ISSN NO. 2320-5407

Journal homepage: http://www.journalijar.com

INTERNATIONAL JOURNAL OF ADVANCED RESEARCH

RESEARCH ARTICLE

MORPHOLOGICAL FEATURES OF OVINE BONE MARROW DERIVED MESENCHYMAL STEM CELLS

Archana Mohapatra[1], Sabiha H Basha[2], T.A.Kannan[3], A.Mangalagowri[4], B. Justin William[5], Geetha Ramesh[6] and M.Parthiban[7]

1,2,6-Department of Veterinary Anatomy Tamil Nadu Veterinary and Animal Sciences University Chennai-600007, India.

3,4,5- Centre for Stem Cell Research and Regenerative Medicine, Madras Veterinary College Tamil Nadu Veterinary and Animal Sciences University Chennai-600007, India.

Manuscript Info

Manuscript History:

Received: 14 September 2015
Final Accepted: 20 October 2015
Published Online: November 2015

Key words:

BM-MSCs, Culture, Morphology, Ovine

***Corresponding Author**

Archana Mohapatra

Abstract

Prospective isolation and morphological study of adult ovine bone marrow derived mesenchymal stem cells (BM-MSCs) was done at different passage levels. MSCs from adult ovine bone marrow was isolated by density gradient centrifugation method. Cells were enriched with DMEM-HG media throughout the study period. After 70-80% confluency, the cells were detached from the culture flask by using 0.25% trypsin/EDTA for 1-2 minutes at 37°C for further passaging upto passage4 (P4). It has been demonstrated that isolation of adult ovine BM-MSCs can be achieved by collecting marrow samples from the femur of adult sheep with a higher cell yield. BM-MSCs which reside in the bone marrow has plastic adherency, non-hematopietic cell population generating colony-forming unit-fibroblasts (CFU-F). Ovine BM-MSCs exhibited a heterogenous morphology with bipolar and polygonal cell bodies ranging from spindle shaped cells to triangular, elongated, spherical and dendritic shape in the primary culture. On subsequent passages (P1-P4) cultured cells changed to homogenous spindle shaped appearance. Among all types of adult stem cells, mesenchymal stem cells (MSCs) obtained from bone marrow (BM) was widely used in regenerative medicine. This was because of their high proliferative and differentiation potential. This study was aimed at sheep, as it 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.

INTRODUCTION

Stem cells are undifferentiated cells which have the ability to divide for indefinite periods in culture and give rise to specialised cells on differentiation. Cells forming a whole embryo/foetus are termed totipotent; whereas pluripotent cells have the ability to contribute several tissues of the foetus. Multipotent somatic stem cells, isolated from foetal and adult tissues, differentiate into effector cells of their tissue. However, recent studies imply that adult stem cells can also generate cell types of heterologous tissues indicating broad differentiation potentials (Bhat *et al.*, 2004 and Sung-Min *et al.*, 2010). Bone marrow is a complex tissue containing stem cells for haematopoietic tissue and non-haematopoietic tissues. The precursors of non-haematopoietic tissues are capable of serving as a feeder layer that supports haematopoietic stem cell growth, self-renewing without differentiation and becoming one of a number of phenotypes. They are initially named plastic adherent cells or colony-forming-unit fibroblasts and subsequently named as marrow stromal cells or mesenchymal stem cells (BM-MSCs) as reported by Hung *et al.*(2002). Although, MSCs have been isolated from many sources, such as peripheral blood, fat, skin, vasculature, muscle, trabecular bone and umbilical cord (Lu *et al.*, 2006), still, bone marrow (BM) is considered as the most popular source of MSCs (Vaananen, 2005; Mafi *et al.*, 2011; Sandhaanam *et al.*, 2013). Although, the optimal dosage of MSCs in therapeutic applications is still unclear and will be dependent upon the type of therapy, $1 - 2 \times 10^6$ MSCs per kg body weight is generally used. Direct collection of such a large number of MSCs from BM is not practical. Therefore, it is necessary to isolate and expand those isolated MSCs *in-vitro* for obtaining a sufficient number for therapeutic approaches (Schallmoser, 2008).

MATERIALS AND METODS

The present study on isolation of bone marrow derived mesenchymal stem cells in adult sheep was carried out in the Centre for Stem Cell Research and Regenerative Medicine, Madras Veterinary College, Chennai-7. Bone tissue was collected from femur of sheep in a sterile manner from Chennai Corporation Slaughter House. It was processed within 30 minutes of collection of sample. Thirty samples were processed for isolation and expansion studies as per Ramakrishnan *et al.* (2013). Adherent soft tissues were debrided from the bone. Bone sample was washed thoroughly with normal saline containing 10% antibiotic. Bone was cut into three pieces at both the ends, near the epiphysis and bone marrow from the epiphyseal end was flushed out with 5ml of Acid Citrate Dextrose (ACD) buffer into a 50ml centrifuge tube.

The harvest was diluted with an equal volume of Dulbecco's Modified Eagle's medium high glucose (DMEM-HG). Large clumps were disaggregated by continuous pipetting and suspension was filtered by 40µm cell strainer. Clear bone marrow suspension was laid over on an equal volume of Percoll density gradient solution in 15ml centrifuge tubes. The cells were fractionated by the density gradient centrifugation at 2500 rpm for 30 minutes at room temperature.

The mononuclear cells which were hazy and whitish, at the interphase of DMEM-HG and density gradient solution was aspirated. Mononuclear cell suspension was centrifuged at 2500 rpm for 10 minutes. The supernatant was discarded and pellet was resuspended with 1ml of 1x RBC lysis buffer for 2-3 minutes over ice. It was centrifuged at 2500 rpm for 10 minutes. Supernatant was discarded and the pellet was reconstituted in 1 ml of DMEM-HG thoroughly.

Cell viability and total cell density were determined by 0.4% Trypan blue exclusion test. The cells were counted manually in Neubauer's haemocytometer. The total cell density for seeding was calculated using the formula: (Total cell number × dilution factor)/10^{-4}. Cells were seeded at recommended levels of 1×10^6 cells in T-25 culture flask for expansion and subculturing. Cells were cultured and passaged under optimal culture conditions. After 70-80% confluency the cells were detached from the culture flask by using 0.25% trypsin/EDTA for subsequent passaging.

RESULTS

Mononuclear cell fraction from the femur was about 4.81×10^6/ml. On seeding $1\text{x}10^6$ cells in T-25 culture flasks, adherence of mononuclear cells was observed within 4-5 hours of seeding. In the primary culture (P0) there was a heterogenous population of cells which were round and glistening in appearance (Figure.1). Non-adherent floating cells from the culture were removed during the change of growth medium after 6 hours of initial seeding. Change of growth medium was done every 12 hours during first two days of primary culture. On the third day post incubation 10-20 percent confluency of cells was observed, few cells got expanded with clear fibroblastoid morphology, most of which acquired the spindle shaped appearance with oval body and flattened cytoplasmic process (Figure.2). From fourth day of post incubation in P0, distinct colony formation was observed with groups of spherical cells clustered together (Figure.3). The colony forming unit (CFU) assumed a less elongated, polygonal appearance and formed aggregates or nodules. Cells started expanding from the CFU on the fifth day of primary culture (Figure.4). On the sixth day of post incubation, the spindle shaped, fibroblast-like cells from the colony forming unit-fibroblasts (CFU-Fs) started to migrate to other areas of the culture flask for further expansion. Migration of cells continued upto twelve days post incubation until 40 % confluency was achieved (Figure.5).

The expanded cells exhibited variable morphology in P0 at twelve days post incubation. Some of the cells were spherical with abundant cytoplasm, few were triangular in shape while many of them appeared to be stellate shaped with cytoplasmic processes, anastomosing with the adjacent cells to form syncytium (Figure. 6). Even few rod shaped or elongated cells and cells with many cytoplasmic processes giving a dendritic cell appearance was also observed in the culture (Figure.7). These heterogenous cell population of P0 with varying morphology also exhibited high mitotic activity which was visualised by their distinct single nucleolus or multilple nucleoli (Figure.8).

In the present study, 40 percent confluency was observed at day 12 of initial seeding which became 70-80 percent confluent on day 14. After 70-80 percent confluency, the heterogenous, primary culture (P0) of BM-MSCs was expanded by passaging in order to inhibit spontaneous differentiation in the split ratio of 1:3 for the next passages. Subcultured cells at various passage levels became confluent after 3-4 days with a consistent homogenous spindle shaped morphology after P2 (Figure.9) and the same was observed upto P4.

List of Figures and Legends:

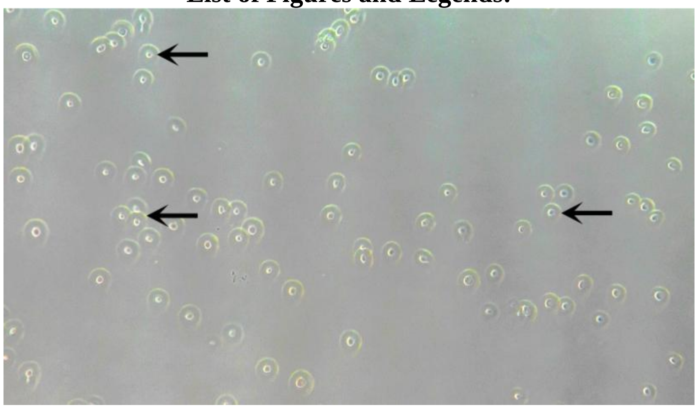

Figure.1 Photomicrograph of Ovine BM-MSCs showing round glistening cells (arrows)

4-5 hrs after initial seeding x 200;

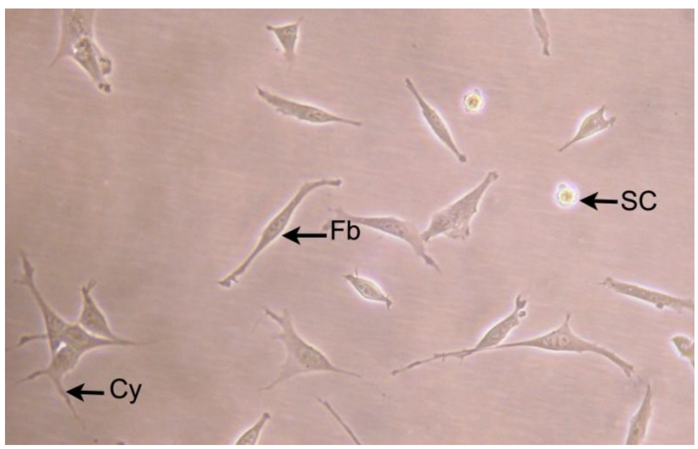

Figure.2 Ovine BM-MSCs after three days of primary culture x 200

Fb- Fibroblast like cells, Cy- Cytoplasmic process, Sc- Spherical cells;

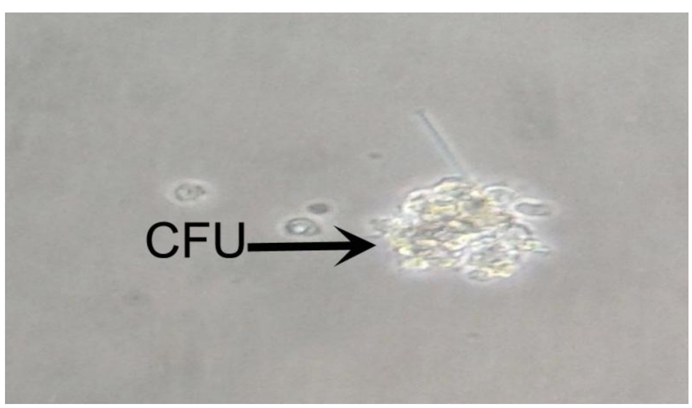

Figure.3 Colony forming unit of Ovine BM-MSCs x 200

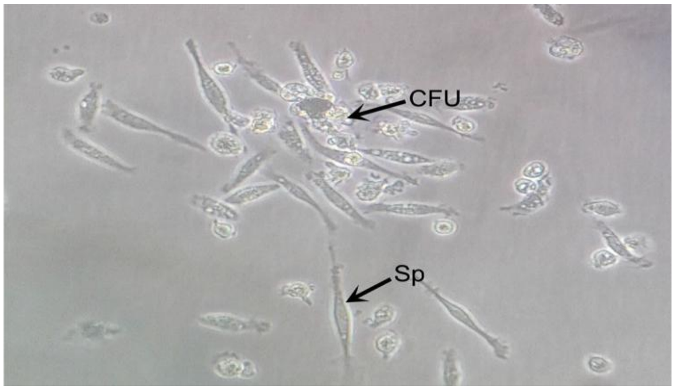

Figure.4 Ovine BM-MSCs primary culture showing expansion of spindle shaped cells (Sp) from colony forming unit (CFU) x 200;

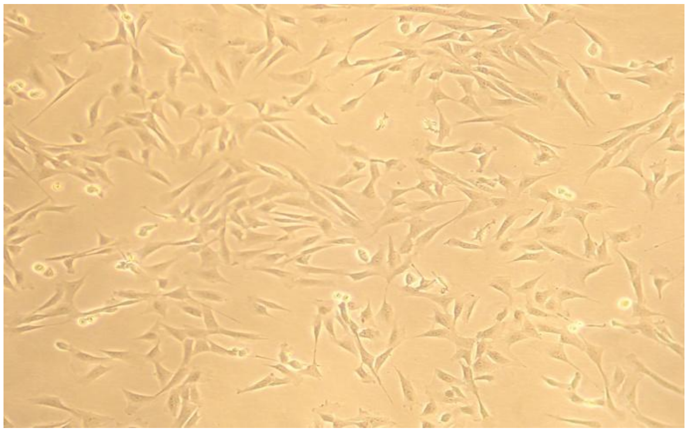

Figure.5 Primary culture showing 30-40 percent confluence at twelveth day x 100;

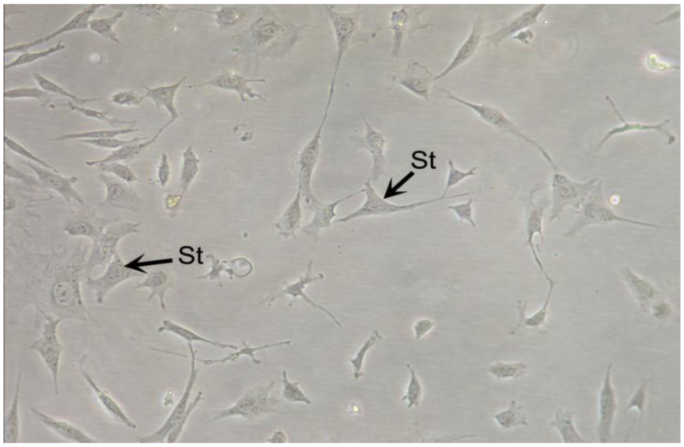

Figure.6 Primary culture showing stellate shaped cells x 200

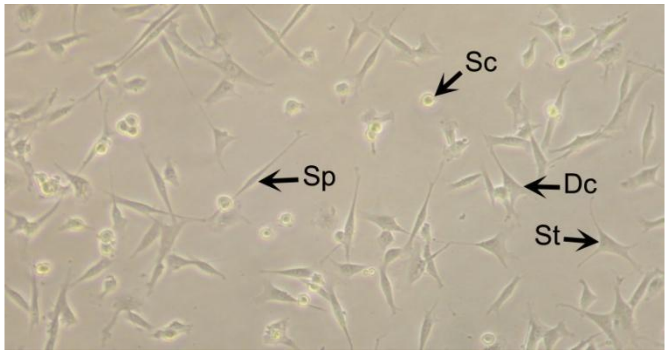

Figure.7 Heterogenous morphology of Ovine BM-MSCs with predominant spindle shaped cells (Sp), Spherical (Sc), Stellate (St) and Dendritic-like (Dc) cells at P1 level x 100;

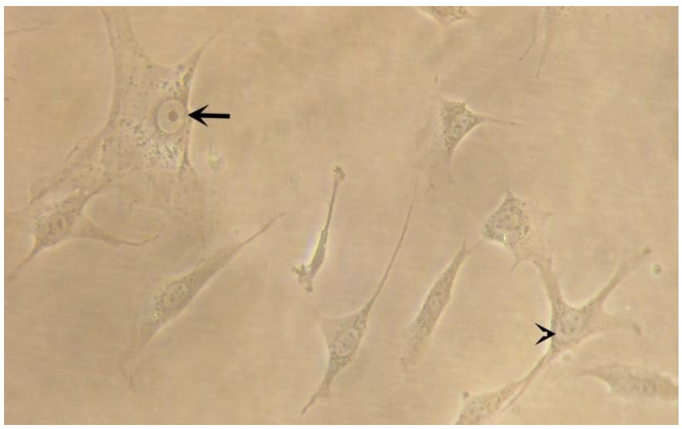

Figure.8 Primary culture of Ovine BM-MSCs showing large, spherical nucleus with single nucleolus (arrow)or multiple nucleoli (arrow head) x 400

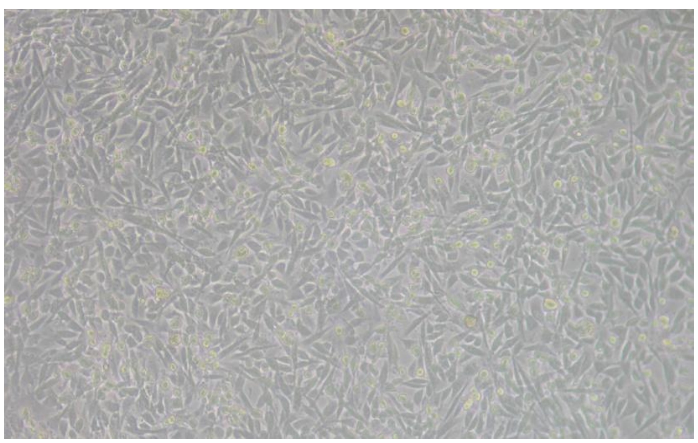

Figure.9 Primary culture showing 70-80 percent confluence after 14 days x 100

DISCUSSION

Mono nuclear cell fraction isolated from femur was $4.81\times10^6 \pm 0.16$/ml of bone marrow suspension which was less as compared to the observed value of those cell fraction in canine bone marrow by Spencer *et al.* (2012) where the yield was $1.6\times10^7 \pm 4.8\times10^6$/ml. However, average cell yield of MNCs was consistent with the observation of who isolated $4\text{-}5\times10^6$/ml of mononuclear cells from femur of rat (Polisetti *et al.*, 2010).

Density gradient separation separates the mono nuclear cell fraction from the red blood cells, granulocytes, platelets and immature precursors in the initial aspirate. As mono nuclear cells have a density of 1.073g/dl, therefore they are commonly isolated in a ring of density gradient centrifugation (1.077g/dl Ficoll and 1.084g/dl Percoll) in equine BM-MSCs (Taylor and Clegg, 2011). Only a small fraction of mononuclear cells is stem cells so, an effective separation procedure for stem cells is always required (Namiri *et al.*, 2011).

Percoll may have better MSC yield and self-renewal, but these differences were seen only in primary cultures of equine BM-MSCs (Bourzac *et al.*, 2010). Seeding density of bone marrow MNCs is one of the important factor to determine the efficiency of MSCs yield as this affects adherence of MSCs, contamination by other cell types and initial growth of adhered MSCs (Ikebe and Suzuki 2014). After initial seeding, the plastic adherence was observed after 4-5 hours which satisfied the first criteria of being mesenchymal stem cells proposed by Dominici *et al. (*2006). Ongoing researches in this field also proved selective nature of plastic adherence of MSCs to culture flask (Rentsch *et al.*, 2010; Fadel *et al.*, 2011; Heidari *et al.*, 2013; AL-Qaisy *et al.*, 2014).

A heterogenous cell population was observed in the primary culture on the day of seeding which were round and bright in appearance in fetal ovine BM-MSCs. Stromal cell isolation did not allow derivation of a pure population of stem cells and therefore cultured bone marrow stromal cells was a mixture of their descendants including progenitors of different types (Rostovskaya and Anastassiadis, 2012) . The heterogenous cell population in the initial culture was due to the presence of erythrocytes, B-lymphocytes, granulocytes, monocytes, adipocytes, very small embryonic- like cells, mesenchymal, haematopoietic and endothelial stem cells as observed by AL-Qaisy *et al.* (2014) in rat BM-MSC culture.

On the third day of post incubation, few cell expansion with 10-20% confluency was observed with predominant fibroblastoid morphology where the same was observed in the first day of culture in case of ovine peripheral blood derived MSCs (Lyahyai *et al.*, 2012) and spindle shaped fibroblast like cells appeared after four days of seeding in case of equine and rat BM-MSCs (Baghaban Eslaminejad *et al.*, 2009 and Zhang *et al.*, 2014) respectively. However, Cortes *et al.* (2013) reported that bovine BM-MSCs attained fibroblast morphology after five to six days of seeding.

Only fibroblast like spindle shaped cells proliferated and formed the colonies representing most highly proliferated cells in the culture as observed by D'Ippolito *et al*. (1999) in human BM-MSCs. Whereas, Chang *et al*. (2009) observed colony forming unit at about one week of culture of human BM-MSCs which became prominent as the incubation time proceeded, whereas in this study, CFU was observed on fourth day of primary culture which later on disappeared as the cells from the CFU migrated to other areas of culture flask on the sixth day. This finding was in accordance with the reports of Baghaban Eslaminejad *et al.* (2009) in equine BM-MSCs.

Various kinds of cell morphology like spindle shaped cells with oval nucleus and flattened cytoplasmic process, rod or elongated cells, spherical cells with abundant cytoplasm, triangular cells and cells with many cytoplasmic processes was observed in the primary culture (P0) of oBM-MSCs. This is in concurrence with the reports of Sung *et al.* (2008) and Cheng *et al.* (2012) who described variable morphological characteristics of BM-MSCs in mice and rat respectively.

In the present study, 40 percent confluency was observed at day 12 of initial seeding which became 70-80 percent confluent on day 14 whereas (Baghaban Eslaminejad *et al.*, 2009) noticed 100 percent confluence on 17-18 days of initial seeding in case of equine BM-MSCs and at this time the culture consisted of a homogenous layer of fibroblastic cells with the appearance of a number of nodule-like cell aggregates which was not evident in this study. According to AL-Qaisy *et al.* (2014), 80 percent confluent monolayer from rat BM-MSC culture was achieved on 5^{th} day of post incubation in case of direct plating whereas the BM-MSCs isolated by density gradient centrifugation showed 50 percent confluency on 9^{th} day of post incubation which corroborated well with the results observed in the present study. After 70-80 percent confluency primary culture (P0) of oBM-MSCs was expanded by passaging in order to inhibit spontaneous differentiation as reported by Taylor and Clegg (2011). Since direct cell to cell contact could induce spontaneous differentiation if they were allowed to become confluent above 80 percent. Bovine BM-MSCs attained confluency after 10-13 days of initial culture which was passaged at a split ratio of 1:3 to 1:4 by using 0.25 percent trypsin and they observed that cells in the first or further passages exhibited uniform monolayer and needed approximately 1 week to reach confluence which was similar to the present study (Bosnakovski *et al.*, 2004).

The initial culture of the isolated BM-MSCs contained a heterogenous cell population with both round and fibroblastic cells, however upon subculture the number of round shaped cells gradually decreased and the growth rate of the fibroblastic cells gradually increased which was also observed by Sung *et al.* (2008) in mouse BM-MSCs. A uniform population of spindle shaped cells predominated from P2 onwards implying the homogeneity of mesenchymal nature.

CONCLUSION

BM-MSCs are an excellent candidate for tissue engineering and cell therapy due to its high proliferation, multilineage differentiation, tissue homing and immunomodulatory properties, hence they are being explored to regenerate damaged tissue instead of just repairing them. Still, little is known about the phenotypic characteristics of MSCs, their developmental origin, their contribution to organogenesis and postnatal tissue homeostasis and their anatomical localization. This study revealed that adult ovine BM-MSCs can be isolated by collecting marrow samples from femur of adult sheep with a higher cell yield. BM-MSCs which reside in the bone marrow compartment, is a plastic adherent, non-hematopietic cell population generating colony-forming unit-fibroblasts (CFU-F) having the ability of plastic adherence, extensive proliferation. *In-vitro* expression of mesenchymal stem cells which are multipotential precursors for diverse tissues forms the present day need in cell based therapies.

Acknowledgement

The authors are thankful to the Indian Council of Agricultural Research, New Delhi for providing Financial support for the study and the Dean, Madras Veterinary College for providing facilities to carry out this work.

REFERENCES

AL-Qaisy, B.A., Yaseen, N.Y., Alwachi, S.N., AL-Shammari, A.M. (2014) Comparison between three different protocols for isolation and culture of mouse bone marrow derived mesenchymal stem cells. *Iraqi J. of Cancer Med. Genet*., 7(1): 26-35.

Baghaban Eslaminejad, M.R., Taghiyar, L., Dehghan, M.M., Falahi, F. and Kazemi, M.H. (2009) Equine marrow-derived mesenchymal stem cells: isolation, differentiation and culture optimization. *Iran J Vet Res*., 10(26): 1-10.

Bhat, G.M., Bhat, M.A. and Kour, K. (2004) Stem Cells: Present Status and Future Prospects. *JIACM*., 5(2): 149-56.

Bosnakovski, D., Mizuno, M., Kim, G., Ishiguro, T., Okumura, M., Iwanaga, T., Kadosawa, T. and Fujinaga, T. (2004) Chondrogenic differentiation of bovine bone marrow mesenchymal stem cells in pellet cultural system. *Exp. Hematol*., 32: 502-509.

Bourzac, C., Smith, L.C., Vincent, P., Beauchamp, G., Lavoie, J.P., Laverty, S. (2010) Isolation of equine bone marrow derived mesenchymal stem cells, a comparison between three protocols. *Equine Vet J.*, 42(6): 519-27.

Chang, Y., Hsieh, P.H. and Chao, C.C.K. (2009) The efficiency of percoll and ficoll density gradient media in the isolation of marrow derived human mesenchymal stem cells with osteogenic potential. *Med J*., 32: 264-75.

Cheng, C.C., Lian, W.S., Hsiao, F.S.H., Liu, I.H., Lin, S.P., Lee, Y.H., Chang, C.C., Xiao, G.Y., Huang, H.Y., Cheng, C.F., Cheng, W.T.K. and Wu, S.C. (2012) Isolation and characterization of novel murine epiphysis derived mesenchymal stem cells. *PLoS ONE*., 7(4): e36085.

Cortes, Y., Ojeda, M., Araya, D., Duenas, F., Fernandez, M.S. and Peraita, O.A. (2013) Isolation and multilineage differentiation of bone marrow mesenchymal stem cells from abattoir-derived bovine foetuses. *BMC Vet. Res*., 9:133.

D'Ippolito, G., Schiller, P.C., Ricordi, C., Roos, B.A. and Howard, G.A. (1999) Age related osteogenic potential of mesenchymal stromal stem cells from human vertebral bone marrow. *J Bone Miner Res*., 14: 115-22.

Dominici, M., Le Blanc, K., Mueller, I., Slaper-Cortenbach, I., Marini, F., Krause, D., Deans, R., Keating, A., Prockop, D. and Horwitz, E. (2006) Minimal criteria for defining multipotent mesenchymal stromal cells The International Society for Cellular Therapy position statement. *Cytotherapy*., 8 : 315–317.

Fadel, L., Viana, B.R., Feitosa, M.L.T., Ercolin, A.C.M., Roballo, K.C.S., Casals, J.B., Pieri, N.C.G., Meirelles, F.V., Martins, D. dos S., Miglino, M.A. and Ambrosio, C.E. (2011) Protocols for obtainment and isolation of two mesenchymal stem cell sources in sheep. *Acta Cir. Bras*., 26(4): 267.

Heidari, B., Shirazi, A., Akhondi, M.M., Hassanpour, H., Behzadi, B., Naderi, M.M., Sarvari, A. and Borjian, S. (2013) Comparison of proliferative and multilineage differentiation potential of sheep mesenchymal stem cells derived from bone marrow, liver and adipose tissue. *Avicenna J Med Biotech*., 5(2): 104-117.

Hung, S.C., Chen, N.J., Hsieh, S.L., Li, H. and Lo, W. (2002) Isolation and Characterization of Size-Sieved Stem Cells from Human Bone Marrow. *Stem Cells*., 20: 249-258.

Ikebe, C. and Suzuki, K. (2014). Mesenchymal Stem Cells for Regenerative Therapy: Optimization of Cell Preparation Protocols. *BioMed Res. Int*. Volume, Article ID 951512, 11 pages.

Lu, L.L., Liu, Y.J., Yang, S.G., Zhao, Q.J., Wang, X. and Gong, W. (2006) Isolation and characterization of human umbilical cord mesenchymal stem cells with hematopoiesis-supportive function and other potentials. *Haematologica*., 91: 1017-26.

Lyahyai, J., Mediano, D.R., Ranera, B., Sanz, A., Remacha, A.R., Bolea, R., Zaragoza, P., Rodellar, C. and Martin-Burriel, I. (2012) Isolation and characterization of ovine mesenchymal stem cells derived from peripheral blood. *BMC Vet. Res*., 8: 169.

Mafi, R., Hindocha, S., Mafi, P., Griffin, M. and Khan, W. S. (2011) Sources of Adult Mesenchymal Stem Cells Applicable for Musculoskeletal Applications - A Systematic Review of the Literature. *Open Orthop J*., 5: 242-248.

Namiri, M., Baharvand, H., Aghdami, N. and Ken, S. (2011) Methods for Isolation of Bone Marrow Stem Cells: Comparative Analysis. *Cell Journal*., 12(4): 439-446.

Polisetti, N., Chaitanya, V.G., Babu, P.P. and Vemuganti, G.K. (2010) Isolation, characterization and differentiation potential of rat bone marrow stromal cells. *Neurol. India*., 58: 201-208.

Ramakrishnan, A., Torok-Storb, B. and Pillai, M.M. (2013) Primary marrow derived stromal cells: isolation and manipulation. *Methods Mol Biol*., **1035**: 75–101.

Rentsch, C., Hess, R., Rentsch, B., Hofmann, A., Manthey, S., Scharnweber, D., Biewener, A. and Zwipp, H. (2010) Ovine bone marrow mesenchymal stem cells: isolation and characterization of the cells and their osteogenic differentiation potential on embroidered and surface-modified polycaprolactone-co-lactide scaffolds. *In vitro Cell.Dev.Biol.- Animal.,* 46: 624–634.

Rostovskaya, M. and Anastassiadis, K. (2012) Differential Expression of Surface Markers in Mouse Bone Marrow Mesenchymal Stromal Cell Subpopulations with Distinct Lineage Commitment. *PLoS ONE*., 7(12): e51221.

Sandhaanam, S.D., Pathalam, G., Dorairaj, S. and Savariar, V. (2013) Mesenchymal stem cells (MSC): Identification, Proliferation and Differentiation – A Review Article. *PeerJ PrePrints*. doi: 10.7287.

Schallmoser, K., Rohde, E., Reinisch, A., Bartmann, C., Thaler, D., Drexler, C., Obenauf, A.C., Lanzer, G., Linkesch, W. and Strunk, D. (2008) Rapid large scale expansion of functional mesenchymal stem cells from unmanipulated bone marrow without animal serum. *Tissue EngC*., 14 (3): 185–196.

Spencer, N.D., Chun, R., Vidal, M.A., Martin, Gimble, J.M. and Lopez, M.J. (2012) In vitro expansion and differentiation of fresh and revitalized adult canine bone marrow-derived and adipose tissue-derived stromal cells. *Vet J*., 191(2): 231–239.

Sung, J.H., Yang, H.M., Park, J.B., Choi, G.S., Joh, J.W., Kwon, C.H., Chun, J.M., Lee, S.K. and Kim, S.J. (2008) Isolation and characterization of Mouse Mesenchymal Stem Cells. *Transplant Proc.*, 40: 2949-2654.

Sung-Min, A., Richard, S. and Bonghee, L. (2010) Genomics and proteomics in stem cell research: the road ahead. *Anat Cell Biol*., 43: 1-14.

Taylor, S.E. and Clegg, P.D. (2011) Collection and propagation methods for mesenchymal stromal cells. *Vet Clin Equine*., 27: 263-274.

Vaananen, H.K. (2005) Mesenchymal stem cells. *Ann. Med*., 37: 469-79.

Zhang, X.Q., Wang, L., Zhao, S.L. and Xu, W. (2014) *In-vitro* cultivation of rat bone marrow mesenchymal stem cells and establishment of Pegfp/Ang-1 transfection method. *Asian Pas J Trop Biomed*., 4(9): 701-706.