B-4752
[1-6]

RESEARCH ARTICLE Indian Journal of Animal Research

In vitro Culture and Morphometry of Porcine Adipose Derived Mesenchymal Stem Cells (pAD-MSCs)

Thokchom Shitarjit Singh[1], O.R. Sathyamoorthy[2], Soundian Eswari[3], Sabiha Hayath Basha[4], M. Parthiban[5]

10.18805/IJAR.B-4752

ABSTRACT

Background: Mesenchymal stem cells are well known for their self-renewal capacity and ability to differentiate into multiple cell lineages. The aim of the study was to develop a simple technique for isolation of mesenchymal stem cells from porcine adipose tissue and to study the morphometric characteristics of porcine mesenchymal stem cells.

Methods: Porcine adipose derived mesenchymal stem cells were isolated *in vitro* by using collagenase type II enzyme. Cell yield and viability of the cells were calculated by using trypan blue exclusion method using Neubauer's chamber. Characterization of MSCs were done by using specific cell markers. The morphological changes, morphometry were analysed in culture using Leishman's stain. The cell doubling (CD) and Population doubling time (PDT) were also calculated.

Result: The isolated adherent cells start forming colony and demonstrated an elongated, round and spindle like fibroblastic morphology by day 1. Almost 80-90 per cent confluency was attained on day 8-9 after the initial seeding and was reduced to day 3-4 in the subsequent passages. RT-PCR reactions revealed positive expression of mesenchymal stem cell markers CD44, CD73 and negative expression of CD34, a hematopoietic cell surface marker. Immunocytochemistry also revealed positive expression for CD44 and negative for CD34. In morphometric studies, the cell length, nucleus length, cell width and nucleus width were increased between 24 and 48 hours in both P2 and P3.

Key words: Immunocytochemistry, Morphometry, MSCs, Porcine adipose tissue, RT-PCR.

INTRODUCTION

Stem cell biology is one of the most vital areas in biomedical research today (Gimble *et al*., 2007). Mesenchymal stem cells (MSCs), also called as mesenchymal stromal cells or stromal stem cells, reside in the stroma of most organs (Wei *et al*., 2013). It offers a great deal of excitement and promise for development of cell-based therapeutic strategies, primarily owing to their intrinsic ability to self-renew and differentiation properties. MSCs are considered as a readily accepted source of stem cells because such cells have already been demonstrated efficacy in multiple types of cellular therapeutic strategies in humans, including applications in treating with hematopoietic recovery (Koc *et al*., 2000), osteogenesis imperfecta (Horwitz *et al*., 2002) and bone tissue regeneration strategies (Petite *et al*., 2000). Adipose tissue, as a stem cell source is available extensively and has several advantages as compared to other sources. It is easily accessible in large quantities with minimal morbidity upon harvest. Adipose derived mesenchymal stem cells (AD-MSCs) shows potential for multiple differentiation (Cowan *et al*., 2004). Culture for AD-MSCs is simple to generate because of their higher intrinsic proliferative rate and maintenance of their phenotypic characteristics (Bunnell *et al*., 2008). MSCs are identified using three characteristics: (1) adherence to the flask bottom (2) characterized the immunoprofile of heterogeneous polyclonal population of undifferentiated cells using different MSC/ADSC markers such as CD44, CD90, CD105 (Dominici *et al*., 2006), CD9, CD10, CD13, CD29, CD54, CD55, CD71, CD91, CD34, CD49d and CD106 (Mauneya *et al*., 2007). Hence, the present experiment is aimed to isolate, characterize and to study the morphology of porcine AD-MSCs under good manufacturing practice (GMP).

[1]Department of Veterinary Anatomy, Madras Veterinary College, Tamil Nadu Veterinary and Animal Sciences University, Chennai-600 007, Tamil Nadu, India.
[2]Department of Veterinary Anatomy, Veterinary College and Research Institute, TANUVAS, Theni-625 602, Tamil Nadu, India.
[3]Centre for Stem Cell Research and Regenerative Medicine, Madras Veterinary College, TANUVAS, Chennai-600 007, Tamil Nadu, India.
[4]Department of Veterinary Anatomy, Veterinary College and Research Institute, TANUVAS, Salem-636 112, Tamil Nadu, India.
[5]Department of Animal Biotechnology, Madras Veterinary College, TANUVAS, Chennai-600 007, Tamil Nadu, India.

Corresponding Author: Soundian Eswari, Centre for Stem Cell Research and Regenerative Medicine, Madras Veterinary College, Tamil Nadu Veterinary and Animal Sciences University, Chennai-600 007, Tamil Nadu, India. Email: drseswari@gmail.com

How to cite this article: Singh, T.S., Sathyamoorthy, O.R., Eswari, S., Basha[4], S.H. and Parthiban, M. (2022). *In vitro* Culture and Morphometry of Porcine Adipose Derived Mesenchymal Stem Cells (pAD-MSCs). Indian Journal of Animal Research. DOI: 10.18805/IJAR.B-4752.

Submitted: 17-08-2021 **Accepted:** 26-11-2021 **Online:** 11-01-2022

MATERIALS AND METHODS

In vitro culture and expansion of pAD-MSCs

Approximately 10 g of subcutaneous fat were harvested from the buccal area from Large White Yorkshire pigs (n=6) between the age of 6-8 months from Department of Livestock Products Technology, Madras Veterinary College, Chennai. Samples were transported on ice in Dulbecco's phosphate-buffered saline (dPBS, Gibco®) containing 200 U/ml of Penicillin and 200 g/ml of Streptomycin and 2.5 g/ml of Fungizone to the GMP facility of Centre for Stem Cell Research and Regenerative Medicine . The tissue was finely minced into fragments of 4-5 mm and washed two to three times with dPBS containing antibiotics and antimycotics (ABAM, Gibco®). Tissue samples were homogenized with collagenase type II powder (Sigma Aldrich, USA, 900 units of collagenase/1.5 ml DMEM/g fat) in incubator at 37°C for 2 hours by shaking the sample at an interval of every 20 minutes.

After digestion the cell suspension was filtered through 100 µm nylon filter system into 50 ml conical centrifuge tubes, centrifuged at 1200 rpm for 10 minutes to collect the pellet of stromal-vascular cells. The supernatant was decanted and 10 ml of dPBS was added into each tube to resuspend the pellet by repeated pipetting and centrifuged at 1200 rpm for 6 minutes twice. The cells were suspended into 5 ml Dulbecco's modified Eagle medium (DMEM, Gibco®) through 70 µm filter system. The cells were counted with a hemocytometer (Neubauer's chamber) with a aliquot 20 µl of cell-containing medium mixed with 20 µl of 0.4% trypan blue solution (1:1 dilution) (Neupane *et al*., 2008). The isolated cells were plated at 1.5×10^6 cells per T_{25} culture flask in DMEM with high glucose supplemented with 10% fetal bovine serum (FBS, Gibco®), 100U/ml of Penicillin and 100 µg/ ml of Streptomycin and 2.5 µg /ml of Fungizone and incubated at 37°C in 5% CO_2.

The culture medium was replaced with fresh medium in every 3-4 days. At 70-90% confluence, adipose derived stem cells were detached, subcultured with 0.25% Trypsin-EDTA solution (Sigma®), re-seeded at an initial concentration of 1.5×10^5 cells per T_{25} culture flask and maintained upto passage 5.

Proliferation assays

The cell doubling (CD) and population doubling time (PDT) were determined with replicate cultures of P2–P3 cells according to standard methods (Rutigliano *et al*., 2006).

$$CD = \ln (Nf/Ni)/\ln (2)$$

$$PDT = CT/CD$$

Where,

CT= Culture time, Nf- Final cell number, Ni- Initial seeding density, In- Natural logarithm.

RNA isolation and reverse transcriptase polymerase chain reaction (RT-PCR)

The messenger RNA (mRNA) from undifferentiated cells were isolated at passage 3 using a Qiagen RNeasy kit (Qiagen, USA) and complementary deoxyribonucleic acid (cDNA) was synthesized using iScript ®(Bio-Rad Laboratories, USA) cDNA Synthesis Kit. PCR was performed with primers for MSC specific marker genes CD 44, CD73, endogenous control β-actin and negative marker CD 34 (Table 1). The PCR program used for the amplification of all genes consisted of the following: a denaturing cycle of 5 min at 95°C, 35 cycles of PCR (95°C for 30 s, 54°C to 58°C for 30 s and 72°C for 60 s) and a step cycle starting at 72°C for 10 min. PCR products were subjected to electrophoresis using a 2% agarose gel, stained with ethidium bromide and illuminated with UV light.

Immunocytochemistry

Immunocytochemistry was carried out to localize the MSC specific marker proteins in the pAD-MSCs using specific primary antibody with its appropriate FITC conjugated secondary antibody. Cells grown in glass chamber slides were fixed with 4% paraformaldehyde for 20 min, washed in DPBS and the cells were then permeabilized with 0.2% Tween 20 and nonspecific binding was blocked with 5% bovine serum albumin by using BSA Blocking buffer for 1 h then washed with DPBS for three times. Then, cultures were incubated with 1:100 dilution of the rat primary antibody for CD44 (ab119335; Abcam) and rabbit primary antibody for CD34 (ab81289; Abcam) and allowed to act for overnight at 4°C. Primary antibody was then removed from wells and plates were washed with DPBS. After washing in DPBS, cell monolayers were incubated with FITC Conjugated Goat Anti-Rat IgG (ab6840; Abcam) and Goat Anti-Rabbit IgG (ab6717; Abcam), 1:1000 dilution for 1 h. Cells were washed in DPBS, stained with 40, 60-diamidino-2-phenylindole (DAPI) 1 µg/ml and mounted with Vectashield mounting medium. Specimens were examined under Inverted Phase Contrast Microscope (Nikon Eclipse T*i*2).

Table 1: Primer sequences for RT-PCR.

Genes	Primer sequences (5′-3′)	Product size (bp)	Ta (C)	Accession no
CD 44	ACATCCTCACATCCAACAC	245	56	NM001206523
	GCCACTGCTCATCTCATC			
CD 73	GAGAACCTGGCTGCTGTGT	411	54	BC114093
	CCGACCTTCAACTGCTGGAT			
CD 34	CCTTAGTTCCAGCGTCTAC	591	54	AB021662
	ACCAGTGCTCTTCCAGAA			
β-Actin	CGCACCACTGGCATTGTCAT	223	59	MN-001009784.3
	TCCAAGGCGACGTAGCAGAG			

Morphometry of pAD-MSCs

The cells were plated at 5 x 10^3 per well in 12 well plate in 20 µl culture medium onto glass coverslips for 60 minutes at 37°C and 5% CO_2 to cellular adherence. After that, 500 µl of culture medium was added on each plate and maintained for 24 h and 48 h (Maciel *et al*., 2014). The culture medium was removed and the coverslips were stained with Leishman's stain and mounted with DPX and observed under Inverted Phase Contrast Microscope (Nikon Eclipse T*i*2).

Fifty cells at P2 and P3 were morphologically evaluated at 200X to measure length and width of the cells and their nuclei. One way ANOVA at 5% level was used between various passage levels. All analysis was done by using SPSS-16. Statistical calculations (mean ± standard error) were recorded according to the standard statistical procedures recommended (Snedecor and Cochran, 1994).

RESULTS AND DISCUSSION

In vitro culture and expansion of pAD-MSCs

In this study, the average number of mononuclear cells was 2.0×10^6/10 g of each fat sample which was successfully established from 6 adult pigs (6-8 month age). The isolated cells were seeded at a density of 1×10^6 cells per T25 culture flask. Out of those mononuclear cells, an estimate of 25-26% adhered to the surface of culture flask and the rest are removed during the medium change after 24h of seeding. However, average mononuclear cells was 2.7×10^6/10g in pig (Williams *et al*., 2008), $5.5 \times 10^4 \pm 3.3 \times 10^4$ from subcutaneous interscapular adipose tissue (Scl-pASCs)/ml and $3.0 \times 10^4 \pm 9.3 \times 10^3$ from buccal fat pad (BFP-pASCs)/ml of raw pig tissue (Niada *et al*., 2013), 2-6 $\times 10^6$ cells per 300 ml of human adipose tissue lipoaspirate (Zuk *et al*., 2001), $2.12 \pm 0.19 \times 10^6$/ ml of ovine subcutaneous fat (Gnanadevi *et al*., 2019). This difference in the number of cells may be due to difference in enzyme used, digestion time and species difference. By day 1, the adherent cells start forming colony and demonstrated an elongated, round and spindle like fibroblastic morphology as in pig (Williams *et al*., 2008; Niada *et al*., 2013 and Liu *et al*., 2016) (Fig 1 and 3). 60-70% confluency was observed on day 4 to 5, cells become large and flatten with enlarge nucleus in the

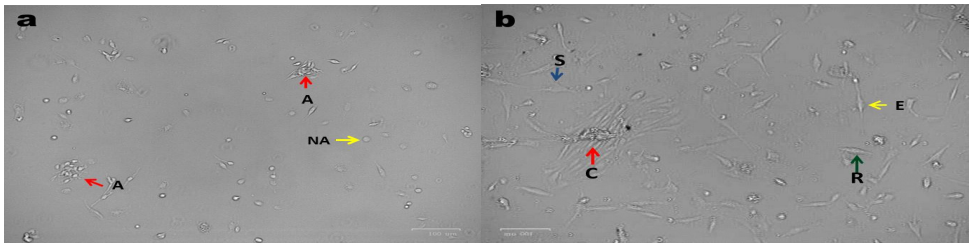

Fig 1: Photomicrograph of pAD-MSCs (a) showing plastic adherence of adhered cells (A) and non-adhered cells (NA) after 24 hour of primary culture (b) showing colony forming unit (C), spindle (S), round (R) and elongated (E) fibroblastic morphology adhered to plastic within 3 day of initial seeding. Scale bar=100 mm.

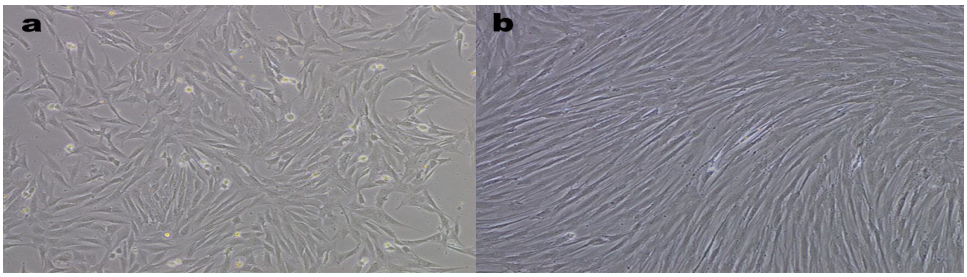

Fig 2: Photomicrograph of pAD-MSCs (a) showing 60-70 percent of confluency after 5 days (b) 90-100 per cent of confluency after 8 days at Passage 0 level (100X).

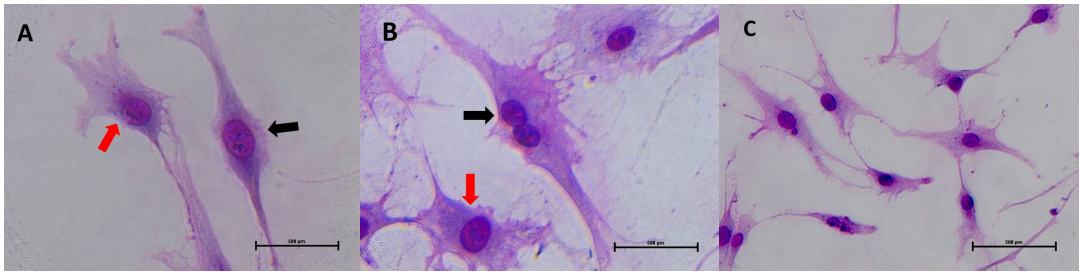

Fig 3: Undifferentiated stromal cell morphology showing (A) elongated shape (black arrow) and spindle shape cell with cytoplasm at one end of the cell (red arrow) (400X) (B) spindle shaped cells with single and two nucleolus (black arrow) and rounded widespread cells with abundant cytoplasm (red arrow) (400x) (C) cells demonstrating different spindle-like fibroblastic morphology adhered to plastic within 24h of Passage 2 (Leishman's stain, 200x).

center of the colony and the colony united together forming a monolayer of pAD-MSCs in the culture flask. Cell culture expanded to 80-90 % confluence by 8-9 days after the initial seeding (Fig 2). This was similar to the observation in pig (Williams *et al*., 2008) and in ovine umbilical cord Wharton's jelly (Eswari *et al*., 2016a and 2016b). However, full confluency was observed on 6th day in ovine (Grzesiak *et al*., 2011).

Cell culture maintenance

The *in vitro* cultured cells after 80-90% confluence were subjected to passage 1 by re-seeding at an initial concentration of 1.5 $\times$ 10^5 cells per T_{25} culture flask. Passage 1 cells showed 80-90 % confluency after 3-4 days and were subjected to passage 2. In this study, pAD-MSCs were subcultered upto passage 5.

Proliferation assays

The proliferation rate of pAD-MSCs was calculated from P1 to P3 culture to access the cell doubling (CD) and population doubling time (PDT). At P1 and P2, cell doubling was found to be same 0.32 ×10^4 at 48 hours. The cell doubling at P3 was higher than P1 and P2 measured about 0.69 ×10^4 at 48 hours. Similarly at 96 h, 144 h and 192 h, the cell doubling of pAD-MSCs increases with increase in time. At P2 and P3 there was a final plateau phase at 96 hours. The population doubling time was found to be similar for P1 and P2 but decreases in P3 at 48 h. At 96 h, 144 h and 192 h, the doubling time of pAD-MSCs decreases with increase in passage level.

Thus when the cell doubling of pAD-MSCs increased, the population doubling time was decreased from P1 to P3. Lesser the population doubling time, greater was the proliferation rate. Hence P3 culture had a higher proliferation rate than P2 and P1. Growth curve was evaluated by counting the harvested cells at the end of each passage level from P1 culture to P3 and found to be increasing in all the passage (Fig 4 and Fig 5). However there were trends for cell doubling time to increase and cell doubling numbers to decrease for both adipose tissue-derived stromal cells and bone marrow-derived stromal cells with increasing cell passages in canine (Spencer *et al*., 2012). These changes in CD and PDT may be due to change with species variation, differences in the culture technique (Kamishina *et al*., 2008) and seeding density (Neuhuber *et al*., 2008) that affect the proliferation rate.

Expression of MSC specific markers by RT-PCR

Gel electrophoresis analysis revealed the presence of both MSC specific markers CD44, CD73 and negative expression of CD34, the hematopoietic surface marker (Fig 6). Previous studies have defined that the undifferentiated human adipose tissue-derived stromal cells demonstrated positive staining for stem cell surface markers CD13, CD29, CD44, CD54, CD55, CD59, CD105, CD106, CD146 and CD166 and an absence of CD14, CD31, CD34 and CD45 expression (Gronthos *et al*., 2001). However, RT-PCR on pig adipose-derived progenitor cells indicated the expression for CD29, CD71, CD73, CD105 and CD166 but didn't express for endothelial marker CD31 (Zhang *et al*., 2016).

Immunocytochemical localization of MSC specific proteins

Immunocytochemistry revealed that mesenchymal stem cells from porcine adipose tissue were positive for the MSC marker CD44 (Fig 7A) and negative for hematopoietic cell marker CD34 (Fig 7B). Similar report was present in goat adipose-derived stem cells (ADSCs) (Ren *et al*., 2012), in human ADSCs (Ghiasi *et al*., 2016) and in pig adipose-derived progenitor cells (Zhang *et al*., 2016). However, it is reported that expression of the MSCs specific markers were

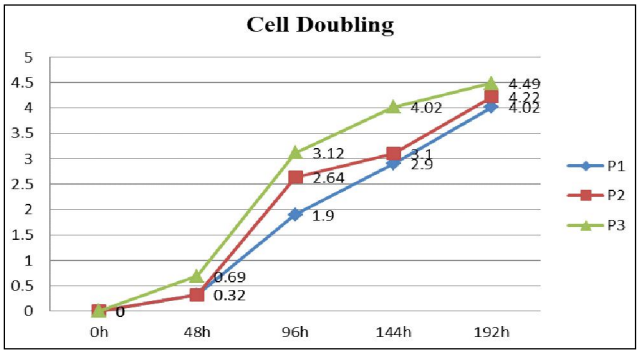

Fig 4: Cell doubling of porcine adipose derived mesenchymal stem cells at different passage (P1-Passage 1, P2-Passage 2 and P3-Passage 3).

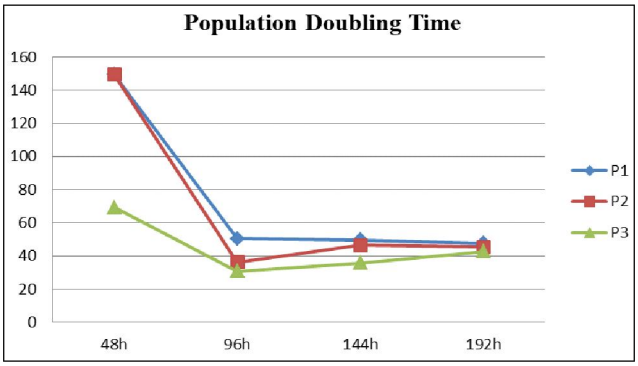

Fig 5: Population doubling time of porcine adipose tissue derived mesenchymal stem cells at different passage (P1-Passage 1, P2-Passage 2 and P3-Passage 3).

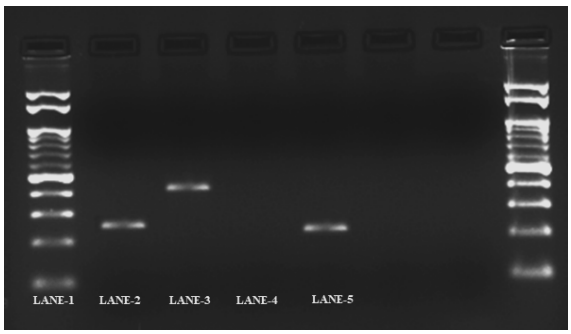

Fig 6: RT PCR analysis of mesenchymal specific marker gene expression in pAD-MSCs. Lane1 - DNA marker (100 bp); Lane 2-CD44 (245 bp); Lane 3 - CD73 (411 bp); Lane 4 – CD34 negative marker; Lane 5 –β actin (223 bp).

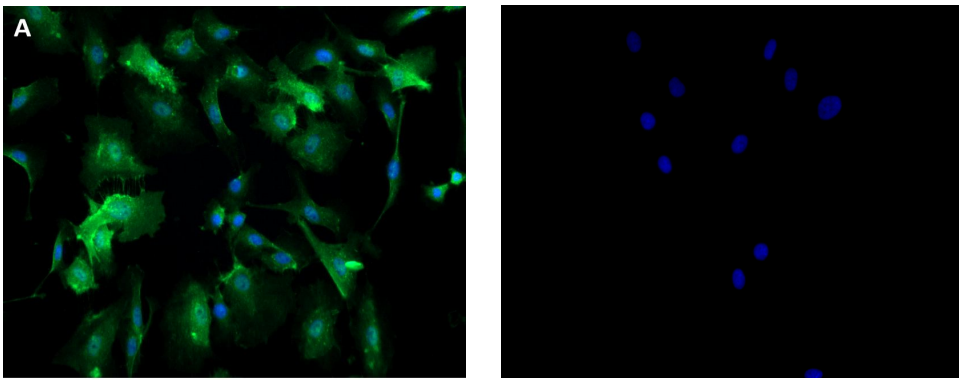

Fig 7: Immunocytochemistry of pAD-MSCs. Photomicrographs show (A) Strong expression for CD44 MSCs specific marker (Green fluorescence) with DAPI (blue fluorescence) the nuclear stain (B) negative expression for CD34 the hematopoietic surface marker with DAPI (200X).

Table 2: Measurements (µm) of pAD-MSCs passage 2 -24 and 48 hours of culture (200x).

Parameter	24 hours	48 hours
Cell length	488.53±20.77	525.08±15.83
Cell width	108.69±5.27	115.70±5.88
Nucleus length	84.01±2.52	88.98±2.08
Nucleus width	58.83±2.46	61.43±1.96

Table 3: Measurements (µm) of pAD-MSCs passage 3 -24 and 48 hours of culture (200x).

Parameter	24 hours	48 hours
Cell length	575.69±22.08	591.42±26.03
Cell width	110.42±3.04	118.80±5.21
Nucleus length	91.53±2.29	92.42±2.42
Nucleus width	61.93±1.79	62.84±2.45

found to differ among passages, adipose tissue-derived mesenchymal stem cells (ATMSCs) at passage 0, expressed higher CD34 and CD45 and lower CD73, CD90 and CD105. So, with the increasing time of ATMSCs in culture, hematopoietic lineage markers (CD34, CD45) were decreased, while expression of CD73, CD90 and CD105 intensified (Yin *et al*., 2014).

Cell morphometry of pAD-MSCs

The results of the statistical inference of the cells with regard to length and width of the pAD-MSCs and nucleus in P1 and P3 were showed in Table 2 and 3, respectively.

From the present study, the morphometric differences were reported for porcine adipose derived mesenchymal stem cells. At passage 2, cell length, nucleus length, cell width and nucleus width increased between 24 and 48 hours. Similarly at passage 3, cell length, nucleus length, cell width and nucleus width increased between 24 and 48 hours. However there was no significant difference noted in both length and width of cell and also length and width of nucleus 24 and 48 hours of culture in passage 2 and 3. Similar finding was reported in human MSCs (Docheva *et al*., 2008) when compared between 24 and 120 hours, in equine (Grzesiak *et al*., 2011) and in feline (Maciel *et al*., 2014) when compared at passage 1 and 3 in 24 and 120 hours.

CONCLUSION

The techniques used in this study can be used to increase the efficiency of isolation and purification of a population of mesenchymal stem cells from easily obtainable adipose tissue. The isolation of a somatic stem cell population from a model animal, such as pig can be used in tissue engineering and applications in regenerative medicine. The availability of such an animal model can be used in determining the true efficiency in comparing the somatic stem cell tissue with embryonic stem cells.

ACKNOWLEDGEMENT

The author acknowledges the Professor and Head, Centre for Stem Cell Research and Regenerative Medicine (CSCR and RM) for permitting to utilize the GMP and other facilities available in CSCR and RM, Madras Veterinary College, Chennai to carry out this work.

REFERENCES

Bunnell, B.A., Flaat, M., Gagliardi, C., Patel B and Ripoll, C. (2008). Adipose-derived stem cells: Isolation, expansion and differentiation. Science Direct Methods. 45: 115-120.

Cowan, C.M., Shi, Y.Y., Aalami, O.O., Chou, Y.F., Mari, C., Thomas, R., Quarto, N., Contag, C.H., Wu, B and Longaker, M.T. (2004). Adipose-derived adult stromal cells heal critical-size mouse calvarial defects. Nat. Biotechnol. 22: 560.

Docheva, D., Padula, D., Popov, C., Mutschler, W., Clausen-Schaumann, H. and Schieker, M.J. (2008). Researching into the cellular shape, volume and elasticity of mesenchymal stem cells, osteoblasts and osteosarcoma cells by atomic force microscopy. Journal of Cellular and Molecular Medicine. 12(2): 537-552.

Dominici, M., Le Blanc, K., Mueller, I., Slaper-Cortenbach, I., Marini, F., *et al.* (2006). Minimal criteria for defining multipotent mesenchymal stromal cells. The International Society for Cellular Therapy Position Statement. Cytotherapy. 8 (4): 315-317.

Eswari, S., Monisha, M., Vijayarani, K. and Gomathy, V.S. (2016a). Isolation of ovine multipotent mesenchymal stem cells from umbilical cord tissue Wharton's jelly. Indian Vet. J. 93(12): 27-29.

Eswari, S., Monisha, M., Vijayarani, K and Kumanan, K. (2016b). Expression of early transcription factors by mesenchymal stem cells derived from ovine umbilical cord Wharton's jelly. Ind. J. Anim. Sci. 86(10): 1132-1135.

Ghiasi, M., Naser, K., Reza, T.Q. and Mohsen, S. (2016). The effects of synthetic and natural scaffolds on viability and proliferation of adipose-derived stem cells. Frontiers in Life Science. 9(1): 32-43.

Gimble, J.M., Katz, A.J and Bunnell, B.A. (2007). Adipose-derived stem cells for regenerative medicine. Circ. Res. 100: 1249-1260.

Gnanadevi, R., Kannan, T.A., Geetha, R. and Sabiha, H.B. (2019). Per cent yield of ovine mesenchymal stem cells (ADMSCs) from different sources of adipose tissue. Int. J. Curr. Microbiol. App. Sci. 8(8): 2620-2624.

Gronthos, S., Franklin, D.M., Leddy, H.A., Robey, P.G., Storms, R.W. and Gimble, J.M. (2001). Surface protein characterization of human adipose tissue-derived stromal cells. J. Cell. Physiol. 189: 54-63.

Grzesiak, J., Marycz, K., Czogala, J., Wrzeszcz, K and Nicpon, J. (2011). Comparison of behavior, morphology and morphometry of equine and canine adipose derived mesenchymal stem cells in culture. International Journal Morphology. 29(3): 1012-1017.

Grzesiak, J., Marycz, K., Wrzeszcz, K. and Czogala, J. (2011). Isolation and morphological characterization of ovine adipose-derived mesenchymal stem cells in culture. Int. J. Stem Cells. 4(2): 99-104.

Horwitz, E.M., Gordon, P.L., Koo, W.K., Marx, J.C., Neel, M.D., *et al.* (2002). Isolated allogeneic bone marrow-derived mesenchymal cells engraft and stimulate growth in children with osteogenesis imperfecta: Implications for cell therapy of bone. Proc. Natl. Acad. Sci. USA. 99: 8932-8937.

Kamishina, H., Farese, J.P., Storm, J.A., Cheeseman, J.A. and Clemmons, R.M. (2008). The frequency, growth kinetics and osteogenic/adipogenic differentiation properties of canine bone marrow stromal cells. In Vitro Cellular and Developmental Biology Animal. 44: 472-479.

Koc, O.N., Gerson, S.L., Cooper, B.W., Dyhouse, S.M., Haynesworth, S.E., Caplan, A.I. and Lazarus, H.M. (2000). Rapid hematopoietic recovery after coinfusion of autologous blood stem cells and culture-expanded marrow mesenchymal stem cells in advanced breast cancer patients receiving high-dose chemotherapy. J. Clin. Oncol. 18: 307-316.

Liu, Y., Ma, W., Liu, B., Wang, Y., Chu, J., Xiong, G., *et al.* (2016). Urethral reconstruction with autologous urine-derived stem cells seeded in three dimensional porous small intestinal submucosa in a rabbit model. Stem Cell Research and Therapy. 8(63): 1-14.

Maciel, B.B., Rebelatto, C.L.K., Brofman, P.R.S., Brito, H.F.V., Patricio, L.F.L., Cruz, M.A and Dittrich, R.L. (2014). Morphology and morphometry of feline bone marrow-derived mesenchymal stem cells in culture. Pesquisa Veterinaria Brasileria. 34(11): 1127-1134.

Mauneya, J.R., Nguyena, Trang, Gillena, Kelly, Kirker-Headb, Carl, Gimblec, Jeffrey, M., Kaplana and David, L. (2007). Engineering adipose-like tissue *in vitro* and *in vivo* utilizing human bone marrow and adipose-derived mesenchymal stem cells with silk fibroin 3D scaffolds. Biomaterials. 28: 5280-5290.

Neuhuber, B., Swanger, S.A., Howard, L., Mackay, A. and Fischer, I. (2008). Effects of plating density and culture time on bone marrow stromal cell characteristics. Experimental Hematology. 36: 1176-1185.

Neupane, M., Chang, C., Kiupe, M. and Burkan, V.Y. (2008). Isolation and caracterization of canine adipose-derived mesenchymal stem cells. Tissue Engineering. 14:1007 -1015.

Niada, S., Ferreira, L.M., Arrigoni, E., Addis, A., Campagnol, M., Broccaioli, E. and Brini, A.T. (2013). Porcine adipose-derived stem cells from buccal fat pad and subcutaneous adipose tissue for future preclinical studies in oral surgery. Stem Cell Research and Therapy. 4: 148.

Petite, H., Viateau, V., V. Bensaid, V., Meunier, A., Pollak, C., *et al.* (2000). Tissue-engineered bone regeneration. Nat. Biotechnol. 18: 959-963.

Ren. Y., Wua, H., Zhou, X., Wena, J., Jin, M., Cang, M., Guo, X., Wang, Q., Liu, D. and Mab, Y. (2012). Isolation, expansion and differentiation of goat adipose-derived stem cells. Res. Vet. Sci. 93: 404-411.

Rutigliano, L., Corradetti, B., Valentini, L., Bizzaro, D., Meucci, A., Cremonesi, F. and Lange-Consiglio, A. (2013). Molecular characterization and *in vitro* differentiation of feline progenitor like amniotic epithelial cells. Stem cell research and therapy. 4: 1-13.

Snedecor, G.W. and Cochran, W.G. (1994). Statistical Methods, 8th Edn. Lowa State University Press, pp. 313.

Spencer, N.D., Chuna, R., Vidala, M.A., Gimbleb, J.M. and Lopeza, 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.

Wei, X., Yang, X., Han, Z.P., Qu, F.F., Shao, L. and Shi, Y.F. (2013). Mesenchymal stem cells: A new trend for cell therapy. Acta Pharmacologica Sinica. 34(6): 747-754.

Williams, K.J., Picou, A.A., Kish, S.L., Giraldo, A.M., Godke, R.A. and Bondioli, K.R. (2008). Isolation and Characterization of Porcine Adipose Tissue-Derived Adult Stem Cells. Cells Tissues Organs. 188: 251-258.

Yin, L., Zhu, Y., Yang, J., Ni, Y., Zhou, Z., Chen, Y. and Wen, L. (2014). Adipose tissue-derived mesenchymal stem cells differentiated into hepatocyte-like cells *in vivo* and *in vitro*. Molecular Medicine Reports. 11: 1722-1732.

Zhang, S., Zheng, C.B., Gao, Y., Fan, Y., Li, L., Guan, W. and Ma, Y. (2016). Identification and characterization of pig adipose-derived progenitor cells. The Canadian Journal of Veterinary Research. 80: 309-317.

Zuk, P.A., Zhu, M., Mizuno, H., Huang, J., Futrell, J.W., Katz, A.J., Benhaim, P., Lorenz, H.P. and Hedrick, M.H. (2001). Multilineage cells from human adipose tissue: implications for cell based therapies. Tissue Eng. 7: 211-228.