Isolation and Characterization of Adipose derived Mesenchymal Stem cells (ADMSCs) in Madras Red Sheep (*Ovis aries*).

**Original Article**

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**Abstract**

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. MSCs can be isolated from the adipose tissue or other tissues like bone marrow and umbilical cord blood. The present study was focused on Madras Red sheep, as sheep is an ideal model for bone tissue engineering and has been proposed as an animal model for a wide range of applications in biomedical research like prion diseases, respiratory diseases, neurological disorders and cardiomyopathies. Characterization of Ovine adipose tissue derived mesenchymal stem cells (ADMSCs) is important before their use for therapeutic purposes. In the present study, ovine adipose tissue samples were processed under sterile conditions for isolation of ADMSCs as per the standard procedures. Yield of ADMSCs was found to be 2.12±0.19 million per gram of adipose tissue. ADMSCs were cultured upto passage 2 (P₂). Characterization was done by using transcription factors namely Oct4 and Sox2 at P₀ level and was demonstrated by immunofluorescence technique. Immunophenotyping of ovine ADMSCs was done by using CD34 and CD44 monoclonal antibodies at P₀ and P₁ levels by using flow cytometry. At P₀ level, 30.14 per cent ADMSCs were positive to CD44 and 13.83 per cent were positive for CD34 antibodies. At P₁ level, 40 per cent and 7.13 per cent cells were positive for CD44 and CD34 respectively. To conclude, Ovine ADMSCs were identified by Oct4 and Sox2 at P₀ level and CD44 positive cells were more at P₀ and P₁ level.

**Keywords:** Mesenchymal stem cells, Adipose tissue, isolation, expansion, characterization, Madras Red sheep

**Introduction**

Mesenchymal stem cells (MSCs) are multipotent non-hematopoietic progenitor cells of stromal origin that can be isolated from the adipose tissue or other tissues like bone marrow and umbilical cord. Adipose tissue can be harvested in large amounts with minimal morbidity. It contains numerous cell types, including adipocytes, pre-adipocytes, vascular endothelial cells and vascular smooth muscle cells; it also contains stem cells that have the ability to differentiate into several lineages, such as fat, bone, cartilage, muscle etc. [1]. Adipose tissue has been proven to serve as an abundant, accessible and rich source of adult stem cells with multipotent properties. Hence, it is used for tissue engineering and regenerative medical applications [2].

Oct 4 is a well-known potent marker of pluripotent stem cells, many reports indicated that Oct4 is also expressed in adult stem cells. Presence of embryonic stem cell markers
such as Nanog, Sox2 and SSEA4 are also seen in stem cells isolated from adipose tissue [3, 4].

Changes in ASCs markers were due to the culture conditions and number of passages had been pointed out as causes of this inconsistency [4]. The expression of CD markers gets changed during the subsequent cell passages in several species and also due to laboratory procedures, biological differences between the donors because of species, age, gender and site of collection [5, 6].

In human and dogs, CD29, CD44 and CD90 were regarded as positive cell surface markers for ADMSCs while CD34 and CD45 were regarded as negative surface markers [7].

Although reports from the literature are available on characterization of ADMSCs in human [5] and different animal species such as rat, dog, equine [3, 5]. There is very little information available on characterization of ADMSCs in Madras Red Sheep. The Madras Red sheep, a native breed of Tamil Nadu in India, is known for its high-quality meat and skin. The animals are medium in size with well-built body having a broad and deep chest. Most of the animals are red in colour which varied from tan to dark brown. The present study on isolation, expansion and characterization of ADMSCs in Madras Red Sheep was undertaken to elucidate the basic understanding of ADMSCs in local breed of Sheep.

**Materials and Methods**

In the present study, adipose tissue samples were collected immediately after slaughter from adult Madras Red Sheep irrespective of sex from Chennai Corporation slaughter house, Perambur. A total of 18 samples were taken for the study. The study was carried out in Centre for Stem cell Research and Regenerative Medicine, Madras Veterinary College, Chennai, India.

**Collection of Adipose tissue**

Adipose tissue samples were collected in normal saline with antibiotic, antifungal solution (Cat No. 15240-062, Gibco®, India). The samples were kept at room temperature and processed within 1 hour. Adipose tissue were weighed by using an electronic weighing balance and washed 3 to 4 times with equal volume of normal saline containing antibiotic and antifungal solution [5]. Storage of tissue samples at 4°C was avoided because it reduced the activity of digestive enzymes [8].

**Isolation of ADMSCs using enzyme digestion**

The weighed tissue samples were teased/separated into small bits of about 3 to 5 mm in size by use of sterile scissors, forceps and surgical blade. Tissue samples were digested with 0.075 per cent collagenase type I (Cat. No. CO130 SIGMA®, India) in a beaker with magnetic stirrer (100 rpm) at 37°C for about one hour. After digestion, two phases were formed namely upper and lower phases. Upper phase contained floating digested adipose tissue and the lower phase contained mesenchymal stem cells, erythrocytes and leukocytes [8]. Lower phase containing stromal vascular fraction (SVF) was pipetted out and 2ml of DMEM-HG (Cat. No.-11320-033 , Gibco, India) with fetal bovine serum (FBS) (Cat. No. 16250-086, Gibco, India) and antibiotic solution was added to stop the enzyme activity. Finally, it was centrifuged at 2500 rpm for 10 minutes to pellet the cells [2]. Supernatant was discarded and the cell pellet was resuspended with 1ml of DMEM-HG with 10 per cent FBS to get cell suspension.

Cell viability and total cell density were measured by Trypan blue (Cat. No. T8154, Sigma-Aldrich, India) exclusion test. The viable cells with transparent cytoplasm were counted manually in Neubauer’s chamber [9].

**Characterization of ADMSCs using transcription factors**

Cultured cells in 12 well plate were washed in Dulbecco’s phosphate buffered saline (DPBS) twice and the cells were fixed in 4% paraformaldehyde for 10 minutes at room temperature. Fixation was confirmed by the appearance of small black dots in the cytoplasm of cells under microscope. After fixation, 400µl of 3 per cent blocking buffer was added to the well and was covered with aluminum foil and stored at room temperature for 90 minutes. After this, 500 µl of Oct4 primary antibody was added to the well and stored at 4°C over night. The cells were washed twice with DPBS and then the secondary antibody (i.e) anti rabbit-FITC was added to the well and covered with aluminum foil and incubated for 90 minutes. Secondary antibody was removed and the cells were again washed with DPBS twice. To this, 400µl of 4’-6-diamidino-2-phenyl indole (DAPI) (1 in 1000 dilution with PBS) was added and covered with aluminum foil and kept at room temperature for 10 minutes. DAPI was removed and PBS washing was done 2 times. To this, 1000 µl of PBS was added and kept at 4°C until the plate was examined under fluorescence microscope. Similar protocol was followed for Sox2 transcription factor [5].

**Characterization of ADMSCS using surface markers**

Characterization of ADMSCs at the level of P0 and P1 was done as per the standard protocol using CD34 and CD44 primary antibodies [10, 11]. Flow cytometric analysis was performed on Becton, Dickinson FACS using a 488nm-
argon-ion laser and 632nm red LASER for excitation. In the histogram, unstained cells were used to mark the P1 and P2 areas. P1 is the unstained population and P2 is the stained population (i.e) positive percentage of cells for the specific marker (CD44).

**Result and Discussion**

**Characterization of ADMSCs using transcription factors**

In the present study, presence of transcription factors *viz.*, Oct4 and Sox2 was studied as intra-nuclear fluorescence in Ovine ADMSCs at P0 level. ADMSCs were subjected to immunofluorescence for determining the expression of transcription factors *viz.*, Oct4 and Sox2. Cells that were positive to Oct4 have been demonstrated at P0 level (Fig.1). Similarly, Sox2 positive cells were also demonstrated at P0 level. In both cases, positive cells for transcription factors showed intra nuclear fluorescence (Fig.2). Presence of Oct4 in *in-vitro* adipocyte cultures denotes the achievement of differentiation in the majority of initial stem cell population. Oct4 and Sox2 are considered as pluripotent capability markers of the cells. In canine, expression of Oct4 and Sox2 factors in ADMSCs has been recorded [12]. In human, Oct4 and Sox2 are naturally expressed in MSCs at low level in early passages and their level gradually decreases as the passage number increases [13].

**Characterization of ADMSCs using surface markers**

In the present study, ADMSCs of Madras Red Sheep at P0 and P1 levels were characterized by surface markers *viz.*, CD34 and CD44 using flowcytometry. However, human ADMSCs at P2 and P3 were used for immunophenotyping [14].

At P0 level, 30.14 per cent of cells were positive to CD44 marker (Fig. 3) whereas 13.83 per cent cells were found to be positive for CD34 marker (Fig. 4). At P1 level, 40 per cent cells were positive for CD44 marker and 7.13 per cent cells for CD34 marker. In the present study, at both P0 and P1 levels, CD44 cells were found to be more when compared to CD34 cells. Whereas, in human, CD44 positive and CD34 negative nature of ADMSCs is typical characteristic feature of the adult mesenchymal stem cells [1, 15, 16].

In the present study, expression of CD34 surface marker decreased from P0-P1 in canine [5]. They reported that CD34 expression was consistently absent or expressed in few cells of canine ADMSCs at higher passage level (P4). Similarly, ADMSCs of human lost their CD34 expression as passage increases [14, 17]. However, the positive
Conclusion

ADMSCs from Madras Red Sheep were characterized by immuno-fluorescence studying the transcription factors like Oct4 and Sox2 at P₀ level. Both Oct4 and Sox2 showed intra nuclear fluorescence. ADMSCs were also characterized by using surface markers like CD44 (positive) and CD34 (negative) by flow cytometry. 30.14 per cent cells showed positive expression for CD44 and 13.83 per cent of cells showed positive expression for CD34 marker at P₀ level. At P₁ level, 40 per cent cells showed positive expression for CD44, whereas, 7.13 per cent cells showed positive expression for CD34 marker. As passage level increased from P₀ to P₁, positive expression for CD44 marker was found to be increased. However, the positive expression of ADMSCs to CD34 marker was decreased as passage level increases.

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Conflict of interest

The authors declare that there is no conflict of interest to reveal.

References


