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## Isolation, Culture and Characterization of New Zealand White Rabbit Mesenchymal Stem Cells Derived from Bone Marrow

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### ABSTRACT

Mesenchymal stem cells are recognised based upon the plastic adherence, fibroblastic morphology, expression of certain surface markers, non-expression of haematopoietic markers and their ability to differentiate into at least three lineages viz., adipogenic, chondrogenic and osteogenic. The rabbit Mesenchymal Stem Cells (rMSCs) though used extensively in research but have not been thoroughly studied and are not compared to other species. The present study was therefore conducted to determine the morphology, surface markers and trilineage differentiation potential of New Zealand white rabbit MSCs. Isolation of rMSCs was done by an established method of density gradient method using Ficoll-hapaque. The cells were characterised by Phase Contrast Microscopy, Reverse Transcription Polymerase Chain Reaction (RT-PCR) and Alkaline Phosphatase (AP) staining. The cells isolated were plastic adherent and had fibroblastic spindle shape with eccentric irregular nuclei. The cells expressed surface markers viz., CD 105 and CD 106 besides expressing genes of collagen type II and I. Haematopoietic markers (CD 34 and CD 45) and aggrecan gene however, were not expressed. The rMSCs showed a moderate alkaline phosphates activity. Trilineage differentiation was conducted utilizing prepared differentiation media and rMSCs were differentiated into corresponding cell lineages based upon the medium used. It was concluded that rMSCs possess morphology similar to other species with good proliferation rate and exhibit the characteristics laid down by International Society for Cellular Therapy (ISCT). The present study provided basic protocols for characterization of rabbit MSCs that should be used before application of these cells for any research or therapy.

**Key words:** Characterization, differentiation, mesenchymal stem cells, rabbit

### INTRODUCTION

Mesenchymal Stem Cells (MSCs) are multipotent stem cells that can differentiate for long periods into different cell lineages depending upon the microenvironment in which they are kept. The cells also modulate immune function extending their potential use in allogenic or xenogenic therapies. Due to these characteristic features, MSCs are currently being increasingly used in tissue engineering and regenerative medicine (Tay *et al.*, 2012; Cutts *et al.*, 2015). These cells have been utilized as therapeutics in both human and animal, for the repair of cartilage, tendon, bone treatment of neurodegenerative disorders, spinal injuries, cardiac defects, facilitate wound healing,

etc. (Caplan, 2005; Ribitsch *et al.*, 2010; Xie *et al.*, 2012; Gugjoo *et al.*, 2015; Cutts *et al.*, 2015). However, MSCs obtained from bone marrow constitute a very small fraction, 0.01-0.001% of the bone marrow cells (Pittenger *et al.*, 1999; Martin *et al.*, 2002; Gugjoo *et al.*, 2015) and many cells obtained during harvesting process may be progenitor cells that mimic the characteristic of MSCs (Chong *et al.*, 2012). Therefore, it is imperative to isolate, culture, expand and characterize MSCs before their effective use.

Currently, rabbit mesenchymal stem cells are very popular among researchers due to their resemblance in cellular and tissue physiology with that of human MSCs (Fox, 1984; Warden, 2007) and due to their easy availability. To evaluate stem cells, it is important to have uninterrupted source of such cells that too with minimal ethical issues. Rabbits are readily available with larger size compared to mouse or rat and easy to handle and cost effective in comparison to dog, sheep or goat (Tan *et al.*, 2013). However, the literature about the basic characteristics of rMSCs is not that much extensive as that of the human Mesenchymal Stem Cells (hMSCs) (Warden, 2007; Amini *et al.*, 2012). So the present study was aimed at culturing, characterization and differentiation of rMSCs.

## MATERIALS AND METHODS

**Collection of bone marrow:** A total of 21 rabbits were used for bone marrow collection and subsequent isolation, culture and characterization of rMSCs. The rabbits were anaesthetized by intramuscular injection of xylazine at 6 mg kg<sup>-1</sup> followed 10 min later, by ketamine at 60 mg kg<sup>-1</sup> in the thigh muscles (Amarpal *et al.*, 2010). The area of the iliac crest on either side was prepared in aseptic manner. The bone marrow aspirate was collected with the help of an 18 G bone marrow biopsy needle from the lateral aspect of the iliac crest. For the collection of Bone Marrow (BM), the needle was inserted through the skin and the muscle with little force. Once the needle (stylet in place) was in contact with the bone, it was advanced by rotating it slowly until the bone cortex was penetrated. As the needle penetrated the cancellous bone, the stylet of the needle was removed and 2.5 mL bone marrow aspirate was drawn/aspirated into a hypodermic syringe containing 2500 IU units of heparin. The needle was then removed and the same procedure was followed in the contra-lateral bone to collect another 2.5 mL bone marrow aspirate in the same syringe (Fig. 1a). Thus, a total quantity of 5 mL of bone marrow aspirate was collected from each animal.

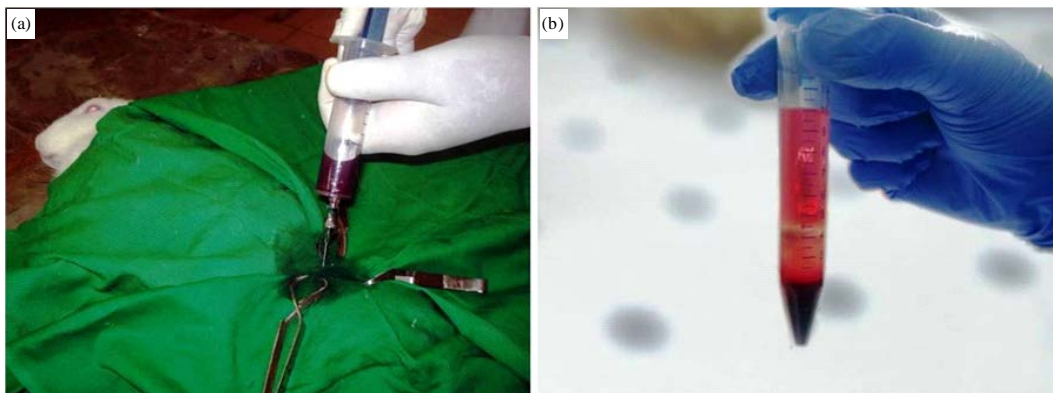


Fig. 1(a-b): (a) Bone marrow collection from rabbit and (b) Mononuclear cell fraction at plasma RBCs interface

**MSCs isolation and culture:** The process of MSCs isolation and culture was carried out on bone marrow samples as per the standard procedure (Udehiya *et al.*, 2013). In brief, the marrow samples were washed with equal volume of Dulbecco's Phosphate Buffered Saline (DPBS) (5 mL) and disaggregated by passing it gently through a 21-gauge intravenous catheter and syringe to create a single cell suspension. Marrow sample with 5 mL of DPBS were loaded onto 5 mL of Ficoll-Paque plus. The mono-nucleated cells were collected from the interface (Fig. 1b) and were washed with RBC lysis buffer. The cells were resuspended in cell growth media, Dulbecco's Modified Eagle's Medium-Low Glucose (DMEM-LG) (Hyclone) containing 10% Fetal Bovine Serum (FBS) (Hyclone) and antibiotics (mixture of 100 units mL<sup>-1</sup> of penicillin and 100 µg mL<sup>-1</sup> of streptomycin). The cells counted by Neubauer's counting chamber method were then plated at an average of 2.2×10<sup>5</sup> cells cm<sup>-2</sup> in T-25 flasks. The cells were maintained at 37°C in a humidified atmosphere of 5% CO<sub>2</sub> and 95% air in a CO<sub>2</sub> incubator. After 3 days of primary culture, the non-adherent cells were removed completely by changing the medium. Upon reaching 80-90% confluency (assessed by visual inspection under inverted microscope), the cells were passaged at lower densities into new culture flasks. For this purpose culture medium was removed and cells were washed with 0.05% trypsin and 0.53 mM ethylenediaminetetraacetic acid (EDTA) for 5 min. The trypsin-EDTA activity was stopped by adding 3 mL of culture medium and the contents were collected in a centrifuge tube and centrifuged at 300×g for 6 min. The supernatant was discarded and the pellet was resuspended in 10 mL of supplemented Dulbecco's Modified Eagle's Medium (DMEM). The suspension was aspirated through a 20 gauge needle three times to obtain a single cell suspension and the cells were replated onto T25 culture flasks at half of their original density. Cultures were maintained at 37°C in a 95% air, 5% CO<sub>2</sub> incubator. Supplemented DMEM was changed every 3-4 days. After 7-10 days, cells upon reaching to their full confluency were again trypsinized as described above. After centrifugation, the supernatant was discarded and the pellet was resuspended in 100 µL of DMEM. After adjusting the average cell count to 2.5×10<sup>6</sup>/50 µL, the cells were characterised and differentiated using specific media.

### **Characterization of rMSCs**

**Alkaline phosphatase staining:** After third passage, the cultured cells were subjected to Alkaline Phosphatase (AP) staining for characterization. Medium was removed from the culture and the cells were fixed for 10 min in 4% paraformaldehyde prepared in DPBS. After fixation, the cells were washed properly with DPBS and incubated with 25 mM Tris-HCl and 150 mM NaCl containing 8 mM MgCl<sub>2</sub>, Naphthol AS-MX phosphate with a concentration of 0.4 mg mL<sup>-1</sup> and Fast Red TR salt at a concentration of 1 mg mL<sup>-1</sup> for 1 h at 37°C.

**Reverse Transcriptase-Polymerase Chain Reaction (RT-PCR):** Once confluent, the MSCs were passaged several times to increase the cell population up to 3rd passage. Third passage confluent MSCs were then characterized for surface markers (positive-CD166, CD105 and negative-CD34, CD45 markers), collagen type I, II and aggrecan by RT-PCR.

Total RNA was extracted from third passaged MSCs (21-24 days) by using RNeasey Micro Kit (Qiagen, USA), which is a silica gel based membrane MinElute spin column based. Total RNA extraction and purification technique was used incorporating a DNase treatment step to prevent any DNA carry over in the final RNA preparation. The procedure employed for the isolation of total RNA was as per manufacturer's recommendation. Concentration of RNA was directly recorded using Nanodrop ND 1000 Spectrophotometer and measured in nano gram per microliter. The first strand cDNA was synthesized using RevertAid™ H Minus Reverse Transcriptase system

(MBI, Fermentas, USA; Cat #EP0451) in a total volume of 20 µL reaction mixture using Molony Murine Leukemia Virus Reverse Transcriptase (MMLV-RT) following the manufacturer's instructions. The cDNA was properly labelled and stored at -20°C for later use. The PCR was performed with cDNA template obtained through reverse transcription for the genes of interest (CD166, CD105, CD34 and CD45, collagen type I, II and aggrecan), with Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) gene as endogenous control. Oligonucleotide primers used and time temperature protocol are given in Table 1-3.

**Confirmation of RT-PCR amplicons:** Confirmation of the amplification for specific RT-PCR amplicons was done by gel electrophoresis on a 1.0% Agarose TBE gel containing 0.5 µg mL<sup>-1</sup> Ethidium Bromide (Cat#H5041, Promega) and visualized on a UV transilluminator as per the standard procedure.

Table 1: Primers, their sequence, product size and melting temperatures used in RT-PCR

Primers for RT-PCR					
Primer name	Primer	Primer sequences	Tm	Product size (bp)	References
CD166	F	GCTCCCCAGTATTTATTGCCTTC	60.6	345	Tan <i>et al.</i> (2013)
	R	GTAGCACCT TCCATTCCCTGTA	58.4		
CD45	F	AGGTAGTAGATGTTTTCCAAGTAGTGA	60.4	130	
	R	ACTTGTCCATTCTGGGCAGGGTAG	64.4		
CD34	F	AGAACTTCCAGCATGTTCCAGTTTATG	62.2	95	
	R	GGCTTGCCACATCTTGCTCGGTGA	66.1		
Sox9	F	AGAGCGAAGAGGACAAGTCCCCCGT	66.3	85	
	R	ATGGGCACCAGCGTCCAGTCGTAG C	69.5		
Ppar-γ	F	AGCAAAGAAGTCGCCATCC	56.7	118	
	R	CGTTCAAGTCAAGGCTCACA	57.3		
RunX2	F	TCAGGCATGTCCCTCGGTAT	59.4	54	
	R	TGGCAGGTAGGTATGGTAGTGG	62.1		
CD105	R	AGGTCAGGTTCCAGGATGGTG	59.4	342	Kumar (2013)
	F	CCGGCGAATACTCTCTCAAG	59.4		
Aggrecan	F	CCAGACCGGCTACCCCGACC	69.6	351	Singh <i>et al.</i> (2012)
	R	CCAAGGGCGGCTTCGTCAGCAA	66.0		
Collagen F	I	ATGCAATGCTGTTCTTGACAG	55.3	401	Im <i>et al.</i> (2001)
	R	CCAGATTGAGACCCTCCTCA	59.4		
Collagen II	F	AGTCGCTGGTGCTGCTGAC	61.0	145/352	
	R	GGGGTCCTTTAGGTCCTACG	61.4		
GAPDH	F	AACATCATCCCTGCCTCTACTG	60.3	196	Kuo and Tuan (2008)
	R	CTCCGACGCCTGCTTAC	60.5		

F: Forward, R: Reverse

Table 2: Polymerase chain reaction programme for amplification of different genes

PCR specifications	Collagen	CD166	CD105	CD34	CD45
	II/aggrecan	CD166	CD105	CD34	CD45
	temperature×time	temperature×time	temperature×time	temperature×time	temperature×time
Initial denaturation	94°C×45 sec	94°C×2 min	95°C×5 min	94°C×2 min	94°C×2 min
Cyclic denaturation (40 cycles)	94°C×45 sec	94°C×15 sec	95°C×45 sec	94°C×15 sec	94°C×15 sec
Annealing	61°C×45 sec	58°C×30 sec	55°C×45 sec	58°C×30 sec	58°C×30 sec
Extension	72°C×90 sec	72°C×30 sec	72°C×45 sec	72°C×30 sec	72°C×30 sec
Final extension	72°C×5 min	72°C×5 min	72°C×10 min	72°C×5 min	72°C×5 min

Table 3: Polymerase chain reaction programme for amplification of differentiation markers and internal positive control

PCR specifications	Sox9	Ppar-γ	Runx2	GAPDH
	temperature×time	temperature×time	temperature×time	temperature×time
Initial denaturation	94°C×2 min	94°C×2 min	95 °C×2 min	95°C×2 min
Cyclic denaturation (40 cycles)	94°C× 15 sec	94°C×15 sec	95°C×15 sec	95°C×15 sec
Annealing	58°C×30 sec	58°C×30 sec	58°C×30 sec	58°C×30 sec
Extension	72°C×30sec	72°C×30 sec	72°C×30 sec	72°C×30 sec
Final extension	72°C×5 min	72°C×5 min	72°C×5 min	72°C×5 min

GAPDH: Glyceraldehyde-3-phosphate dehydrogenase

### **Multilineage differential potential**

**Osteogenic differentiation:** For osteogenic differentiation, the bone marrow cells were seeded with a density of  $2 \times 10^4$  cells per well in 24 well plates and were cultured in growth media (DMEM+15% FBS) until 70-80% confluency. Later growth medium was replaced by the osteogenic differentiation medium (StemPro-Gibco). As a negative control, an equal number of cells were maintained in the expansion medium. All the cells were cultured for 18 days with medium changes every 3-4 days and calcium deposition was evaluated by Alizarin Red staining. Osteogenic differentiation marker (Runx2) expression was confirmed by RT-PCR.

**Adipogenic differentiation:** Prior to addition of differentiation medium, the cells were seeded as discussed for osteogenic differentiation. Adipogenesis differentiation kit (StemPro, Gibco) was later used as per manufacturer's instructions for the adipogenic induction of rMSCs. The MSCs were cultured for 18 days, medium was changed every third day and differentiation was assessed by the presence of lipid droplets that were recorded after staining with Oil Red O stain. As a negative control, an equal number of cells were maintained in the expansion medium for 18 days. Expression of adipogenic differentiation marker (Ppar- $\gamma$ ) was confirmed by RT-PCR.

**Chondrogenic differentiation:** The rMSCs were seeded ( $2 \times 10^4$  viable cells  $\text{mL}^{-1}$ ) in culture plates as mentioned above. Chondrogenesis differentiation medium (StemPro, Gibco) was used as per manufacturer's instructions for chondrogenic induction. The cells were cultured for 18 days, medium was changed every third day and differentiation was assessed by Toluidine blue staining. As a negative control, an equal number of cells were maintained in micromass culture supplemented with expansion medium up to 18 days. Chondrogenic differentiation marker (Sox9) expression was confirmed by RT-PCR.

## **RESULTS**

### **Mesenchymal stem cells culture**

***In vitro* culture of Mesenchymal stem cells:** Out of 21 samples, 13 rMSCs culture showed excellent to good quality culture characteristics, while 8 samples showed poor to no growth in tissue culture flasks. The rMSCs showed excellent to good culture rate in 61.9% samples.

**Morphology of mesenchymal stem cells at different developmental stages:** On day 0, heterogeneous population of the nucleated cells depicting oval to round shape was observed (Fig. 2a). By day 2, many cells from the heterogeneous cell population started adhering to the tissue culture flask base. The floating cells (haematopoietic cells) were removed in first media change with prospective MSCs adhered to the flask bottom on day 3. The adhered cells started to change their morphology from round to spindle shape on day four to six (Fig. 2b). After seven to ten days, the homogenous spindle shaped cells formed 6-8 small colonies in the tissue culture flasks (Fig. 2c). These homogenous cells were grown further and after 10-14 days almost 70-80% area of the tissue culture flasks was covered by these cells (Fig. 2d). After 15-18 days, on reaching 80-90% confluency, as assessed by visual inspection under inverted microscope, the cells were passaged at lower densities onto new culture flasks. After first passage, the cells grew uniformly throughout the surface of the tissue culture flask.

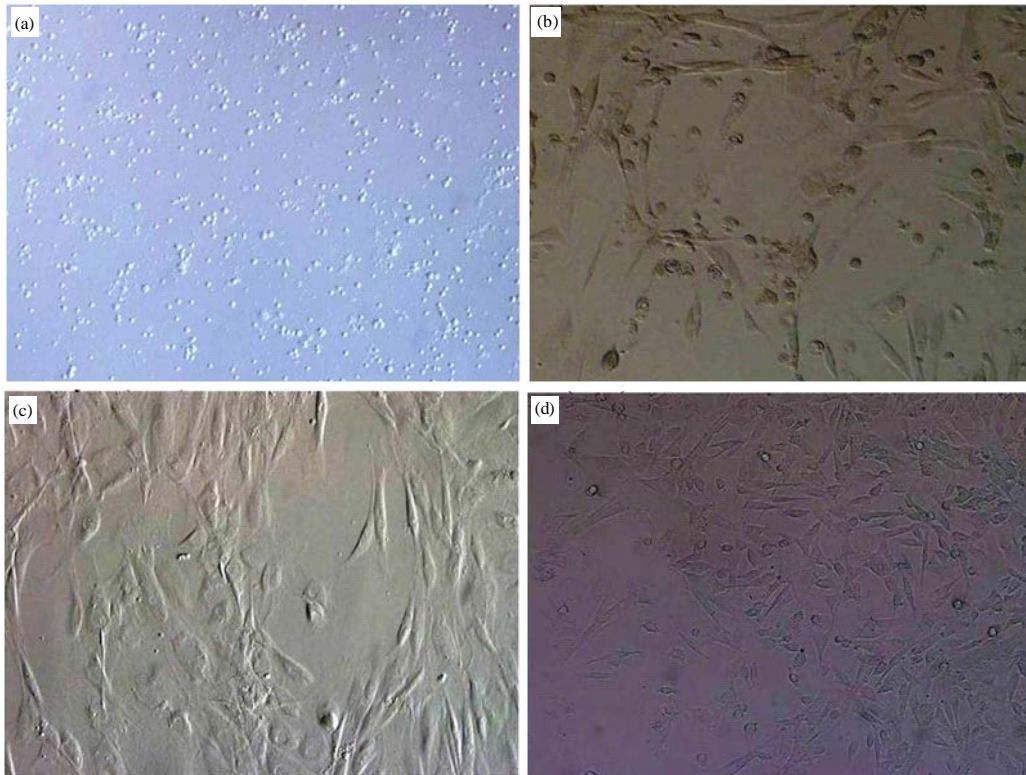


Fig. 2(a-d): Rabbit mesenchymal stem cells in culture flask at different morphological stages and confluency with different time intervals, (a) Round nucleated cells depicting heterogenous cell population on day 0 (X10), (b) Cell depicting changed morphology to spindle shape on day 4-6 (X40), (c) Spindle shaped cells in 4-6 cell colonies in tissue cultured flask on 7-9 days (X40) and (d) Day 10-14, the cells reached 70-80% confluency (X10)

**Characterization of rMSCs:** Characterization was performed upon third passage rMSCs as per the criteria laid down by International Society for Cellular Therapy (ISCT) viz., expression of different surface markers (CD105 and CD166) and non-expression of haematopoietic surface markers (CD34 and CD45) and chondrogenic (Sox9), adipogenic (Ppar- $\gamma$ ) and osteogenic (Runx2) markers, collagen type I, II and aggrecan. Apart from phenotypic and genotypic characterization, MSCs were evaluated for alkaline phosphatase activity.

**Phenotypic characterization by RT-PCR:** The rMSCs expressed mRNA transcripts for CD166 and CD105 markers, collagen type II (Fig. 3a) and collagen type I (Fig. 3b). There was lack in the expression of Haematopoietic surface markers (CD34 and CD45) (Fig. 3a) and aggrecan (Fig. 3b). Housekeeping gene GAPDH was included as an endogenous control to evaluate the quality of cDNA synthesis.

**Alkaline phosphatase enzyme activity:** Mesenchymal stem cell colonies were subjected to the localization of Alkaline Phosphatase (AP) enzyme activity on third passage of the culture. The AP activity was confirmed in rMSCs that appeared red after staining (Fig. 4a).

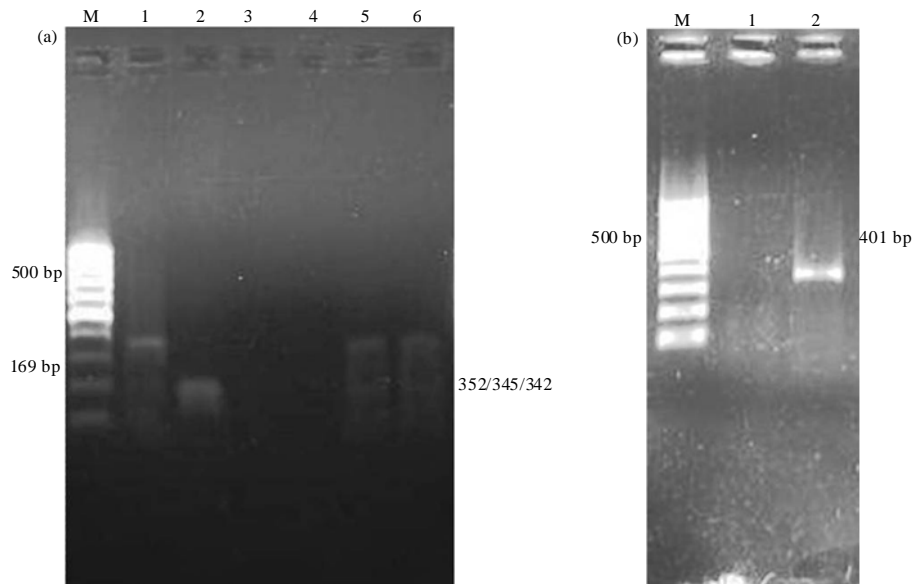


Fig. 3(a-b): (a) RT-PCR expression of different genes of rMSCs and (b) Expression of collagen type I and aggrecan genes in rMSCs, Lane M: 100 bp DNA ladder, Lane 1: Aggrecan (351 bp), Lane 2: Collagen type I (401 bp)

### **Tri-lineage differentiation potential of MSCs**

#### **Differentiation of BM-MSCs into the respective lineages was observed as under**

**Osteogenic differentiation:** After 18 days of culture in specific induction medium, osteogenic differentiation of MSCs was confirmed by the presence of calcium oxalate crystals as evident on staining with Alizarin Red stain (Fig. 4b). The appearance of the cells changed in the first 3-5 days from long spindles to polygonal or irregularly shaped conformations. Further, confirmation of osteogenic marker gene expression (Runx2) was by done by RT-PCR which showed an amplicon of 54 bp (Fig. 5).

**Chondrogenic differentiation:** Sphere-like aggregates and cartilage specific proteoglycans were secreted by rMSCs that stained positive with Toluidine blue (Fig. 4c), but stained negative in control group. Chondrogenic differentiation marker (Sox9) (Fig. 5) and collagen type II and aggrecan genes expression was confirmed by RT-PCR.

**Adipogenic differentiation:** The cells changed in appearance from long spindles to polygonal shapes and became enlarged following 3-5 days incubation in a differentiation medium. After 7 days of culture in adipogenic induction medium, the rMSCs started detaching from each other and formed isolated cell clusters. The fibroblast morphology of rMSCs was lacking and also the cells contained small number of intracytoplasmic Oil-red O positive lipid droplets (Fig. 4d). Such changes were absent in the undifferentiated MSCs in control group. Adipogenic marker (Ppar- $\gamma$ ) gene expression was also detected by RT-PCR which showed 118 bp amplicon size (Fig. 5).



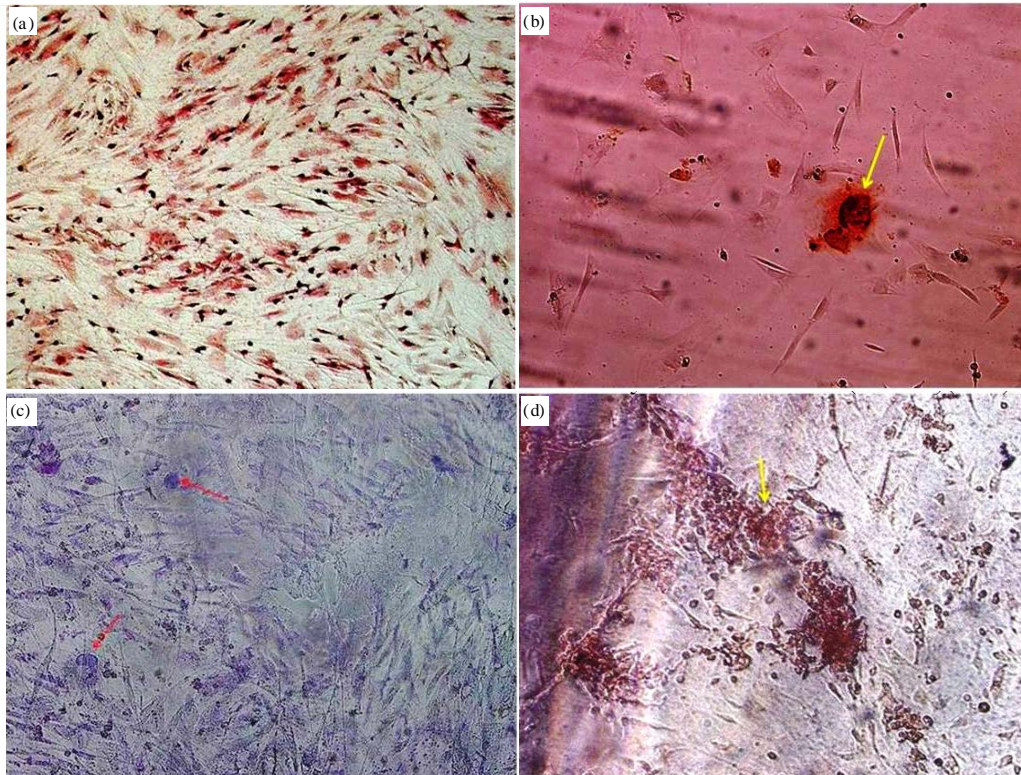


Fig. 4(a-d): (a) Characterization of AP staining, rMSCs, appeared (10X), differentiation of rMSCs into, (b) Osteogenic lineage demonstrating calcium-rich deposit stained by Alizarin red S (Arrow) (10X) (c) Chondrogenic lineage demonstrating blue colour ECM stained by Toluidine blue (arrow) (10X) and (d) Adipogenic lineage demonstrating red colour fat droplets stained by oil red O (arrow) (10X)

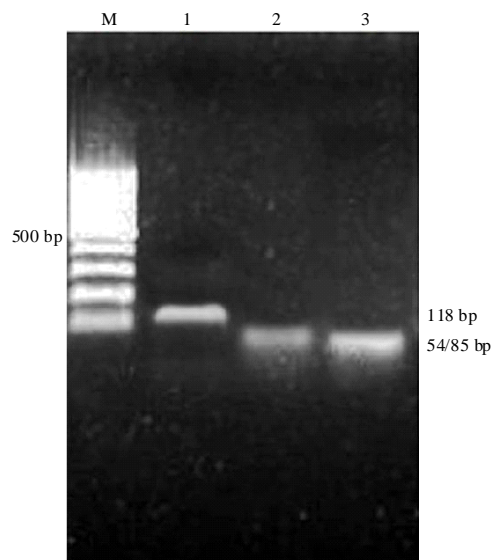


Fig. 5: Gene expression of differentiation markers by RT-PCR

## DISCUSSION

Stem cells, capable of self-renewal, multiplication and differentiation are currently seen as a potential tool for regenerative therapy both in human and veterinary medicine (Gade *et al.*, 2013; Gugjoo *et al.*, 2015). The cells being readily available including the patient himself make their utility to one and all barring the cost factor. The cells have been utilized as therapeutics in both human and animal with encouraging results for the repair of cartilage, tendon, bone, neurodegenerative disorders, spinal injuries, cardiac defects, facilitate wound healing, etc. (Caplan, 2005; Ribitsch *et al.*, 2010; Xie *et al.*, 2012; Gugjoo *et al.*, 2015; Cutts *et al.*, 2015). However, such a potential is yet to be harnessed due to the lack of proper understanding of the cell-tissue interactions, their differentiation and the immune reactions that cells may encounter in host environment. Besides, MSCs obtained from bone marrow constitute a very small fraction, 0.01-0.001% of the bone marrow cells (Pittenger *et al.*, 1999; Martin *et al.*, 2002; Gugjoo *et al.*, 2015) and many cells obtained during harvesting process may be progenitor cells that mimic the characteristic of MSCs (Chong *et al.*, 2012). Further, isolation and culture methodologies may affect the characteristics of stem cells. Therefore, it is imperative to standardize the process to isolate, culture, expand and characterize MSCs before their effective use.

Currently, rabbit Mesenchymal Stem Cells (rMSCs) are very popular among researchers due to their resemblance in cellular and tissue physiology with that of human MSCs (Fox, 1984; Warden, 2007) and due to their easy availability. As per the International Society for Cell Therapy (ISCT), cells that are plastic adherent, express CD105, CD73 and CD90, lack expression of CD45, CD34, CD14 or CD11b, CD79a or CD19 and HLA-DR surface molecules and can differentiate into osteogenic, chondrogenic and adipogenic lineages are described as Mesenchymal Stem Cells (MSCs) (Dominici *et al.*, 2006).

Isolation of rMSCs was performed by a standard method reported earlier (Pittenger *et al.*, 1999; Woodbury *et al.*, 2002; Ansari *et al.*, 2013; Udehiya *et al.*, 2013). The rMSCs on the basis of morphology, cell frequency rate and molecular marker profile were similar to the canine, murine, porcine, rodent or human MSCs (Castro-Malaspina *et al.*, 1980). On morphological basis, MSCs were spindle-shaped with high cell density and had individual colonies that appeared at 7-10 days, as also reported by others (Lapi *et al.*, 2008; Tan *et al.*, 2013; Udehiya *et al.*, 2013). Morphological heterogeneity in MSCs is generally associated with the presence of cells at different differentiation levels and not with the distinct cell culture subtype (Docheva *et al.*, 2008). Besides, large flat MSC is described as mature senescent cell with low mitotic potential (Neuhuber *et al.*, 2008; Fu *et al.*, 2012). Proliferation rate in mesenchymal stem cells is very high and one MSC can proliferate to  $2 \times 10^6$  cells in a single passage in rabbits (Lapi *et al.*, 2008). However in human, this potential is as high as  $5.5 \times 10^8$  to  $1.2 \times 10^9$  that can proliferate up to 10-25 passages (Conget and Minguell, 1999). In the present study, proliferation rate was also higher with 80-90% cell confluency obtained within a period of 18-21 days. These observations were in concurrence with the findings of others (Zhou *et al.*, 2010; Udehiya *et al.*, 2013).

Expression of cell surface markers (CD105 and CD166) was confirmed by RT-PCR as has also been reported in previous studies (Gade *et al.*, 2013). However, there are contrasting reports about the MSCs expression of collagen type II gene. Findings of current study were similar to the observations reported for horse MSCs (Guest *et al.*, 2008) but a contrasting finding was reported in other study (Fortier *et al.*, 2011). This may be due to their study on early passage cells (passage 1) and at an early culture period (11 days). The present study used cells of passage 3 for the analysis of collagen expression. This longer time frame may have allowed more enrichment

for dividing MSCs (Guest *et al.*, 2008). The report from Fortier *et al.* (2011) did not utilize a ficoll or histopaque gradient in the isolation of their MSCs. In the present study, the mononuclear cells were collected following the centrifugation of bone marrow over a histopaque gradient. The different isolation methods could be responsible for the observed differences in collagen type II expression (Guest *et al.*, 2008). Expression of the genes of haematopoietic stem cell markers (CD34 and CD45) was lacking in present study as reported by others (Guest *et al.*, 2008; Gade *et al.*, 2013; Ansari *et al.*, 2013). However, there are reports that showed MSCs expression of the CD34 and CD45 surface markers, with one report showing their dim expression (Tan *et al.*, 2013) and other reported that MSCs have heterogeneous CD34 and CD45 phenotype that changes under in vitro conditions (Kaiser *et al.*, 2007). Tri-lineage differentiation potential of MSCs was observed at about 18 days. Chondrogenic, osteogenic and adipogenic potential was confirmed by special stain viz., Toluidine blue, Alizarin blue and Oil Red O, respectively. Further, RT-PCR was also used to confirm expression of the specific genes like Sox9, collagen type II, Runx2 and Ppar- $\gamma$  for chondrogenic, osteogenic and adipogenic, respectively. Similar findings have also been reported by other researchers (Fortier *et al.*, 2011; Gade *et al.*, 2013; Tan *et al.*, 2013; Gao *et al.*, 2014; Gong *et al.*, 2014). In a report that compared tri-lineage differentiation potential of rMSCs with that of human showed higher osteogenic and adipogenic differentiation potential in later with no difference in relation to chondrogenic differentiation potential (Tan *et al.*, 2013).

In hMSCs, alkaline phosphatase activity is present at baseline levels in undifferentiated cells (Pittenger *et al.*, 1999) and increased in a time dependent manner (Sun *et al.*, 2008). Alkaline Phosphatase (AP) activity has been detected in rMSC either prior to or following induction to the osteocytic phenotype (Lee *et al.*, 2011). Alkaline phosphatase thus, can be considered as an early and requisite marker for characterization of MSCs. In the present study third passage cells showed moderate alkaline phosphates activity, which was considered as a positive marker for rMSCs.

## CONCLUSION

The rMSCs can be isolated using methods described previously and conform to most of the standards set by ISCT. The present study provided basic protocols for characterization of rabbit MSCs that should be used before application of the se cells for any research or therapy.

## ACKNOWLEDGMENT

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