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Isolation and culture of porcine mesenchymal stem cells from adipose tissue

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Abstract
Adipose tissue derived mesenchymal stem cells are of considerable interest because of its easily accessible and can generate a huge proliferation of cells with minimal morbidity upon harvest. In the present study, adipose tissue samples from porcine were isolated using 0.1% collagenase type I enzyme. Cell yield was an average of 1.8 x10^6 cells/10g of adipose tissue. Most of the adhered cells demonstrated an elongated, spindle-like fibroblastic morphology. Acridine orange/Ethidium bromide dual fluorescent staining showed no significant difference in the different passages but with increasing in the passage level, the number of viable cells decreased but increase in the number of necrotic cells.

Keywords: Adipose tissue, Porcine, Acridine orange, ethidium bromide

1. Introduction
Stem cells are the undifferentiated, primitive cells that retain the capability of indefinitely reproducing themselves ("self-renewal") and also have the ability to generate multiple types of cells upon proper signals from internal and external cues ("pluripotency" or "multipotency") (Ahn *et al*., 2010) [1]. Adipose tissue as a stem cell source is ubiquitously available and has several advantages as compared to other sources. It is easily accessible in large quantities with minimal morbidity upon harvest, yields higher intrinsic proliferative rate and maintenance of their phenotypic characteristics (Bunnell *et al*., 2008) [6]. It has the potential for self-renewal and proliferation with low immunogenicity and significant anti-inflammatory properties (Wang and Sun, 2017) [19]. Adipose derived mesenchymal stem cells (AD-MSCs) have the following properties like anti-apoptotic, proangiogenic, anti-inflammatory, immunomodulatory and anti-scarring effects that makes these cells promising candidate for cellular therapy in regenerative medicine (Bertolini *et al*., 2012) [5]. It had a great deal of interest in clinical situations and have been utilized to treat defective fracture healing, osteoarthritis (Guercio *et al*., 2012) [9], myocardial repair (Miyahara *et al*., 2006) [14], and in neurodegenerative disease or traumatic events such as stroke or spinal cord injury (Joyce *et al*., 2010) [10].

2. Materials Methods
2.1 Collection of porcine adipose tissue
The porcine buccal subcutaneous adipose tissue was collected from Large White Yorkshire pigs (n=6) between the age of 6-8 months from Post- Graduate Research Institute in Animal Sciences, Kattupakkam. Samples were transported on ice in Dulbecco's phosphate-buffered saline with Antibiotic.

2.2 Establishment of Primary Cultures
Approximately 10 gram of adipose tissue was rinsed with Dulbecco's phosphate-buffered saline (dPBS) with antibiotic to remove blood, small vessels and connective tissue. The tissue was finely minced by using sterile forceps and surgical blade until mush is obtained. The minced tissue was incubated, then an equal volume of 0.1% collagenase type I (240 IU/ml; Gibco) in dPBS in a shaker for 2 hours, diluted with 1:1 in DMEM and 10% fetal bovine serum (FBS). The cell suspension was filtered through 100 and 40µm filters. Adipocytes were separated from the stromal-vascular fraction by centrifugation at 1,200 rpm for 7 min. The supernatant was decanted and added 10 ml of dPBS to resuspend the pellet by repeated pipetting, and centrifuged at 1200 rpm for 7 minutes twice.

Cell yield was determined by 0.4 per cent trypan blue dye exclusion test. 20µl of cell suspension was added to 20µl of trypan blue dye and mixed well. From that mixture 20µl of cell suspension was loaded in Neubauer's haemocytometer and checked for viability of cells in light microscope (Neupane *et al*., 2008) [15].

$$\text{Cell yield} = \frac{\text{Average cell count}}{10^{-4}} \times \text{Dilution factor}$$

The isolated cells were plated at a density of 1.5x 10^6 cells per T_{25} culture flask in DMEM with high glucose supplemented with 10% fetal bovine serum (FBS, Gibco®), 1% antibiotic and incubated at 37 ° C in 5% CO_2. The spend medium was replaced with fresh medium in every 3–4 days.

2.3 Subculture of Cells

At 70–90% confluence, the medium was removed from the flask and washed with dPBS. Appropriate volume of 0.25% trypsin was added and incubated for 5 min at 37° C to detach the cells. Complete detachment was checked by gently tapping the side of the flask and observed under the microscope. Inactivated trypsin by adding equal volume of pre-warmed basal medium. Cell pellet was prepared by centrifugation at 1,500 rpm for 15 min. Then the pellet was reconstituted with 1 ml basal medium and seeding density was maintained after counting the cells. The cells passaged were maintained upto passage 4.

2.4 Acridine orange (AO)/Ethidium bromide (EB) Fluorescent Staining

Acridine orange is taken both by viable and nonviable cells, emits green fluorescence if intercalated into double stranded nucleic acid (DNA) or red fluorescence if bound to single stranded nucleic acid (RNA). Ethidium bromide is taken up only by the nonviable cells and emits red fluorescence. We distinguished three different types of cells based on fluorescence emission and the morphological aspect of chromatin condensation in the stained nuclei i.e. viable cells (VC), late apoptotic (LA) and necrotic cells (NC).

One microlitre of dye mixture (100 mg/ml AO and 100 mg/ml EB in distilled water) was mixed with 25µl of cell suspension (2 x 10^4 cells/ml) on a clean microscope slide and then covered with a coverslip. The suspension was immediately examined by fluorescence microscopy at 400X magnification using Inverted Phase Contrast Microscope (Nikon Eclipse T*i*2). A minimum of 300 cells were counted in every sample (Kuan *et al*., 2015) [11]

3. Results and Discussion

3.1 Source of porcine adipose tissue derived mesenchymal stem cells

In this study, the sterile buccal subcutaneous adipose tissue was collected from the healthy animals in line with the procedure followed by (Chen *et al.*, 2016). The porcine adipose tissue samples were finely minced and subjected to 0.1% collagenase type I in dPBS for isolation of stromal vascular fraction (SVF) as per (Williams *et al.*, 2008; Niada *et al*., 2013) [20, 16] (Fig. 1, 2 and 3). However, there have been report that SVF was obtained by using 0.75% collagenase type I (Marappagoundar *et al.*, 2010) [13] in Human, 900 units of collagenase type II /1.5 ml DMEM/g fat (Liu *et al.*, 2016) [12] and 0.2% collagenase type I (Almalki and Agrawal, 2017) [2] in porcine. The difference in the concentration and type of collagenase used varies between the species (Barberini *et al.*, 2016). Viability of the isolated cells was checked by Trypan blue exclusion test (Neupane *et al.,* 2008) [15].

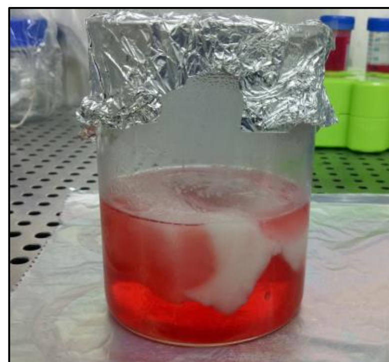


Fig 1: Photograph showing the collection of porcine subcutaneous adipose tissue.

Fig 2: Photograph showing the mincing of porcine subcutaneous adipose tissue.

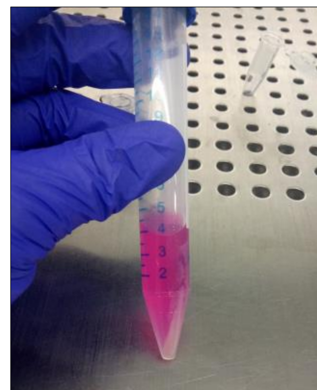


Fig 3: Photograph showing the cell pellet after enzymatic digestion.

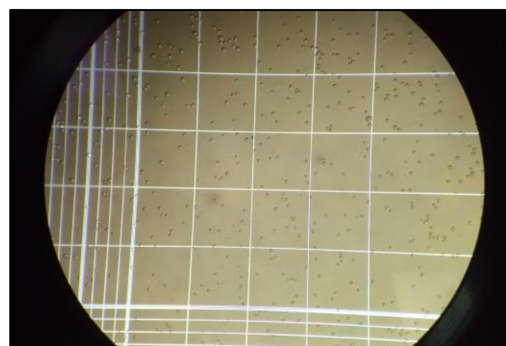


Fig 4: Photograph showing the viability of the isolated cells by Trypan blue exclusion test (100X).

3.2 Seeding of pAD-MSCs

In the present study, the average number of isolated cells was

1.8 x10^6 cells/10g of porcine subcutaneous adipose tissue. However, an average of 2–6 x 10^6 cells per 300 ml of processed lipoaspirate in human (Zuk *et al*., 2001) [21], 2.7 x 10^6 /10 g from 6-8 months porcine adipose tissue (Williams *et al*., 2008) [20], 7.7 × 10^5 cells/fat pad from canine (Spencer *et al*., 2012) and 2 x 10^8 cell were harvested from 60 g obtained from the 7 to 9 days old dorsal fat depot (Liu *et al*., 2016) [12]. The viable pAD-MSCs were seeded at the density of 1×10^6 cells per T_{25} culture flask in DMEM with 10 per cent FBS at 37 ºC with 5 per cent CO_2. The cells are left undisturbed for 24 hours. An estimate of 25% adhered to the surface of culture flask and the rest are removed during medium change after 24 hours of seeding as in pig (Williams *et al*., 2008) [20]. In the present study, adherent cells demonstrated fibroblastic morphology and 90-100 per cent confluency was observed on 8-9 day (Fig. 5). Similar finding was reported in pig by Williams *et al*. (2008) [20]. However, 100 per cent confluency by 7 days of post incubation in feline adipose tissue (Shazia *et al*., 2018) [17].

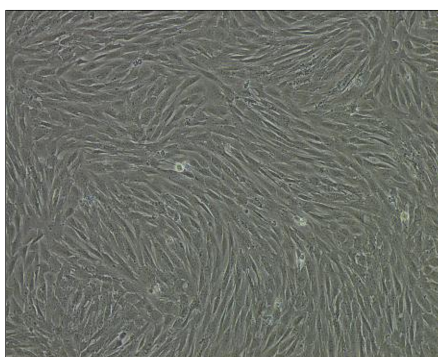


Fig 5: Photomicrograph of pAD-MSCs (a) showing 90-100 percent of confluency after 8 days at Passage 0 level (100X).

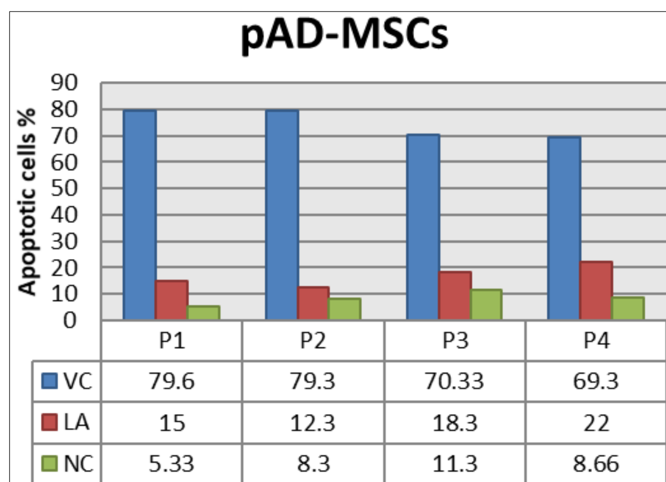
3.3 Subculture of pAD-MSCs

In the present study**,** the cultured cells from P0 on day 8 post incubation with 80-90 per cent confluency were subjected to passage1. The cells were seeded into the new flask at 1:3 ratio. The cells got adhered by day one and start showing expansion with spindle shape morphology. On third day of post incubation, the cells exhibited varied morphology such as elongated, stellate, triangular and polyhedral (Fig. 6). On day five post incubation, the cells attained about 70-80 per cent confluency at passage 1 (P1) and cells were subcultured upto passage 4. However, Madras Red sheep adipose tissue derived mesenchymal stem cell took six days to reach 60-70% cofluency (Beaulah *et al*., 2016) [4] and took five days to reach same percentage to P1 in feline adipose tissue (Shazia *et al*., 2018) [17].

Fig 6: Photomicrograph of pAD-MSCs showing elongated (E), stellate (S), triangular (T) and polyhedral (P) shape morphology at P1/Day 3 (100X)

3.4 Acridine orange/Ethidium bromide Dual Fluorescent Staining

In the present study, dual stained cells were examined under a fluorescent microscope. There was no significant difference detected in the different passages (Fig. 7). Viable cells have uniform bright green nuclei with organized structure, late apoptotic cells have orange to red nuclei with condensed or fragmented chromatin and necrotic cells have uneven orange to red nuclei with organized structure (Fig. 8). With increasing passage level the number of viable cells decreased but increase in the number of necrotic cells. This is similar with (Kuan *et al*., 2015) [11] in human osteosarcoma cells in which increasing the concentrations and treatment lengths, the number of early-stage apoptotic cells and necrotic cells increased. Fluorescent staining using AO alone has been used in the past. However, AO penetrated only normal and early apoptotic cells with intact membranes, giving green colour fluorescent when bound to DNA. Detection of cell apoptosis using AO/EB is relatively a new approach. So, comparison to AO staining, the AO/EB method improves the detection of apoptosis and can distinguish between late apoptotic and dead cells.



	P1	P2	P3	P4
VC	79.6	79.3	70.33	69.3
LA	15	12.3	18.3	22
NC	5.33	8.3	11.3	8.66

Fig 7: Different subsets of apoptotic cells as percentages of all cells measured by AO/EB fluorescence staining in different passages (P1-Passage 1, P2-Passage 2, P3-Passage 3 and P4-Passage 4 in percentage of viable cells (VC), late apoptotic (LA) and necrotic cells (NC).

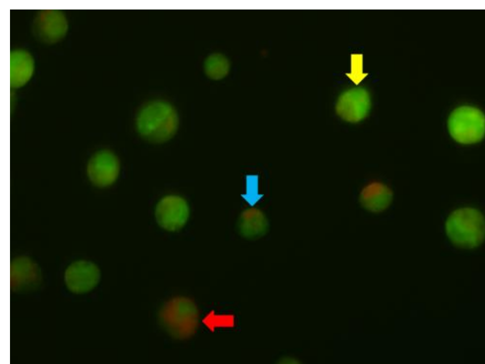


Fig 8: Fluorescent staining showing viable cells (Yellow arrow), late apoptotic (Blue arrow) and necrotic cells (Red arrow) in passage 1 porcine adipose tissue derived mesenchymal stem cells (Acridine orange/Ethidium bromide stain x400)

4. Conclusions

The techniques used in this study can be used to isolate MSCs from porcine adipose tissue. It also suggested that adipose

tissue derived mesenchymal stem cells can be used as a useful tool for donor cells because of ease of adipose tissue recovery, simplicity and rapid growth rate.

5. Acknowledgement

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6. References

1. Ahn SM, Simpson R, Lee B. Genomics and proteomics in stem cell research: the road ahead. Anatomy & cell biology 2010;43(1):1-14.
2. Almalki SG, Agrawal DK. ERK signaling is required for VEGF-A/VEGFR2-induced differentiation of porcine adipose-derived mesenchymal stem cells into endothelial cells. Stem Cell Research & Therapy 2017;8(113):1-14.
3. Barberini JD, Natália PPF, Mariana SM, Leandro M, Amanda JL, Marta CH *et al*. Equine mesenchymal stem cells from bone marrow, adipose tissue and umbilical cord: immunophenotypic characterization and differentiation potential. Stem Cell Research & Therapy 2014;5:1-11.
4. Beaulah JV, Ushakumary S, Kannan TA, Justin William B, Geetha R. Isolation and Characterization of Adipose derived Mesenchymal Stem cells (ADMSCs) in Madras Red Sheep (*Ovis aries*). Adv. Biomed. Pharma 2016;3(3):156-160.
5. Bertolini F, Lohsiriwat V, Petit JY, Kolonin MG. Adipose tissue cells, lipotransfer and cancer: A challenge for scientists, oncologists and surgeons. Biochim. Biophys. Acta 2012;18(26):209-214.
6. Bunnell BA, Flaat M, Gagliardi C, Patel B, Ripoll C. Adipose-derived stem cells: Isolation, expansion and differentiation. Science direct Methods 2008;45:115-120.
7. Chen YJ, Liu HY, Chang YT, Cheng YH, Mersmann HJ, Kuo WH *et al*. Isolation and Differentiation of Adipose-Derived Stem Cells from Porcine Subcutaneous Adipose Tissues. J. Vis. Exp 2016;109:1-10.
8. Dejan B, Suzana P, Petar R, Nebojs NA. Analysis of cycloheximide-induced apoptosis in human leukocytes: Fluorescence microscopy using annexin V/propidium iodide versus acridin orange/ethidium bromide. Cell Bi. Int 2006;30:924-932.
9. Guercio A, Marco PD, Casella S, Cannella V, Russotto L, Purpari G *et al*. Production of canine mesenchymal stem cells from adipose tissue and their application in dogs with chronic osteoarthritis of the humeroradial joints. Cell Biol. Int. 2012;36:189-194.
10. Joyce N, Annett G, Whlin L, Olson S, Bauer G, Nolta JA. Mesenchymal stem cells for the treatment of neurodegenerative disease. Regen Med 2010;5(6):933-946.
11. Kuan L, Peng-cheng L, Run L, Xing W. Dual AO/EB Staining to Detect Apoptosis in Osteosarcoma Cells Compared with Flow Cytometry. Med. Sci. Monit. Basic Res 2015;21:15-20
12. Liu Y, Ma W, Liu B, Wang Y, Chu J, Xiong G *et al*. 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 2016;8(63):1-14.
13. Marappagoundar D, Indumathi S, Rajkumar S, Pachaiyappan V, Sangeeth Kumar G, Vidya V, *et al*. Characterization of human adipose tissue derived hematopoietic stem cell, mesenchymal stem cell and side population cells. Int. J Biol 2010;2:71-78.
14. Miyahara Y, Nagaya N, Kataoka M, Yanagawa B, Tanaka K, Hao H *et al*. Monolayered mesenchymal stem cells repair scarred myocardium after myocardial infraction. Nat. Med 2006;12(4):459-465.
15. Neupane M, Chang CC, Kiupe M, Burkan VY. Isolation and Characterization of canine adipose–derived mesenchymal stem cells. Tissue Engineering 2008;14:1007-1015.
16. Niada S, Ferreira LM, Arrigoni E, Addis A, Campagnol M, Broccaioli E *et al*. Porcine adipose-derived stem cells from buccal fat pad and subcutaneous adipose tissue for future preclinical studies in oral surgery. Stem Cell Research & Therapy 2013;4:148.
17. Shazia N, Geetha R, Sabiha HB, Balachandran C, Leela V. Isolation of Feline Adipose Tissue Derived Mesenchymal Stem Cells. Int. J Curr. Microbiol. App. Sci 2018;7(3):553-557.
18. Spencer ND, Chuna R, Vidala MA, Gimbleb JM, Lopeza MJ. *In vitro* expansion and differentiation of fresh and revitalized adult canine bone marrow-derived and adipose tissue-derived stromal cells. Vet. J 2012;191(2):231-239.
19. Wang Z, Sun D. Adipose-derived mesenchymal stem cells: a new tool for the treatment of renal fibrosis. Stem Cells and Development 2017. DOI: 10.1089/scd.2017.0304.
20. Williams KJ, Picou AA, Kish SL, Giraldo AM, Godke RA, Bondioli KR. Isolation and Characterization of Porcine Adipose Tissue-Derived Adult Stem Cells. Cells Tissues Organs 2008;188:251-258.
21. Zuk PA, Zhu M, Mizuno H, Huang J, Futrell JW, Katz AJ *et al*. Multilineage cells from human adipose tissue: implications for cell-based therapies. Tissue Eng 2001;7:211-228.