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Isolation, Enrichment and characterisation of Caprine bone marrow derived mesenchymal stem cells

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Abstract
Mesenchymal stem cells are now- a-days treated as the cornerstone of the cellular therapy and tissue engineering arena. These omnipresent cells can be isolated from a wide range of sources and can be differentiated and trans-differentiated into many cell types based on specific cellular command. In this study, we had isolated bone marrow derived mesenchymal stem cells from 1-2 years old apparently healthy goats. The heterologous cell population then subjected to magnetic activated cell sorting against $CD73^+$, $CD90^+$, $CD105^+$, $CD271^+$ and $CD34^-$ to exclude mesenchymal stem cells from haematopoietic stem cells. The cells were then cultured and incubated at 37 °C in humidified atmosphere with 5% CO_2. After 3 passages the cells were tested for alkaline phosphatase activity. Furthermore, the cells were also evaluated based on their morphometric analysis.

Keywords: Mesenchymal stem cells, Caprine, CD markers

Introduction
Stem cells play a vital part of cellular therapy and tissue engineering. Among the cluster of stem cell types, mesenchymal stem cells represents a lions share due to its wide range of beneficial effects which it secrets around the cellular niche. In recent past the researchers around the world are working in the field of mesenchymal stem cells to give newer insights like cell signalling, bio-mechanism, potential active biomolecules which it secretes etc. These cells lack tissue-specific characteristics but under the influence of appropriate signals they can be differentiated into specialized cells with a phenotype distinct from that of the precursor. It may be that stem cells in adult tissues are reservoirs of reparative cells, ready to mobilize and differentiate in response to wound signals or disease conditions (Barry *et al*., 2004) [1]. The MSCs can be isolated from a wide range of sources like bone marrow (Ninu *et al*., 2017) [13], adipose tissue (Elkhenany *et al*., 2016) [4], amniotic fluid (Tsai *et al*., 2004) [18], amniotic membrane (Cai *et al*., 2010) [2], dental tissues/ pulp (Seifrtova *et al*., 2012) [16] etc. However, although MSCs can be isolated from these varied sources, the nature and differentiation potential and specificity towards CD markers vary from source to source. Dominici and co-workers (2006) stated that these cells must have three properties like adherence to plastic, specific surface antigen (Ag) expression and multipotent differentiation potential in order to term them as mesenchymal stem cells. These cells can be characterised in many ways starting from staining to molecular expression. For use in cellular transplantation, these cells can be characterised through Magnetic Activated Cell Sorting (MACS). Similarly, few staining techniques like Alkaline Phosphatase staining is definitive staining for osteogenic potential determination of MSCs.

Materials and Methods
All the chemicals and the biologicals used in the study were of molecular and standard analytical grade procured from reputed international and national firms *viz.* Sigma Aldrich (USA), Merck (India), Thermo fisher scientific, Invitrogen etc.

Collection of biological samples and place of study
The area of study was Female Reproduction Lab, ICAR-Central Institute for Research on Goats, Farah, Mathura, U.P. apparently healthy goats free from any concurrent diseases aged between 1-2 years were selected for the bone marrow aspiration.

Isolation of bone marrow from goats

For the collection of bone marrow, the pelvic area including iliac crest was shaved and cleaned thoroughly with 70 % alcohol. It was followed by administration of xylazine IM (@ 0.05 mg/kg body weight) to achieve sedation and the animal were placed in lateral recumbency with incision site on top once sedation was developed. Subsequently, 2 mL of 2% Lignocaine hydrochloride was injected around the iliac crest. After that 1 cm stab incision of was given on iliac crest to insert bone marrow aspiration needle (16G). After sufficient depth was achieved, the stylet was removed. The bone marrow was collected to a syringe with anticoagulant (EDTA) to prevent coagulation of marrow. At the end of the collection procedure, the puncture area was disinfected with antiseptic lotion and the animals were administered broad spectrum antibiotic.

Processing and culture of aspirated bone marrow

The bone marrow was transferred to a sterile falcon tube pre-loaded with histopaque and was centrifuged at 2800 rpm for 30 minutes. After centrifugation, the middle buffy coat layer containing mesenchymal stem cells was taken with utmost care for further processing. It was again subjected to centrifugation at 7000 rpm for 10 minutes with PBS to harness cell pellet. The cell pellet was washed with RBC lysis buffer at 7000 rpm for 3 minutes to exclude RBC. It was followed by washing with PBS twice through centrifugation at 7000 rpm for 10 minutes. At the culmination of processing, the cell pellet was suspended in DMEM supplemented with FBS (15%), antimycotics- antibiotic (100 IU/ ml) and non-essential amino acids (5µl/mL), low glucose, L-glutamine (2 mM) and gentamicin (50mg/100ml) in a25 CM^2 tissue culture flask and incubated at 37°C in humidified atmosphere with 5% CO_2. The non-adherent cells were removed in every 48-72 h of incubation and the media was changed with fresh growth media in order to propagate the plastic adherent MSCs.

Passaging of adherent BMMSCs

The cells were passaged once the cells (BMMSCs) attained 70-80% confluency. In order to do that, the old culture media was removed and the culture flasks were washed with DPBS (without Ca^{++} and Mg^{++}) followed by trypsinisation (0.25%) of the cells for 3-5 minutes. AT the end of incubation the detached cells were transferred into a centrifuged tube with washing media and centrifuged at 7000 rpm for 10 minutes at RT. After centrifugation, supernatant was removed very carefully and cell pellet was resuspended in culture media. Finally the suspended cells were seeded in to culture flasks and incubated at 37°C in humidified atmosphere with 5% CO_2.

Characterisation of BMMSCs

Characterisation through morphology

The morphological characteristics of BMMSCs were assessed inverted microscope at different magnifications like 10× and 40×. The cells were observed at different time points to assess the typical fibroblast like structure having spindle like processes.

Characterisation through Alkaline Phosphatase (ALP) Staining

The ALP staining was done with Alkaline Phosphatase Kit (Sigma Aldrich) in accordance with the manufacturer's protocol. Briefly, 45 ml deionized water was measured and brought to RT for preparation of diazonium salt solution. For the said purpose 1 ml Sodium Nitrite solution was added to 1 ml of FBB-Alkaline solution and mixed gently and incubated for 2 minutes. Later on, 1 ml of Naphthol AS-BI Alkaline solution was added to diluted diazonium salt solution and mixed thoroughly. The Citrate-Acetone-Formaldehyde Fixative (2.5 ml: 6.5 ml: 0.8 ml) was used for the fixation of wells by immersing the wells in fixative solution for 30 seconds. It was followed by rinsing gently in deionized water for 45 seconds. Immediately alkaline-dye mixture was added and incubated at 18–26°C for 15 minutes. Utmost care was taken to protect wells from direct light and after 15 minutes of incubation, alkaline-dye mixture was discarded and rinsed for 2 minutes in deionized water. It was closely followed by counterstain with Neutral Red Buffered Solution for 2 minutes. The wells were rinsed thoroughly in distilled water and air dried followed by microscopic evaluation for staining of BMMSCs.

Magnetic Activated Cell Sorting (MACS)

MACS was conducted against $CD73^+$, $CD90^+$, $CD105^+$, $CD271^+$ and $CD34^-$ with MACS MicroBeads (Miltenyi Biotec) as per manufacturer's protocol. Briefly, after getting the bone marrow sample, it was subjected to density gradient centrifugation through Histopaque-1077^{TM}to get buffy coat. It was further centrifuged with PBS to get cell pellet. The cell suspension was centrifuged at 300×*g* for 10 minutes and the BMMSCs number was determined manually by haemocytometer. The cell suspension then diluted in 90 µl of buffer solution (phosphate-buffered saline, pH 7.2, 0.5% bovine serum albumin (BSA), and 2 mM EDTA) per 10^7 total cells. After that 10 µl of CD marker MicroBeads per 10^7 total cells was added and mixed thoroughly and incubated for 15 minutes in the dark in the refrigerated temperature (4°C). At the end of incubation period, the cells were washed with buffer and centrifuged at 300×*g* for 10 minutes. After discarding supernatant cell pellet was resuspended in buffer for magnetic separation. The cell suspension was applied onto the column placed in a suitable MACS separator in a magnetic field. Flow-through was collected containing un-labelled cells. After pressing the final volume, the magnetic column was washed with buffer. At the end, the column was separated and placed on a suitable collection tube. 1 ml of buffer was pipetted onto the column and flushed out the magnetically labelled cells. After collection of positive CD marker cells, they were seeded into different flasks/plates and incubated at 37°C in humidified atmosphere with 5% CO_2.

Results and Discussion

Average 10 ml of bone marrow was collected after anaesthetisation and BMMSCs were isolated through density gradient centrifugation. The average number of heterogeneous cells obtained was 10-15×10^6/ ml.

Culture of BMMSCs

The isolated BMMSCs were cluttered in culture flasks (T25/T75) and incubated at 37°C in humidified atmosphere with 5% CO2. The non-adherent cells were removed by every 48-72 h of incubation and the media was changed with fresh growth media in order to propagate the plastic adherent MSCs. The cells attained typical fibroblast like structure after few days and can be demonstrated in the figures 1.

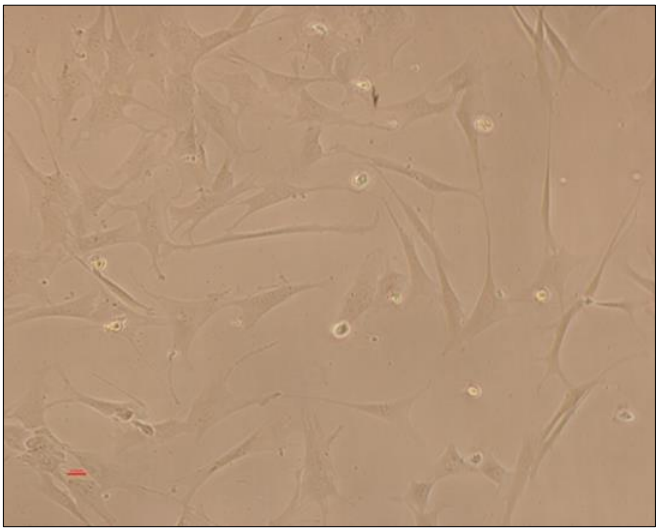

Fig 1: The BMMSCs with typical fibroblast like structure as observed in normal culture condition after one week.

Characterisation of BMMSCs

Morphologic characterisation

The BMMSCCs showed round shaped shape when seeded in the culture flask. However, as the culture times passed, the cells showed a typical spindle like appearances with narrow body and tapering ends much like that of fibroblast cells. The morphological features were observed at 10× and 40× magnifications using inverted microscope (Nikon and ZEISS).

Apart from the typical fibroblast like structure some peculiar shapes were also observed like triangular or star-like cell shape and cuboidal or flattened pattern (Fig 2).

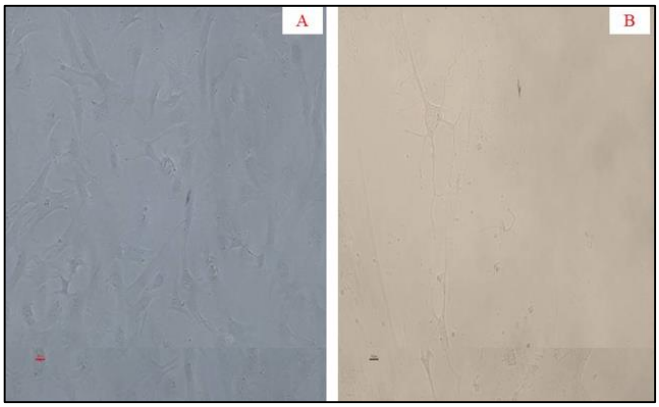

Fig 2: The BMMSCs with typical cuboidal or flattened (A) and triangular or star-like (B) cell shape as observed in culture condition

Characterisation through Alkaline Phosphatase (ALP) Staining

The BMMSCs were found to be positive for ALP staining and the intensity was clearly observed in the BMMSCs monolayer which established the alkaline phosphatase activity and osteogenic differentiation ability of the MSCs. This was observed in all passages of BMMSCs. The BMMSCs monolayer took the characteristics red colour signifying the osteogenic capacity of the cells which can be observed in Fig 3.

Magnetic Activated Cell Sorting (MACS)

MACS was performed against $CD73^+$, CD90+, $CD105^+$, $CD271^+$ and $CD34^-$ along with FC blocker with MACS MicroBeads (Miltenyi Biotec) to separate BMMSCs from haematopoietic stem cells which are very common when the source of MSCs is bone marrow. The flow through was discarded and cells clung to the magnetic column were seeded into different flasks/plates and incubated at 37°C in humidified atmosphere with 5% CO_2. These cells were further passaged to increase the number of cells and used for various experiments. Approximately average 1×10^6/ml cells were isolated as MACS positive against above mentioned antibodies out of 20×10^6 cells/ml. The morphological illustration of propagation of BMMSCs after MACS separation is given in Fig 4.

MSCs represent minor fraction (0.01–0.001%) of the total population of nucleated cells in bone marrow. These cells can be found in a heterogeneous cell population along with hematopoietic stem cells. So it is imperative to segregate the hematopoietic stem cells from the BMMSCs in order to facilitate them for further uses like cellular transplantation or other routine culture works. Various studies reported different techniques to differentiate between these two categories like hematopoietic stem cells and mesenchymal stem cell like FACS and MACS (Jena *et al*., 2020) [7]. In MACS, the sorted cells can be utilised for further culture works or transplantation purpose also. In our work we had collected roughly 10 ml of bone marrow from the iliac crest of the goats and for characterisation purpose we had gone for MACS against specific cluster of differentiation (CD) markers like CD73, CD90, CD105 and CD 271 as positive markers and CD34 as negative markers. Very few studies have reported the isolation and culture of caprine bone-marrow derived mesenchymal stem cells. The standard culturing techniques in combination of density gradient centrifugation were commonly used for the purpose (Remya *et al*., 2014; Silva Filho OF *et al*., 2014; Elkhenany *et al*., 2016; Kumar *et al*., 2016) [15, 17, 4,8]. There are different protocols reported previously in terms of isolation, characterization and expansion of MSCs and mostly ficoll density gradient method with small modifications are commonly used (Wang *et al*., 2015; Mamidi *et al*., 2012; Raynaud *et al*., 2012) [19, 11, 14] and seeded into culture plates. By culturing them they can be isolated and purified, due to their surface adherent property (Nadri *et al*., 2007; Remya *et al*., 2014) [12, 15]. MSCs from other tissue sources shared many characteristics with bone marrow MSCs, but still showed some differences amongst each other in phenotype, proliferation and the capacity of differentiation (Chen *et al*., 2008) [3].

Alkaline phosphatases are the iso-enzymes present on the outer layer of the cell membrane. Their presence can be found in decreasing concentrations in placenta, ileal mucosa, kidney, bone and liver. The majority of alkaline phosphatase in serum (more than 80%) is released from liver and bone, and in small amounts from the intestine (Lowe *et al*., 2020) [10]. The bone marrow MSCs shows strong positivity towards ALP staining. In our studies we had observed highly positive BMMSCs as ALP staining was concerned. The results were in line with earlier findings with Jaiswal *et al*., 2000; Latifpour *et al*., 2014; Ghasemzadeh-Hasankolaei *et al*., 2014 [6, 9, 5].

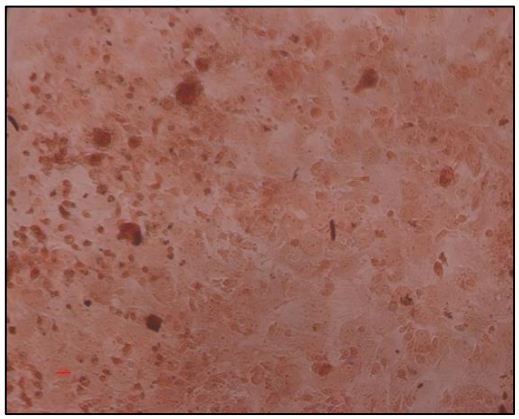

Fig 3: The characteristics red colour of the BMMSCs monolayer after ALP test signifying Osteogenic activity of the cells

Fig 4: The morphological illustration of propagation of BMMSCs after MACS separation.

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