffin *et al.*, 2013). Mesenchymal stem cells which are isolated from bone marrow have exhibited to infer a skeletal myogenic outcome (Mizuno, 2010).

Moreover, substantiation that MSCs subpopulations may as well attribute diverse qualities and regeneration possibility has cropped up (Pérez-Silos *et al.*, 2016). The trans-differentiated skeletal myocytes can be utilised as veterinary therapeutics for the use in inherited muscular disorders and also patients suffering from trauma due to accidents and other pathological condition concerning muscles. The differentiated cells from the same donor might help in early recovery from the pathological or inherited condition as compared to mesenchymal stem cells.

Materials and Methods

The present research was carried out in the Department of Veterinary Surgery and Radiology, College of Veterinary and Animal Science on a mongrel, intact, male adult dog of 3 years of age weighing 20 kg with apparently good general condition.

After keeping the dog off feed for atleast 12 hours prior to surgical manipulation, the required bone marrow aspirate was taken from the ileac crest or femur in either sternal or lateral recumbency respectively. The dog was subjected to the administration of atropine @ 0.04mg/kg B.W and diazepam @ 1mg/kg B.W intramuscularly as pre-anesthetic medications followed by 2.5% thiopental sodium intravenously to the effect as a general anesthetic for intubation and maintenance (Jadon *et al.*, 1998). With a small incision, the ileac crest of the bone got exposed the marrow aspirate was collected with the help of 18 G bone marrow aspiration/biopsy needle (Rosantheel needle). The aspiration of 2.5 ml of bone marrow sample into the cold heparinized low glucose-Dulbecco's modified Eagle's medium (L-DMEM) syringe locked to the bone marrow aspiration needle comprising of 1000 IU heparin was done.

A total quantity of 5 ml marrow aspirate was collected from the animal. The buffy coat method of density gradient centrifugation technique was used for separation of mesenchymal stem cells in the bone marrow aspirate. The cells were cultured in T-25 flasks after formation of a pellet which was suspended in culture medium [(DMEM-LG containing 10% fetal bovine serum (FBS) and Antibiotic-Antimycotic solution (A mixture of 10,000 units/ml of Penicillin, 10 mg/ml Streptomycin and 25µg/ml Amphotericin B in 0.9 % normal saline)]. The tissue culture flasks were maintained at 37⁰C in a humidified atmosphere of 5% CO₂ and 95% air in CO₂ incubator. After the cells reached 80-90% confluence cells were passaged using Trypsin-EDTA and they were observed for distinct morphological features and viability by "trypan blue exclusion test" (Strober, 2001).

The trypan blue dye solution was filtered and 10µl of the single cell suspension was pipetted in an eppendorf tube and amalgamated with 10µl of 0.4% trypan blue dye solution in ratio of 1:1 (dilution factor 2). The suspension was kept at room temperature for 5 minutes and 10ul of the suspension was encumbered into haemocytometer chamber. The cells were focused under a microscope with 40x and 10x lens and the counting of cells was done in the four WBC squares. Separate counting of the viable and non-viable cells was done. The number of cells per ml and percentage of viable cells were given by the formula (Monica *et al.*, 2012):

Number of the cells per ml = (Total no. of cells/4) x dilution factor x 10⁴

Viable cell (%) = (No. of the viable cells per ml) / (total no. of the cells per ml) x 100

The cells were counted for their viability after first passage.

After the cells reached about 60% confluence after 4th passage the cells were exposed to skeletal myogenic differentiation media. For 48 hours the cells were grown in the mesenchymal stem cell culture medium, thereafter, the skeletal myogenic differentiation medium enhanced with 10 µmol/L 5-azacytidine (Sigma-Aldrich, Bangalore, USA), 2.5 µg L⁻¹ basic fibroblast growth factor (bFGF, Sigma-Aldrich, Bangalore, USA), 2% horse serum (HS) (Invitrogen, Carlsbad, CA, USA) was added to the tissue culture flasks to proceed for the differentiation process. The medium was changed after every 3 days. The cells were harvested at day 7, 14 and 21. The cells which were grown to about 90% confluence without induction were considered as the control sample. In vitro differentiation of canine MSCs was characterized by Hoechst 33342 staining, polymerase chain reaction and scanning electron microscopy.

Hoechst 33342 staining

The dye stains the A and T segment of DNA present in the nucleus of skeletal myocytes and hence stain nucleus blue. The staining was performed at 21st day of differentiation and cells were evaluated under inverted microscope.

Preparation of dye from stock solution

Hoechst 33342 dye stock solution was prepared by mixing the fillings of one vial (100 mg) in 10 ml of deionized water (diH₂O) to generate a 10 mg/ml (16.23 mM) solution.

Protocol for staining

The trans-differentiated cells were fixed by 4% paraformaldehyde after removal of culture medium from the flask. The staining solution was then poured into the culture flask. The

culture flask was then incubated for 5-10 min at 37°C away from light. The cells were imaged under fluorescent microscope after removal of stain and washing it with DPBS.

RNA extraction, one step reverse transcriptase-Polymerase chain reaction (RT-PCR)

In a sterile nuclease free PCR tube a total volume of 50 µl was formulated by adding nuclease free water in a amalgam of 4 µg total RNA, 1 µl of Reverse transcriptase enzyme, 1 µl random hexamer primer, 25 µl 5X reaction mixture. The mixture was centrifuged for 30 seconds. Real Time - Polymerase Chain Reaction with the acceptable cycle condition was determined and utilized for each primer pair as follows: cDNA synthesis and pre-denaturation performed with 1 cycle of: 45-60°C for 15-30 minutes and 94°C for 2 minutes, PCR amplification performed with 40 cycles of: denature, 94°C for 15 seconds anneal, 55-66°C for 30 seconds extend, 68°C for 1 minute/kb, finally extension was done with 1 cycle of 68°C for 5 minutes. The oligonucleotide sequences of the gene used in PCR were as follows:

Myf5 (Mizuno *et al.*, 2002) Forward 5⁰ - CTGTCTGGTCCCGAAAGAAC-3⁰ Reverse 5⁰ -TGATTCGATCCAC TATGCTG-3⁰ having amplicon length 500 bp, Myogenin (Ueda *et al.*, 2002) (F) 5'- AGTGAC TGCAGCTCCACAG -3' (R) 5'- GACG TGAGAGAGTGCAGGTT -3' having amplicon length 438 bp GAPDH -5'- CCCATC ACC ATC TTC CAG GA -3' 5'- TTG TCA TAC CAG GAA ATG AGC -3' having amplicon length 731 bp.

PCR products were analyzed by electrophoresis on 2% ethidium bromide incorporated-agarose gel in Tris-Boric-EDTA (TBE) buffer under UV transilluminator.

Scanning electron microscope

The cells were harvested on day 21 and fixed in Karnovsky's Fixative for 24 hours. Further the cells were rinsed in 0.1 M PBS and dehydrated in ascending series of graded ethyl alcohols viz. 30%, 40%, 50%, 70%, 80%, 90% and absolute alcohol for 10 minutes in each and critical point drying was carried out. The sample was dehydrated and dried the sample was partitioned in 1mm to 2mm sizes and put onto the stabs with the help of an adhesive tape. The stabs were then placed into the gold plating machine so as to cover the surface of the sample with gold to prevent any damage to the sample. After the gold plating was performed the samples were visualised under the scanning electron microscope to characterise the trans-differentiated skeletal myocytes. Data collected were expressed as per the method described by Snedecor and Cochran (1994).

Results and Discussion

The anaesthetic amalgamation of atropine, diazepam and thiopental sodium produced satisfactory plane of anaesthesia which confirms the findings of Jadon *et al.*, (1998). Mesenchymal stem cells were isolated from the bone marrow aspirate by 'buffy coat' protocol as previously also demonstrated by Klepsch *et al.*, (2013) although some modifications were made in this protocol. The adhered cells were considered as the population of mesenchymal stem cells as it is previously also observed by Ghiban *et al.*, (2010). Trypan blue dye exclusion test was also performed for determining the number of viable and non-viable cells in the cell suspension and also to determine the number of cells per ml in the suspension.

The dilution factor used in this experiment was 2. The cell viability % of 4 samples of canine MSCs was calculated at passage 1.

The average cell viability % was found to be 98.15 % and thus good to be used in this study. The shape of the isolated stem cells was elongated, which was found in accordance with the results found out by Huang *et al.*, (2015). Multiple cell layers and dense cell nodules were formed in some areas of the culture dish which confirm the findings of Huang *et al.*, (2015).

The variation in the shape of cells was observed at every passage which revealed that there is an increase in cells width at centre at every growing passage. The cells from elongated in 1st passage transformed to polygonal shape in 4th passage.

The canine BM-MSCs were cultivated in the differentiating medium for a period of 21 days as previously described by Du *et al.*, (2014). The mesenchymal stem cells in the differentiation media assumed a fairly uniform elongated, bipolar or tubular morphology within 48-60 hours of addition of differentiation media and by 5-7 days these cells assumed a sequence of linear adjacent arrangement with the appearance of bands of trans-differentiated skeletal myocytes across the flask (Figure 1). The cells grew long fusiform gradually and were neatly arranged as a muscular tube like structure, the cells also assumed multinucleated structure which is also a sign of differentiation of skeletal myocytes. This arrangement was found alike to that of myotubes formed by fusion of myoblasts (Merrison *et al.*, 2012).

Hoechst 33342 stain binds into the minor furrow of DNA and demonstrates discrete fluorescence emission spectra that are reliant on dye:base pair ratios. The trans-differentiation assessment of cells by Hoechst 33342 stain was done at 21st day. The staining of nuclei was performed and all nuclei were visualized by Hoechst 33342 staining (Figure 2).

RNA of the trans- differentiated cells was extracted using HiPura mini kit .The samples were tested for Myf5 gene (Mizuno *et al.*, 2002), Myogenin (Ueda *et al.*, 2002) and GAPDH (a housekeeping gene), after 14 and 21 days. The primers for the genes Myf5 and myogenin being the markers of early myogenesis in the cell cultures gave strong bands against their respective base-pairs after 14 days of their treatment in the skeletal myogenic differentiation medium. Hence it was observed that the cells trans-differentiated into primitive skeletal myocytes

as they expressed these 2 genes as previously reported by Gang *et al.*, (2004). The gene expression of the marker related with skeletal myogenic differentiation Myf5 and myogenin was manifested in trans-differentiated cells and not in undifferentiated mesenchymal stem cells while GAPDH, being a housekeeping gene was apparent in both trans-differentiated and undifferentiated cells (Figure 3). The Polymerase chain reaction result of Myf5 and myogenin genes revealed a sturdy band in the trans-differentiation induced cells which validate the findings of Zuk *et al.*, (2002).

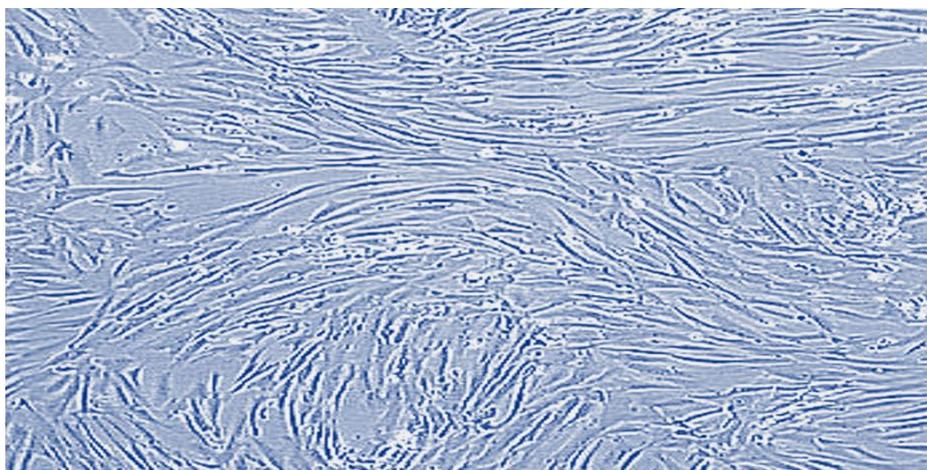


Fig.1 Morphology of of canine bone marrow derived mesenchymal stem cells in T-25 culture flasks after addition of skeletal myocyte differentiation medium, the cells are visible with striations and aligned longitudinally to each other (at 10x plus 10x magnification)

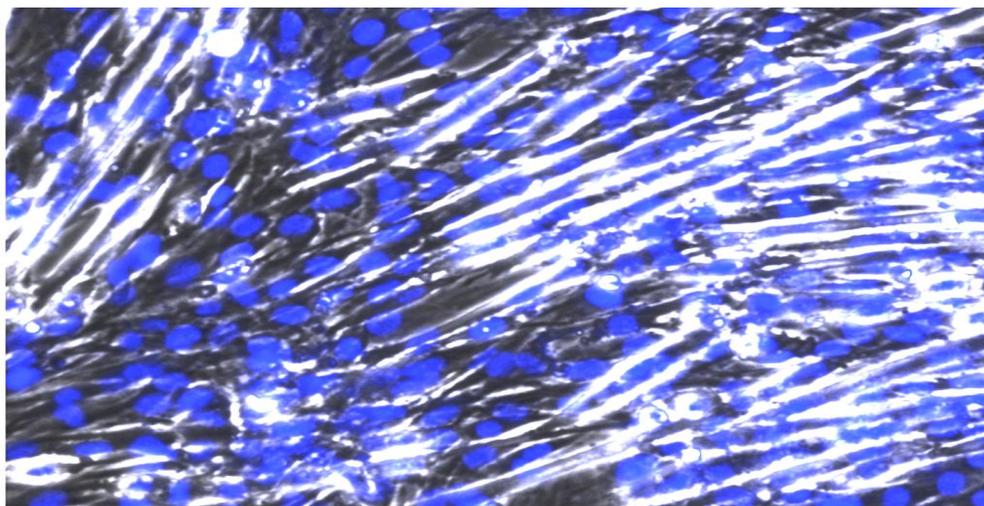


Fig.2 The staining of nuclei by Hoechst 33342 staining revealed the presence of multinucleated cells (the blue coloured are nuclei)

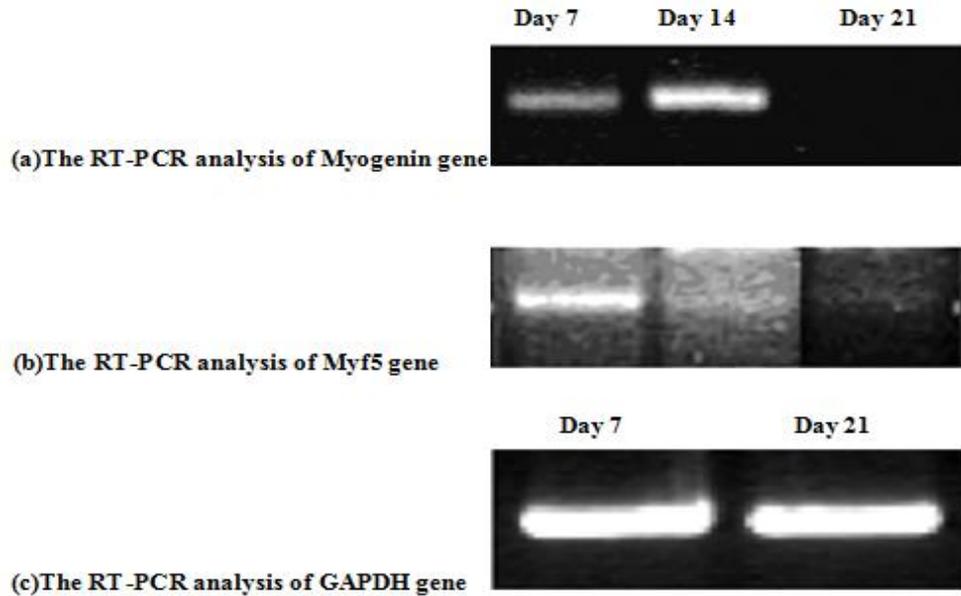


Fig.3 The RT-PCR analysis of the differentiating samples at day 7, day 14 and day 21 for the genes Myogenin and Myf5(a & b) in differentiating samples. The RT-PCR analysis of house keeping gene GAPDH at day 7 and day 21

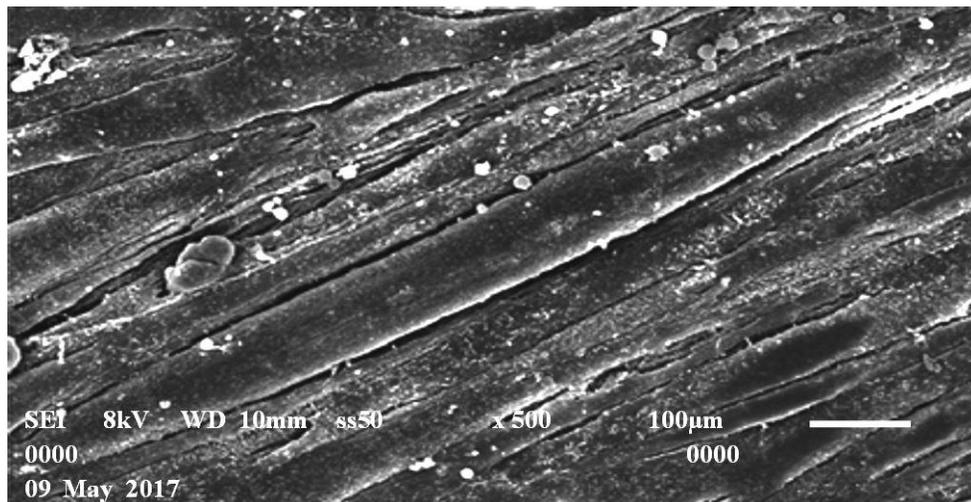


Fig.4 The Scanning electron microscopy of the trans-differentiated skeletal myocyte grown on gelatin coated coverslips reveals the presence of striations and adherence to the gelatin scaffold

The electron microscopy for the scanning of the cells was performed after 24 days of the trans-differentiation procedure to observe and validate that the cells trans-differentiated to skeletal myocytes. The scanning electron microscopy after 24 days of incubation of mesenchymal stem cells in the skeletal myogenic differentiation media revealed the presence of trans-differentiated skeletal

myocytes. The cells when cultured in the skeletal myogenic media on the gelatin coated coverslips revealed the morphology of cells as flattened and with more than 2 nuclei in one cell. The cells aligned in longitudinal manner with each other in the tissue culture flasks and proliferated well. The cells were seen as longitudinal bands with striations (Figure 4). The majority of the skeletal myocytes formed

were in clusters as they aligned in a longitudinal manner with each other. The cells were seen as longitudinal bands with striations and they were aligned to each other.

With this research study it might be concluded that the mesenchymal stem cells aspirated from bone marrow of the animal can be trans-differentiated to skeletal myocytes *in vitro*. These skeletal myocytes can be utilised to treat the inherited diseases of muscles and traumatic conditions which cause muscular loss in animals.

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