ISSN NO. 2320-5407

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

INTERNATIONAL JOURNAL OF ADVANCED RESEARCH

RESEARCH ARTICLE

ISOLATION, CULTURE AND CHARACTERIZATION OF HUMAN OMENTUM ADIPOSE TISSUE DERIVED STEM CELLS

Dr.V.Pushpa Rani & J.Jones Jemima
P.G. & Research Department of Advanced Zoology & Biotechnology,
Loyola College, Chennai-600 034. Tamil Nadu, India.

Manuscript Info

Manuscript History:

Received: 11 January 2014
Final Accepted: 26 February 2014
Published Online: March 2014

Key words:
Omentum Fat, Flow cytometry, PBS, Ficoll, Hemocytometer, Surface Markers

Corresponding Author

Dr.V.Pushpa Rani

Abstract

The growth, characterization, expression, expansion and plasticity of adipose derived stem cells in relation to the markers used at various passages of omentum fat sample of human. Assess the proliferation potential and viability of adipose derived stem cells in the culture medium. Analyze the proliferation potential of adipose derived stem cells and side population cells under laboratory conditions. Determine the efficacy of cell adhesion molecules and their relevance in a differentiation towards adipose derived stem cells. Specify the mediation of cell migration and expression profile for cell adhesion using receptors. Determine the differentiation potential of adipose derived stem cells in terms of antigenic expression of CD34+, CD45+, CD133+, CD31+, HLA-DR+, CD29+, CD90+, CD105+ in different cell cycles / passages P_1, P_3 and $_{P5.}$ Assess the growth rate of adipose derived stem cells..

.

INTRODUCTION

Stem cells are undifferentiated cells that are able to differentiate into specialized cell types. Both types are generally characterized by their potency, or potential to differentiate into different cell types (such as skin, muscle, bone, etc.).Adipose tissue is specialized connective tissue that functions as major storage site for fat in the form of triglycerides. Adipose tissue plays a central role in energy homeostasis and was found to be a highly active metabolic organ (**Rosen ED *et al*, 2007**).Adipose tissue is found in mammals in two different forms, white and brown adipose tissue. Adipose tissue also serves as important endocrine organ by producing hormones such as leptin, resistin and cytokine. The information of adipose tissue appears to be controlled by the adipose gene. Adipose tissue is located beneath the skin (subcutaneous fat), around internal organ (visceral fat) and in bone marrow. Adipose tissue is found in specific location which is referred as adipose depots. Adipocytes comprise roughly two third of cells found in adipose tissue and remaining cell population collectively referred to stromal vascular fraction which includes smooth muscles cells, mast cells, nerve cells, endothelial cells and other more (**Hemmrich K *et al*., 2007)**

Adipose tissue contains several cell types, in which adipocytes is the highest percentage of cells being occupied which also contain fat droplets, adipocytes which also contain fibroblast, macrophages and endothelial cells to originate from fibroblast like precursor cells that differentiate into adipocytes under the appropriate stimulatory conditions. The precursor cells do not possess any morphological or enzymatic marker that can be used to determine whether they will become adipocytes. Adipose tissue cell populations represented in tissue also comprise mesenchymal and hematopoietic population **(Windsor *et al*., 2009).** The criteria used to identify adipocytes depend upon lipid accumulation within the cell after proliferation has stopped, making early

identification of adipocytes difficult. An increase in adipose tissue masses can occur by hyperplastic growth, which is an increase in the number of adipocytes. This increase in number occurs primarily by mitotic activity in precursor cells.

The omentum easily stores fat, since it is readily accessible to the body. When people lose weight, the omentum shrinks, helping to reduce risks for a number of conditions. Dr. Oz contends that the great concern with a fatty omentum is that it starts inflammatory processes, which can lead to diabetes, high blood pressure and hardening of the arteries. Essentially the bigger the omentum, the more you are at risk for a variety of difficult illnesses. The omentum also receives and stores hormones like cortisol, called a stress hormone. High stress can stimulate its growth. People who are under a great deal of stress may find that reducing the size of this organ is very difficult, and they are often advised not simply to diet, but also to reduce stress through a variety of therapies, relaxation techniques and lifestyle changes. Stress and tummy fat are inexorably connected.

MATERIALS AND METHODS

SURGERY AND COLLECTION OF OMENTUM FAT:

Abdominoplasty operations vary in scope and frequently subdivided into categories. Depending on the extent of the surgery, a complete and abdominoplasty can take 1 to 5 hours. A partial abdominoplasty (Mini-Tuck abdominoplasty) can be completed between 1 to 2 hours.Reconstruction of the umbilicus (belly button) following an abdominoplasty surgery. The original umbilicus is sutured into a new hole created by the surgeon.

A comple abdominoplasty follows these steps: An incision was made from hip to hip just above the pubic area. Another incision was made to free the navel from the surrounding skin. The skin is detached from the abdominal wall to reveal the muscles and fascia to be tightened. The muscle fascia wall tightened with sutures. The remaining skin and fat are tightened by removing the excess and closing the defect. The old belly button stalk is brought out through a new hole and sutured into place. Liposuction is often used to refine the transition zones of the abdominal sculpture. A dressing and sometimes a compression garments are applied and any excess fluid from the site is drained. Full abdominoplasty consisting of musculofascial placation abdominal dermal lipectomy, suction-assisted lipectomy of hips.

WASHING STEP:

Solid fat adipose tissue 50 mg was placed in the conical tube containing 1X PBS 1 with 1% antibiotic solution and washed thoroughly to remove the erythrocytes and leukocytes from the tissue. 1X PBS along with the erythrocytes and other contaminants are removed using 10ml pipette. The washing step was repeated until all the blood vessels and connective tissues appear to have been liberated (usually 2 washes).Place small pieces of adipose tissue in a sterile culture dish. Mince all adipose tissue into small pieces of 2-3mm in diameter. Transfer the chopped tissues into fresh sterile conical tube. Add Collagenase solution of 0.075% concentration prepared with PBS to the chopped adipose tissue.

COLLAGENASE DIGESTION

Dispersion of adipose tissue was achieved by collagenase digestion. Collagenase has the advantage over other tissue digestive enzymes that it can efficiently disperse adipose tissue while maintaining high cell viability. Make up Collagenase solution just prior to digestion. The final volume required is half that of the washed adipose tissue volume. Add powdered Collagenase to PBS at a final concentration of 0.075%. The required amount of Collagenase was dissolved in 100mi of PBS, then filter sterilize into the remaining working volume. Add prepared collagenase solution to the minced adipose tissue and keep for incubation for 1 to 2 hours at 37°C. Resuspend the adipose tissue by shaking the tubes vigorously for 5-10 seconds every 15 minutes. On completion of the digestion period, the digested adipose tissue should have a “soup-like” consistency. Add FBS to final concentration of 10% to stop collagenase activity. Dispense the collagenase-digested tissue into 50 ml tubes. Avoid dispensing undigested tissue. Centrifuge at room temperature at 400x g for 10 min. After centrifugation, use a 50 ml pipette to aspirate the floating adipocytes, lipids and the digestion medium. Leave the SVF pellet in the tube. The SVF predominantly contains erythrocytes, leukocytes, endothelial cells and stromal stem cells. Erythrocytes are removed first, using the red blood cell lysis buffer. The cells were counted by using hemocytometer. The viable cells in the chamber were counted to calculate the % viability of the suspension. The % viability of cell suspension was calculated using the formula:

$$\% \text{ viability} = \frac{\text{No of viable cells X 100}}{\text{Total number of cells}}$$

Cells are suspended in the DMEM / 10% FBS and anti solution as 1 x 10^6 cells. Samples were then maintained in the humidified incubator at 37°c and 5% CO_2 Cells after 72 hours, entire medium from well was aspirated, trypsinization and FACS characterization for SVF and Ps_0

HARVESTING CELLS

Remove medium completely from the culture plate by keeping the plate at 45º angle, slowly pipeting out with a Pasteur pipette. Add small volume of sterile PBS to wells and allow PBS to remain on cells for two minutes. Replace PBS with 500µl of 0.5% trypsin / EDTA. Incubate for 5 minutes. Verify under microscope whether 90% of cells have detached from plate. Add 500µl of stromal medium to allow the serum contained in the solution to neutralize the trypsin reaction. Transfer the medium containing the suspended cells from the well to the sterle 2ml tube. Centrifuge at 300g for 5 minutes. Aspirate the pellet and proceed to the cell counting and by taking an alliquote of cells diluted in tryphan blue for 1:5 dilutions. Count cells using hemocytometer.

Characterization of SVF cells using flowcytometry 1 X 10^6 cells from stromal vascular fraction and P_0 cells were taken and characterized for various hematopoietic, mesenchymal and side population markers using BD FACS Aria.

FLOWCYTOMETRIC PROTOCOL FOR CHARACTERIZATION

Flowcytometry was performed on a Becton, Dickinson FACS Aria using a 488-nm argon-ion LASER for excitation; Fluorescence emission was collected using its corresponding detectors.$1X10^6$ cells were stained with appropriate amount of conjucated antibodies in each of 12x75mm falcon polystyrene FACS tube. The quantity of each antibody conjugated with fluorochromes added to the cells in each tube was added respectively. All tubes were incubated for 20 minutes in dark. After incubation, cells were washed in phosphate buffer saline to remove the unbound antibodies. The pellet was further resuspended to 500µl. Data analysis and acquisition was performed using DIVA software, Becton Dickinson. Flow cytometer instruments were set using unstained cells. Cells were gated by forward versus side scatter to eliminate debris. The number of cells staining positive for a given marker was determined by the percentage of cells present within a gate established. A minimum of 10 000 events was characterized and recorded.

A dot plot was created with FSC vs. SSC on X axis and Y axis, a polyglonal gate was drawn and labeled the population as LYMPHOMONONEUTRO in the population hierarchy. A second plot was created with FITC-A in the X-axis vs. SSC-A in the Y-axis. Sample was loaded and when events begin to appear in the plots adjusts the LYMPHOMONONEUTRO gate such that the LYMPHOMONONEUTRO population was covered within the gate. After recording the required number of events, right clicks on the second plot and select show population LYMPHOMONONEUTRO. Draw a polygonal gate next to the cluster of events appearing in this plot such that the gate covers no events or few events. Labeled the gate as $ALDH^+$ cells in the population hierarchy.

RESULT

Isolation, growth and culture of Mesenchmal Stem Cells

The omentum fat samples were collected with clinical care and the mononuclear layer was separated and then cultured and subsequently subjected to different passages after attaining confluence. The P_0 stage had MNC with a mixed population of MSC and SP (Side Population). This population of cells was once again transferred to another flask in P_1 after attaining the confluence level (fig: 6) The above procedure was repeated for couple of times to determine the cell viability and the cell growth rate was maintained constant in P_3 (fig:7) and P_5 as well where the cells showed constant growth rate (fig: 8)

Characterization of adipose derived stem cells

The different stages of passages were chosen to study the characterization of adipose derived stem cells using different markers with FACS at the P_1 stage. This stage has MNC populations which are marked with CD34+, CD133+, CD29+, CD90+ , this showed 55.2%, 61.4%, 74.0% and 72.7% respective to the markers used **(Table: 1).** At passage 3 (P_3), MNC populations which are marked with CD29+, CD90+, CD105+ has showed the best results. This showed 68.3%, 68.0%, and 68.2% respective to the markers used **(Table: 2).** At passage 5 (P_5), MNC populations which are marked with CD29+, CD90+, CD105+ has showed the best results. This showed 71.4%, 71.4%, and 71.4% respective to the markers used **(Table: 3)**

To investigate the cells under *ex vivo* expansion conditions, a few tests were conducted to show the undifferentiated phenotype of omentum fat derived MSCs by specific surface markers and gene expression profiles. Proliferation of MSCs was arrested without change in morphology and differentiation capacity at first passage (P1). At a confluence rate of 70 to 80 % the cultivated cell suspension was made and flow cytometric analysis was performed to verify the purity of cell population devoid of contamination, if any. Subsequently, the following surface marker profiles were detected: CD34+, CD45+, CD133+, CD31+, HLA-DR+, CD29+, CD90+ and CD 105+

(Graph 1-6). Later a cell aliquot was seeded out for further testing of their differentiation ability. Each investigated stages (S1-S3) exhibited homogenous cell population for the entire cell surface antigen (Marker) and demonstrated that MSCs did not alter their physical and morphological properties during *ex vivo* expansion under the culture conditions tested. Flow cytometry and functional tests confirmed that more than 71.4 % to 74% of omentum fat derived stem cells were isolated, expanded. 4.3% to 13.5% of MSCs were destined to express the markers for mesenchymal lineages. Hence compatibility of omentum fat derived stem cell was standardized using the elucidated protocol.

Table 1: Surface Marker Profiles of MSCs at Passage 1 by Flow Cytometry

Sl. No	CD Markers	Passage 1 (%)
1	CD34+	55.2
2	CD45+	36.5
3	CD133+	61.4
4	CD31+	5.6
5	HLA-DR+	3.4
6	CD29+	74.0
7	CD90+	72.7
8	CD105+	0.4

Table 1: comparison of freshly isolated MSCs using different markers, showing best results in CD34+, CD133+, CD29+, CD90+, CD10

Table 2: Surface Marker Profiles of MSCs at Passage 3 by Flow Cytometry

Sl. No	CD Markers	Passage 2
1	CD34+	1.7
2	CD45+	4.7
3	CD133+	4.7
4	CD31+	0.3
5	HLA-DR+	0.4
6	CD29+	68.3
7	CD90+	68.0
8	CD105+	68.2

Table 2: comparison of MSCs using different markers, showing best results in CD29+, CD90+, CD105+

Table 3: Surface Marker Profiles of MSCs at Passage 5 by Flow Cytometry

Sl. No	CD Markers	Passage 3
1	CD34+	2.7
2	CD45+	3.8
3	CD133+	4.0
4	CD31+	2.8
5	HLA-DR+	1.5
6	CD29+	71.4
7	CD90+	71.4
8	CD105+	71.4

Table 3: comparison of MSCs using different markers, showing best results in CD29+, CD90+, CD105+

Figure 1: Extraction of omentum fat by surgical method from abdomen

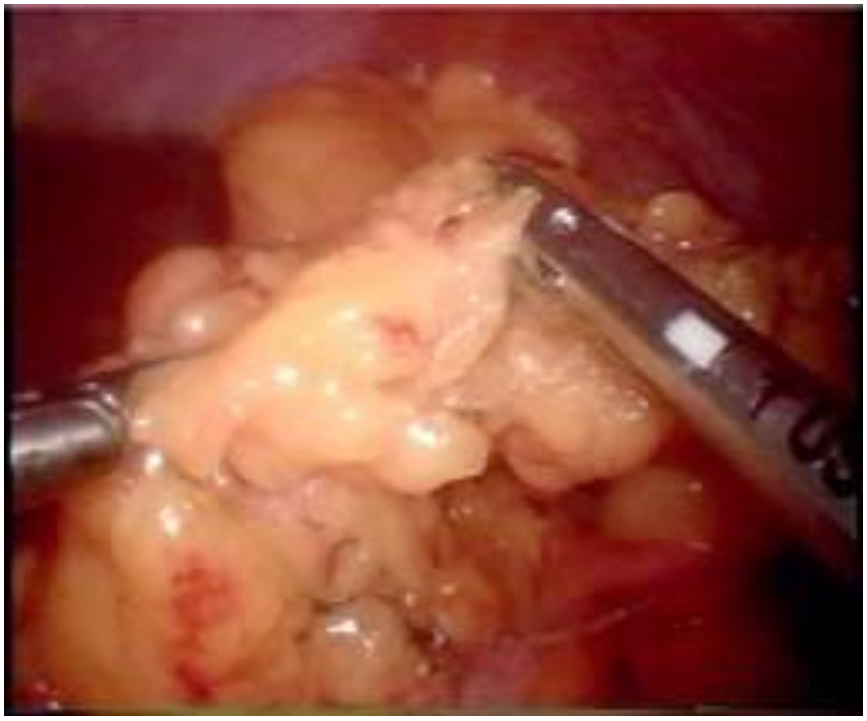
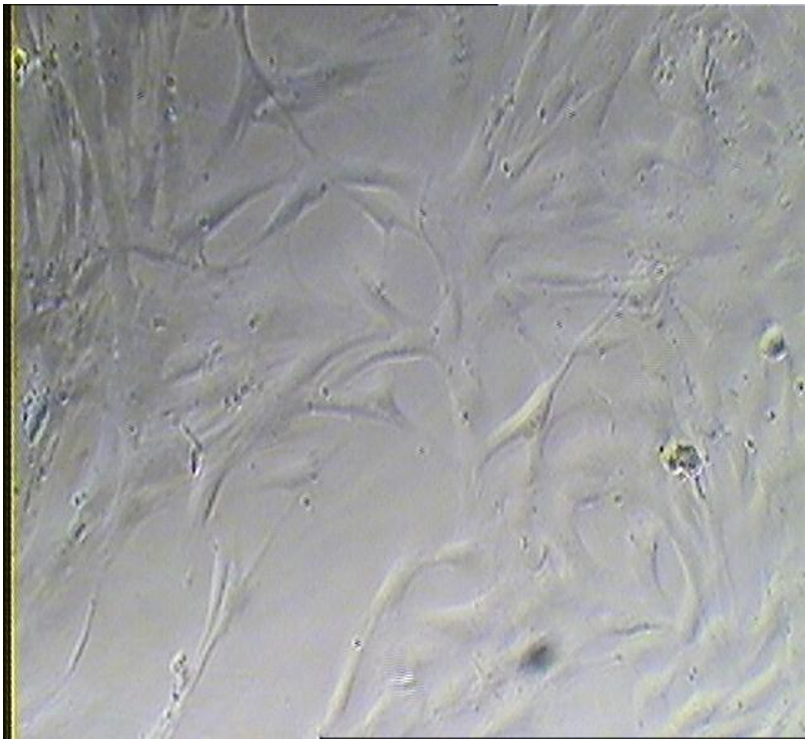
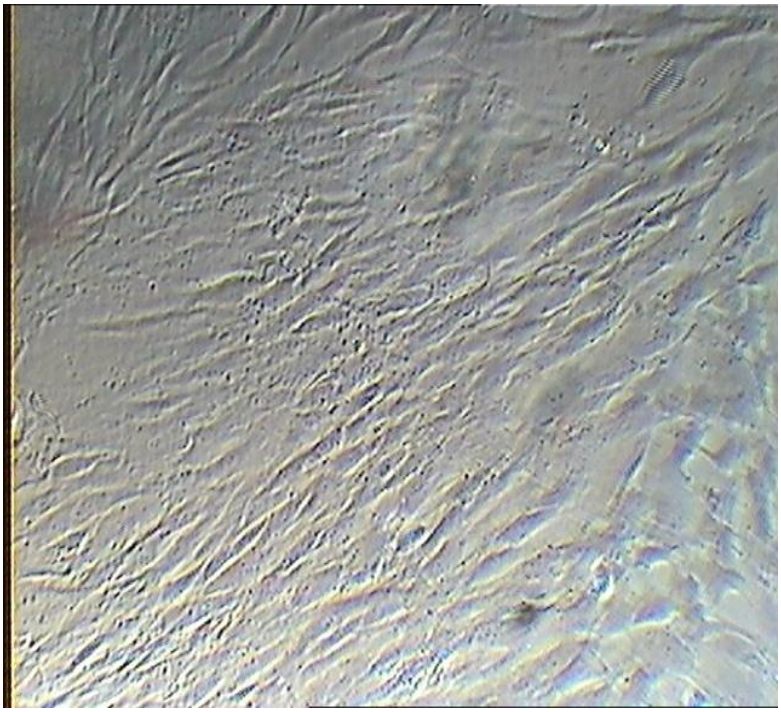


Figure 6: Passage 1

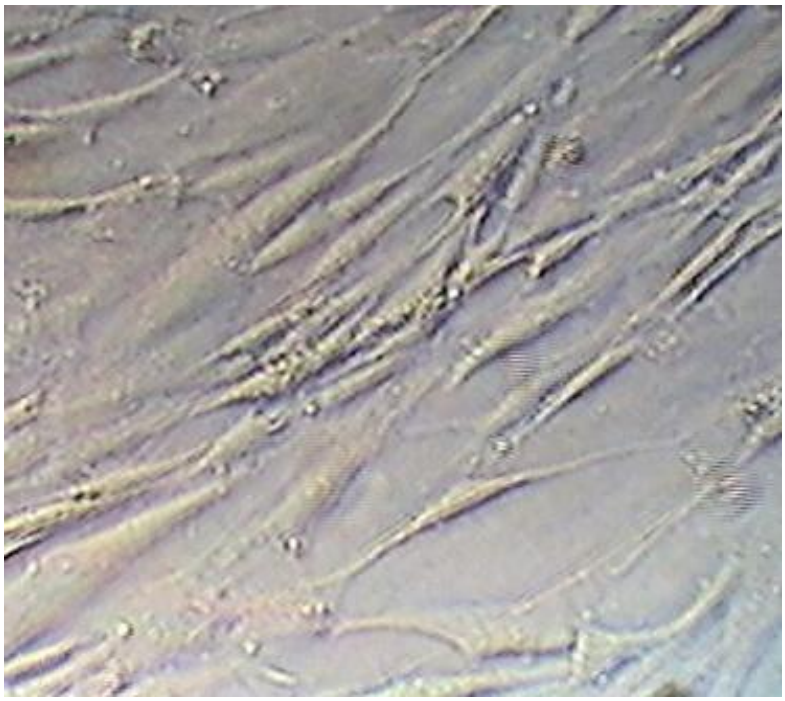


Omentum fat derived MSCs after 3 days of growth
Figure 7: Passage 3



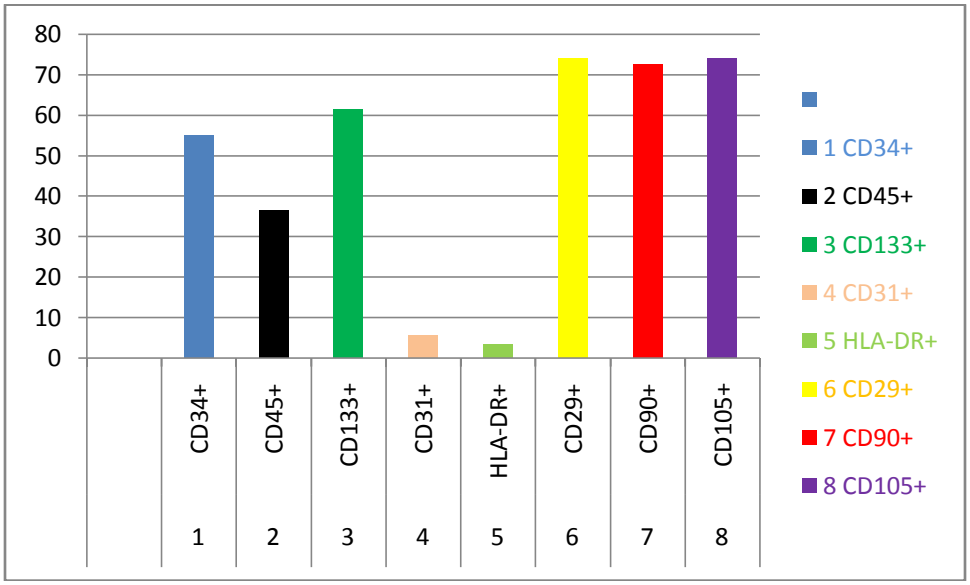
Omentum fat derived MSCs at confluence stage

Figure 8: Passage 5

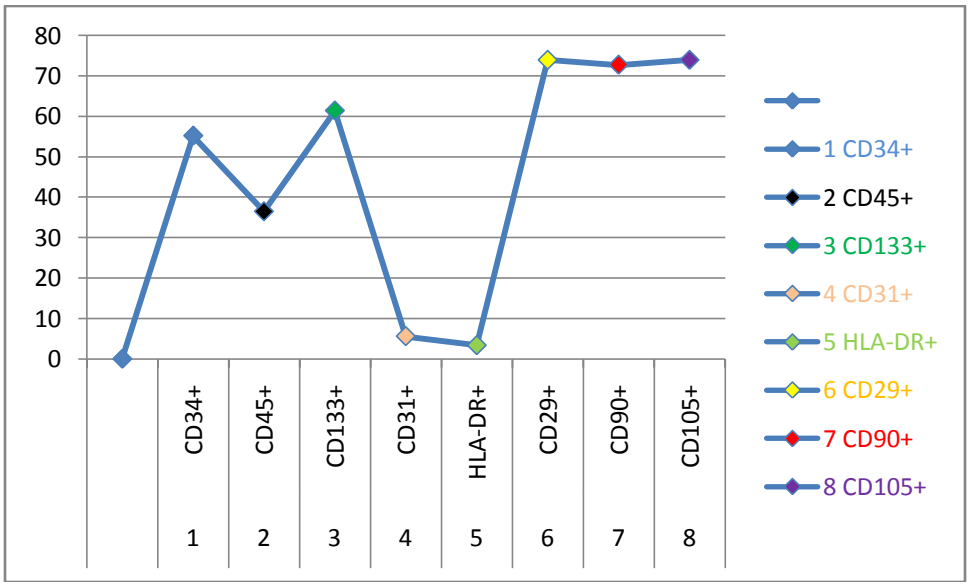


Omentum fat derived MSCs fully expanded in the culture media

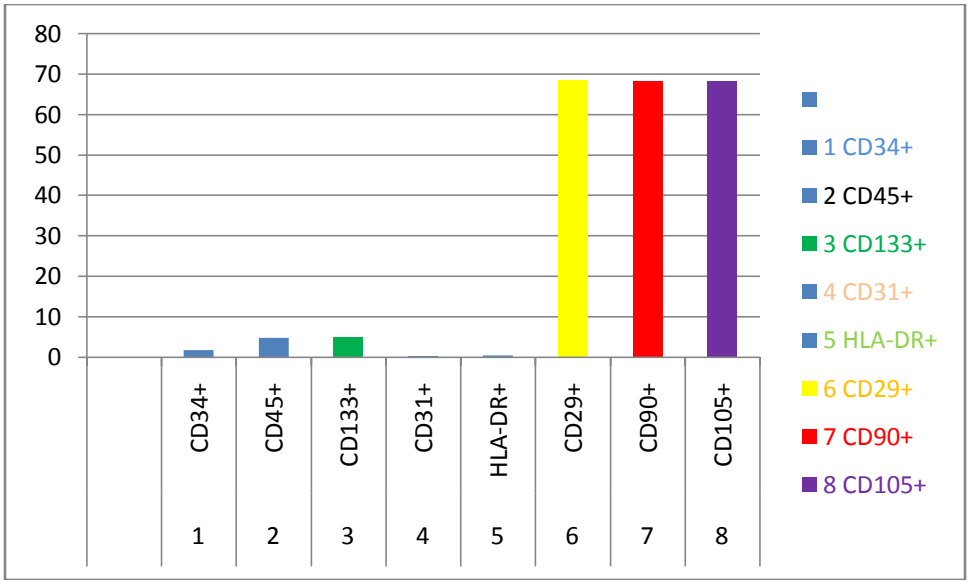
Graph 1: Surface Marker Profiles of MSCs at Passage 1 by Flow Cytometry



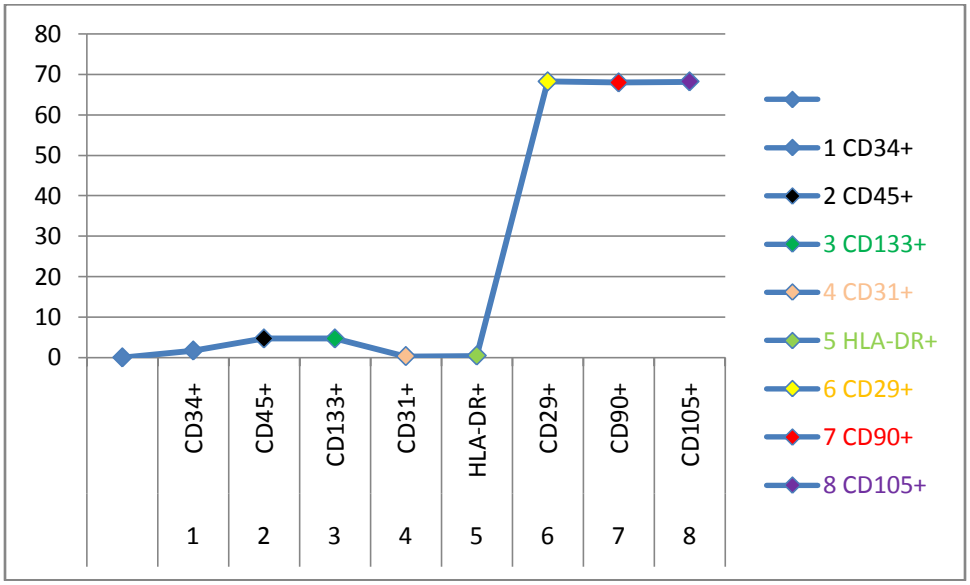
Graph 2: Distribution of surface Marker Profiles of MSCs at Passage 1 by Flow Cytometry



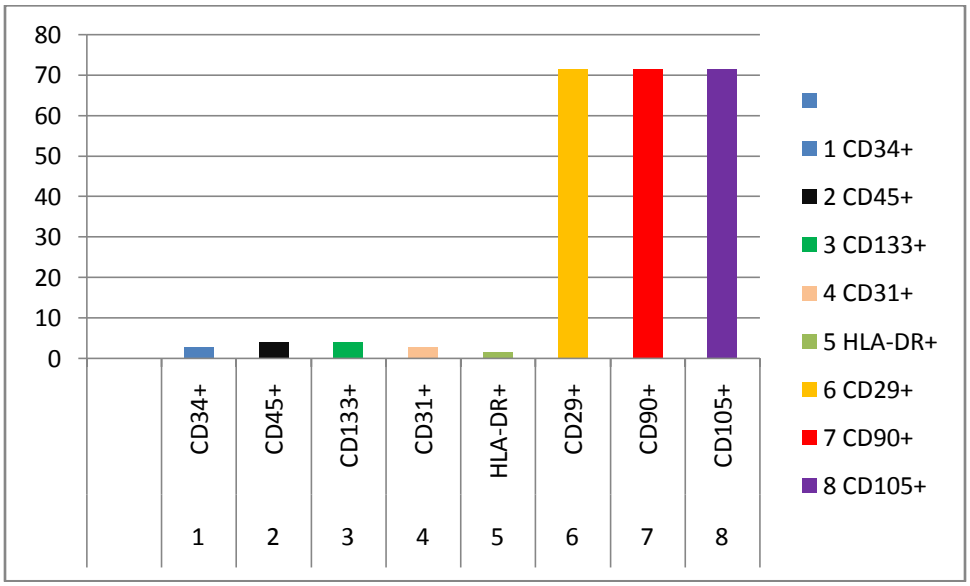
Graph 3: Surface Marker Profiles of MSCs at Passage 3 by Flow Cytometry



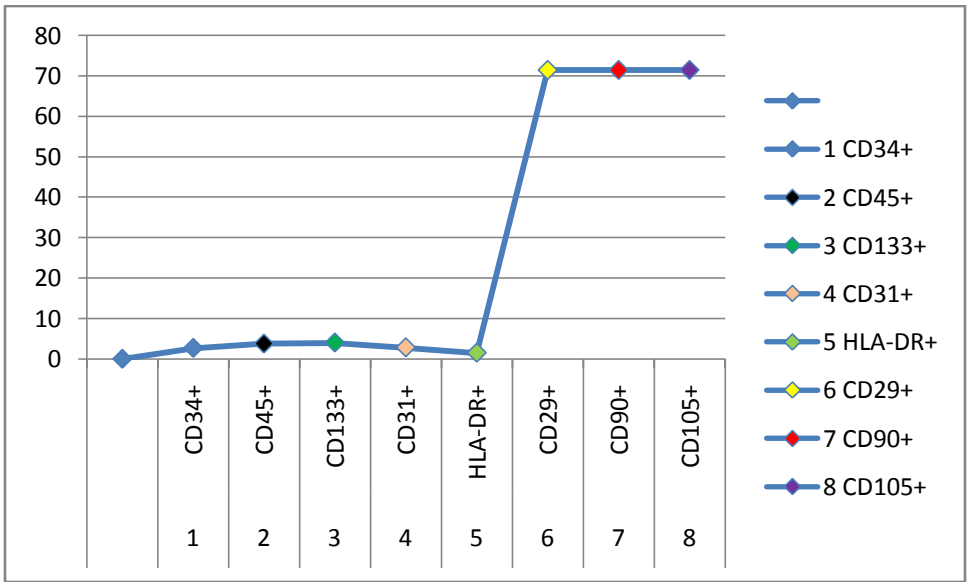
Graph 4: Distribution of Surface Marker Profiles of MSCs at Passage 3 by Flow Cytometry



Graph 5: Surface Marker Profiles of MSCs at Passage 5 by Flow Cytometry



Graph6: Distribution of Surface Marker Profiles of MSCs at Passage 5 by Flow Cytometry



DISCUSSION

Adipose tissue represents an abundant and accessible source of adult stem cells with the ability to differentiate along multiple lineage pathways. The isolation, characterization, and preclinical and clinical application of adipose-derived stem **cells (JM.Gimble, 2007)** were well known through the years. In furtherance of this study, the present investigation aimed at studying the complete profile of the ASCs. In contrary to the previous works freshly isolated ASCs were shown to be highly positive for CD34, and positive for HLA-DR. On the other hand, expression of CD105 and other freshly isolated ASCs was relatively low (Varma, 2007)present study showed a high in the rates of cells marked with CD 29+, CD 90+ and CD 105 + in the fresh sample as well as in the Passages . Wherein, the passages 1-5 showed a positive for the above mentioned markers. Passages P1, P3 and P5 were chosen for FACS characterization to optimize the cell plate capacity and to study the morphological characteristics at different growth rates.

With all the chosen markers being receptive for the mesenchymal stem cells, best of results were obtained from the expansion specific markers. This could be identified from the **(Table 1, 2 &3).** After an evaluation of the current treatment options, discussion studies of adipose-derived stem cell (ADSC) therapy**,** (Damien, 2008)a novel approach for treating complex perianal fistula, Crohn's disease etc.,

Present study showed a highly positive results for the stem cells cultured, characterized and harvested. This would be a breakthrough in accruing adipose derived stem cells for therapeutic standardization. Based on this background knowledge, the primary purpose of this concise review is to summarize and describe the underlying biology of ADSCs and their proliferation and differentiation capacities, together with current preclinical and clinical data from a variety of medical fields regarding the use of ADSCs in regenerative medicine (Mizuno 2012).

During the initiation and progression of breast cancer, the tumor cells reorganize the tissue microenvironment to support their proliferation and invasion into the surrounding tissue (Pupa *et al.*, 2002). However, the effect of ASCs against cancer tumor cells is controversial. Several studies have reported that implanted ADSCs inhibited breast cancer metastasis and growth in a murine model (Sun *et al.*, 2009) present work supported with this wherein the marking potential of CD 90+ which is supposedly used as an carcinomic surface marker showing an impressive results. . Currently, an ongoing clinical trial using ADSCs for cardiovascular treatment has been reported. The clinical trial is being carried out in patients with acute myocardial infarction (AMI) (Sanz-Ruiz *et al.*, 2009), and it is a prospective, double-blind, randomized, placebo-controlled, sequential dose-escalation trial including up to forty-eight patients, If proven so, this findings would support the claim of ADSCs being used as an potential neocardiogenic infuser . Freshly-isolated ADSCs are delivered through intracoronary infusion in patients with AMI and left ventricular (LV) ejection fraction impairment results of the present study

showed the higher marking capability in freshly isolated ASCs which backs the earlier work. Considering the above facts ADSCs can be used for multiple applications.

In conclusion, the data support the hypothesis that a human lipoaspirate contains multipotent cells and may represent an alternative stem cell source to bone marrow-derived MSCs (Zuk 2004).ASCs were used for spinal cord injury in rats after *in vitro* differentiation into Schwann cells (Ohta *et al.*, 2008). But in this study, the authors stated that functional improvement was not observed despite the significant histological improvement achieved at the primary injury site by the transplantation of Schwann cells. Present study showed the higher marking capability in freshly isolated ASCs which backs the earlier work. Considering the above facts ADSCs can be used for multiple applications.

Conclusion

With applications ranging from carcinogenic to cardiovascular to enteric disorders, ASCs hold a key in the future unraveling of therapeutic applications. The volatility of ASCs being tremendous and promising this could be used in *in vivo* infusion in broadly classified disorders. ASCs exhibit stable growth and proliferation kinetics and can differentiate toward osteogenic, chondrogenic, adipogenic, myogenic, or neurogenic lineages *in vitro*. Anticipating this to be a prospective replacement for the painful procedure of harvesting Bone marrow derived stem cells. This is an ideal source of stem cell collected from procedure like liposuction. Freshly isolated ASCs which show a highly positive adhesion and expandability could be used instead of cultured cells in case of necessity unlike BMSCs. The fields of tissue engineering, cell therapy, adipose biology, stem cell physiology, and growth and development, ASCs are uniquely positioned to translate findings into new medical treatments.

BIBLIOGRAPHY

1.Anita H Undale et al.,(2009). Mesenchymal stem cells for bone repair and metabolic bone diseases. Mayo clin proc; 84(10): 893-902

2.Ashok K Singh et al., (2009). Stromal cells cultured from omentum express pluripotent markers, produce high amount of VEGF, and engraft to injured sites. Cell tissue res; 332: 81-88

3.Aust L et al., (2004). Yield of human adipose derived adult stem cells from liposuction aspirates: Cytotherapy; 6: 7-14

4.Bensinger W et al.,(1993). Bone marrow transplant; 11: 369-374

5Cinti S, Morroni M., (1995). Brown adipocyte precursor cells: a morphological study. Ital J Anat Embryol;100 Suppl 1:75-81

6.Damien et al., (2008). Expanded adipose-derived stem cells for the treatment of complex perianal fistula including Crohn's disease. Expert Opin Biol Ther; 8(9): 1417-1423

7.De Ugarte et al.,(2003). Comparison of multi-lineage cells from human adipose tissue and bone marrow. Cell tissues organs; 174(3): 101-109

8.Dhanasekaran M et al.,(2013). Human omentum fat-derived mesenchymal stem cells transdifferentiates into pancreatic islet-like cluster. Cell Biochem Funct.

9.Dhanasekaran M et al.,(2013). Effect of high glucose on extensive culturing of mesenchymal stem cells derived from subcutaneous fat, omentum fat and bone marrow. Cell Biol Int; 36(11): 1029-36

10.Dhanasekaran M et al.,(2012). Long-term culture optimization of human omentum fat-derived mesenchymal stem cells. Cell Biol Int; 36(11): 1029-36

11.Festy et al., (2005). Surface protein expression between human adipose tissue derived stromal cells and mature adipocytes. Histochem. Cell Biol. 124, 113-121

12.Giuseppe Astori et al., (2007). "In vitro" and multicolor phenotypic characterization of cell subpopulations identified in fresh human adipose tissue stromal vascular fraction and in the derived mesenchymal stem cells. Journal of Translational Medicine; 5: 55

13.Hausman D.B. et al., (2001). The biology of white adipocyte proliferation. Obes. Rev.2:239-254

14.Hemmrich K et al., (2007). Isolation and expansion of primary preadipocytes. Int J Adipose tissue: 1(1): 24-33

15.JM Gimble et al., (2003). Adipose derived adult stem cells: isolation, characterization and differentiation potential: Cytotherapy 5: 362-369
16.JM Gimble et al., (2007). Adipose deived stem cells for regenerative medicine. Circulation research; 100:1249-1260
17.Kirkland JL et al., (1990). Age, anatomic site and the replication and differentiation of adipocyte precursors; Am J Physiol; 258: 206-210
18.Miranville A et al., (2004). Improvement of postnatal neovascularization by human adipose tissue-derived stem cells. Circulation 110, 349- 355
19.Mizuno et al., (2012). Adipose-derived stem cells as a novel tool for future regenerative medicine; 804-10
20.Ohta et al., (2008). Mature Adipocyte-Derived Cells, Dedifferentiated Fat Cells (DFAT), Promoted Functional Recovery From Spinal Cord Injury-Induced Motor Dysfunction in Rats. Cell Transplantation; Vol(17): 877-886

21.Patrick CW Jr., (2000). Adipose tissue engineering: the future of breast and soft tissue reconstruction following tumor resection. Semin surg oncol; 19(3):302-11

22.Poznanski WJ et al., (1971). Differences in vitro of adipose cells from normal and obese patients; Nature; 231:445
23.Rodriguez A M et al.,(2005). The human adipose tissue is a source of multipotent stem cells. Biochimie; 87: 125-128
24.Rosen ED, Spiegelman BM., (2000). Molecular regulation of adipogenesis. Annu Rev cell Dev Biol.;16:145-171
25.Rupnick et al., (2002). Adipose tissue mass can be regulated through the vasculature. PNAS; vol 96(16): 10730-10735
26.Sanz-Ruiz et al., (2009). Early translation of adipose-derived cell therapy for cardiovascular disease. Cell Transplantation; 18(3): 245-254
27.Sengenes C et al., (2005). Native human adipose stromal cells: localization, morphology and phenotype. International journal of obesity; 35(9): 1141-1153
28.Seo MJ et al., (2005). Differentiation of human adipose stromal cells into hepatic lineage in vitro and in vivo. Biochem Biophys Res Commun; 328: 258-264
29.Soufiane Ghannam et al., (2010). Immunosuppresion by mesenchymal stem cells: Mechanisms and clinical applications. Stem cell research and therapy 1:2
30.Tamara C. Otto and M. Daniel Lane., (2005). Adipose Development: From stem cell to adipocyte. Vol 40: 229-242
31.Varma MJ et al., (2007). Phenotypical and functional characterization of freshly isolated adipose tissue derived stem cells. Stem Cells Dev; 16(1): 91-104
32.Windsor et al., (2009). Human adipose-derived stem cells: isolation, characterization and applications in surgery. ANZ Journal of Surgery; Vol (79): 235-244

33.Zuk PA et al., (2001). Multilineage cells from human adipose tissue: implications for cell based therapies. Tissue Eng; 2001; 7:211-228
34.Zuk PA et al., (2002). Human adipose tissue is a source of multipotent stem cells. Mol Biol cell; 13:4279-4295
35.Zuk PA et al., (2004). Osteogenic Potential of Human Adipose Tissue-Derived Stromal Cells as an Alternative Stem Cell Source. Cell Tissues Organs; 178: 2-12