AIM OF THE WORK

The aim of the study is to describe the isolation procedures, differentiation of stromal vascular fraction (SVF) into mesenchymal stem cells (MSCs) and identification of MSCs by immunophenotyping.

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SUBJECTS

The study was conducted on **Adipose tissue** obtained from **12 subjects. All subjects were middle-aged females.**

Inclusion criteria:

Subjects (18-60 years) with normal platelets count who were doing;

1- Breast reduction surgery.

2- Abdominoplasty.

3- Liposuction.

Exclusion criteria:

The subjects were free from:

1- Breast cancer,

2- Diabetes mellitus,

3- Admission of corticosteroids therapy.

All subjects signed a written informed consent according to Ethics Committee For Human Research In Alexandria Faculty of Medicine.

They were subjected to the following:

1- Adequate medical history taking:

- To exclude medical illness e.g. diabetes mellitus or cancer breast,
- Drug history: to exclude the admission of corticosteroids (CST) therapy for any cause.

2- Laboratory investigations:

- Complete blood picture (CBC): to assess platelets count.

METHODS

Samples:

Adipose tissue was either obtained from:

- ***Liposuction***: 40 ml of lipoaspirate material collected in a syringe was obtained from clinic from patients undergoing laser-free liposuction followed by lipo-injection. *(figure 3)*

Figure (3): Lipoaspirate material.

- ***Abdominoplasty or breast reduction***: 2-4 g of adipose tissue obtained from patients undergoing abdominoplasty or breast reduction were cut and placed into sterile cups in operating room.

Equipments:

1. Biological safety cabinet class II (NuAire ,Germany) *(figure 4a)*.
2. Water jacket CO_2 incubator (NuAire, Germany) *(figure 4b)*.
3. Centrifuge. (Hettich, Germany)
4. Inverted microscope (Olympus).
5. Water bath with shaker. (GFL 1083, Germany)
6. Falcon tubes (15, 50 ml).
7. Vacutainer tubes with sodium citrate anti-coagulant (3.2%).
8. Sterile petri dishes.
9. Sterile cups.
10. T-25 cm^2 Nunc culture flasks.
11. Pasteur pipettes.
12. Automatic pipettes (1-10 ul and 100-1000 ul).
13. Sterile ophthalmic scissors and forceps.
14. BD FACSCalibur. (BD biosciences, California, USA)

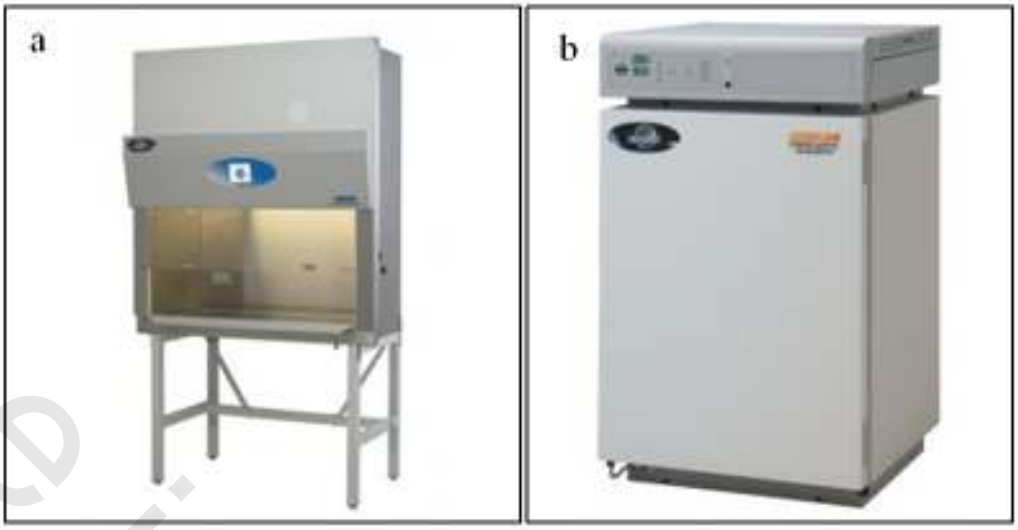


Figure (4): (a) Biological safety cabinet class II (NuAire, Germany), (b): Water jacket CO_2 incubator (NuAire, Germany).

Reagents:

1. Foetal bovine serum (FBS). (Biochrom, Germany)
2. Sterile phosphate buffer saline (PBS tablets -Sigma Aldrich, Germany); each tablet is dissolved in 200 ml de-ionized water to yield 0.01 M phosphate buffer, 0.0027 M potassium chloride and 0.137 M sodium chloride, pH 7.4, at 25 °C.
3. 0.075% collagenase solution; collagenase enzyme (Type I; Sigma-Aldrich, Germany) was prepared at a concentration of 1mg/ml sterile TESCA buffer (50mM TES, 0.36 mM calcium chloride, pH 7.4 at 37^0C). Working solutions of collagenase were then prepared by diluting 75 ul of already prepared collagenase solution in 100 ml sterile PBS. Collagenase is a mixture of enzymes secreted by ***Colistridium histolyticum***, with different products differentiated by the relative ratios of the 10-18 components found in the secreted enzymes. The main components are two collagenases, clostripain, and a neutral protease. The synergistic action of these enzymes degrades collagen and other intracellular material. The action of both collagenase enzymes and the neutral protease is necessary for effective release of cells from tissue.
4. 0.05% Trypsin/EDTA. (Euroclone, Italy)
5. Penicillin- Streptomycin (P/S) (10, 000 U/ml). (Euroclone, Italy)
6. Low glucose (1 g/l) Dulbecco's modified Eagle's medium (Lonza, Belgium); for each 100 ml low glucose DMEM: 10 ml FBS and 8 ml P/S were added.
7. High glucose (4.5 g/l) Dulbecco's modified Eagle's medium with L-glutamine (Lonza, Belgium) ; for each 100 ml high glucose DMEM with L-glutamine: 10 ml FBS and 8 ml P/S were added.
8. Flourescence- labeled antibodies:
   - CD45 –FITC.
   - CD 34-PE.
   - CD90- FITC.
   - CD73-FITC.
   - CD105-FITC (R&D systems, USA).
   - CD 49d-FITC.

- CD 14- FITC.
- CD19- FITC.
- CD3- FITC.
- HLA-DR- FITC.

All the used fluorescence-labeled antibodies were from (Immunostep company, Spain) except CD105-FITC was from (R&D systems, USA).

Protocols used for isolation and expansion of MSCs:

Three protocols were used for isolation and expansion of MSCs;

I. **Mechanical protocol** for isolation of MSCs from adipose tissue and MSCs expansion using **low glucose DMEM with 10% FBS**.

II. **Chemical protocol** for isolation of MSCs from adipose tissue and MSCs expansion using **DMEM with 10% FBS**; this was further classified into:

Protocol IIa: Chemical isolation of MSCs from adipose tissue obtained from abdominoplasty, breast reduction and liposuction and MSCs expansion using high glucose DMEM with L-glutamine and 10% FBS.

Protocol IIb: Chemical isolation of MSCs from adipose tissue obtained from abdominoplasty, breast reduction and MSCs expansion using low glucose DMEM with 10% FBS.

III. **Chemical protocol** for isolation of MSCs from adipose tissue, followed by their expansion using a **FBS-free culture media**; this was further classified into:

Protocol IIIa: Using platelets-rich-plasma (PRP).

Protocol IIIb: Using platelet lysate.

I. Mechanical protocol for isolation of MSCs from adipose tissue, followed by their expansion using low glucose DMEM with 10% FBS:[98]

1- Fresh adipose tissue obtained from subjects undergoing abdominoplasty and breast reduction was washed in sterile petri dishes with sterile PBS at room temperature to clean surface of adipose tissue from blood clots, red blood cells (RBCs) and local anesthetics. This was repeated twice.

2- Adipose tissue near blood vessels and fascia was then cut into small pieces using sterile pair of ophthalmic scissors and forceps.

3- The little pieces were transferred into T- 25cm^2 Nunc culture flasks and were aligned so that stem cells could spread and grow from pieces of adipose tissue,

4- 2 ml of 100% FBS were then added into the culture flasks to nourish pieces of adipose tissue and incubated in a humidified atmosphere containing 5% CO_2 at 37^0 C for 12 hours.

5- The culture flasks were then turned on their sides to allow pieces of adipose tissue to attach to the wall of culture flasks and continued to incubate for another 12 hours. Finally, culture flasks were turned again and restored to their original position for continuous culture.

6- After 24 hours, adipose tissue pieces and FBS were then discarded and culture flasks were washed with sterile PBS, then 4 ml low glucose DMEM with 10% FBS was added.

7- Culture flasks were incubated in a humidified atmosphere containing 5% CO_2 at 37 °C. They were examined under inverted light microscope for cell growth.

II. Chemical protocol for isolation of MSCs from adipose tissue, followed by their expansion using DMEM with 10% FBS:

- ***Protocol IIa:***

A. Isolation of MSCs from adipose tissue:

- Lipoaspirate:

1- Lipoaspirate was poured in two 50 ml falcon tubes.
2- Equal volume of PBS was added to each tube at room temperature and gently mixed in a rotator way for one minute.
3- The falcon tubes were allowed to stand for 2 minutes until two layers appeared ; *upper layer* representing the lipoaspirate mass and *lower layer* representing red-tinged PBS.
4- The pasteur pipette was gently passed from the upper layer and the lower layer was aspirated and discarded in the waste.
5- Repeat until the color of lower layer changed to be transparent, this usually occurred after 3-4 times washing with sterile PBS.
6- The upper layer was poured in T-25 cm^2 Nunc culture flasks and equal volume of 0.075% working collagenase solution was added for 1 hour in water bath at 37 °C with shaking 500 rpm.
7- The collagenase enzyme was then deactivated with equal volume of high glucose DMEM with L-glutamine with 10% FBS. Pour and distribute all the contents of flasks in 15 ml falcon tubes. The mixture was then centrifuged at 3000 rpm for 10 min at 4^0 C.
8- The supernatant was then discarded and the pellet, was re-suspended in 5 ml sterile PBS and centrifuged at 2000 rpm for 5 minutes.
9- Red cell lysis buffer was then added for 5 minutes at room temperature to lyse RBCs, then mixture was centrifuged at 2000 rpm for 5 minutes. The obtained pellet was re-suspended in 5 ml sterile PBS and centrifuged at 2000 rpm for 5 minutes. The resulted pellet represents the initial SVF.
10- The initial SVF pellet was then re-suspended in 2 ml high glucose DMEM with L-gluatmine and 10% FBS.

- Adipose tissue from breast reduction or abdominoplasty:

1- Fresh adipose tissue near blood vessels and fascia was cut into tiny pieces using sterile forceps and a pair of scissors in a sterile cup.
2- Sterile PBS was added and gently mixed in a rotator way for one minute.
3- The sterile cups were allowed to stand at room temperature for two minutes, two layers will appear ; *upper layer* containing tiny adipose tissue pieces and *lower layer* representing red-tinged PBS.
4- The pasteur pipette was passed from the upper layer and the lower layer was aspirated and discarded in the waste.
5- Steps (2 & 3) were repeated until the color of lower layer changed to be transparent. This usually occurred after 3-4 times.
6- Then we proceeded as lipoaspirate.

B. Cell count and cell viability:

1- 50 ul of fluid containing cells suspended in 2 ml high glucose DMEM with L-glutamine and 10% FBS was taken. Equal volume of trypan blue was added, mixed thoroughly and allowed to stand for 5 to 15 minutes.
2- **Cell count**: Each square of the haemocytometer (*figure 5*) with cover-slide in place, represents a total volume of 0.1 mm^3 or 10^{-4} cm^3, since 1cm^3 is approximately equivalent to 1ml, the subsequent cell concentration per 1 ml will be calculated as the following:

Cells per ml = average number of viable cells (unstained) per one square x dilution factor x10^4.

Total number of cells = number of cells per ml x original volume of fluid from which cells were taken.

3- **Cell viability (%):**

Total viable cells (unstained) / total cells (stained and unstained) x 100.

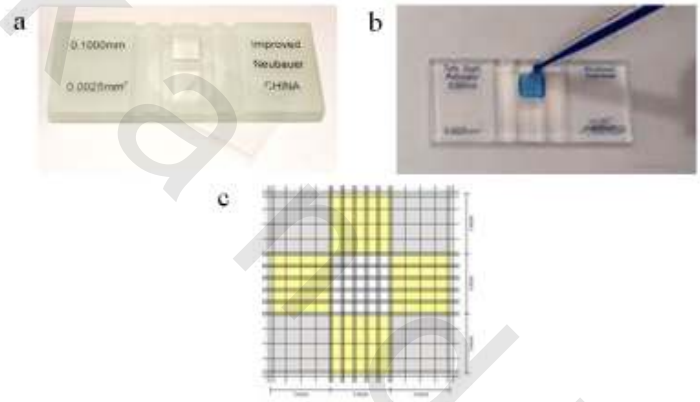


Figure (5): **(a)** haemocytometer and cover slide. **(b)**: demonstrating how to place the cell suspension and trypan blue mixture in haemocytometer chamber by carefully touching the edge of the cover-slide by the pipette tip and allow each chamber to fill by capillary action. **(c):** illustrative drawing of haemocytometer chamber.

C. Culture of isolated stromal vascular fraction (SVF):

1- 2 ml of SVF cells suspended in high glucose DMEM with L- glutamine and 10% FBS with cell count of 3.2 x 10^5 cells/ml was plated in T-25 cm^2 Nunc culture flasks.
2- 2 ml of pre-warmed high glucose DMEM with L- glutamine and 10% FBS to 37^0 C was added.
3- The flasks were then incubated in a humidified atmosphere containing 5% CO_2 at 37^0C.

D. Expansion of isolated cells:

1- After overnight incubation, flasks were examined and washed with sterile PBS to remove non-adherent cells and 4 ml of pre-warmed high glucose DMEM with L-glutamine and 10% FBS to 37^0 C was added.

2- Culture flasks were maintained until reaching 80-90% confluence with culture media being changed every 48 hours.

E. Cell harvest:

Trypsin is used to remove adherent cells from a culture surface. The cells were harvested using 0.05% trypsin /1.0mM EDTA as following:

1- The media was removed from culture flasks by aspiration and the monolayer was washed with PBS to remove all traces of serum, then PBS was removed by aspiration.
2- Trypsin /EDTA was put in culture flasks to completely cover the monolayer of cells.
3- The flasks were placed in incubator at 37^0 C for 10 minutes.
4- Progress can be checked by examination with an inverted microscope.
5- The ***time required*** to remove cells from the culture surface depends on; cell type, population density, serum concentration in the growth medium, potency of trypsin and time since last subculture. Trypsin causes cellular damage and time of exposure should be kept to a minimum.
6- When trypsinization process is complete, the cells will be in suspension and appear rounded.
7- Equal volumes of pre-warmed high glucose DMEM with L-glutamine and 10% FBS to 37^0 C were added to inactivate the trypsin.
8- The mixture was then centrifuged at 1300 rpm for 15 min at 4^0 C.
9- The supernatant was then discarded and pre-warmed high glucose DMEM with L-glutamine and 10% FBS to 37^0 C were added to the cellular pellet and then cultured in T-25 cm^2 Nunc culture flasks. Cultures were expanded for 2-8 passages.

- ***Protocol IIb:*** [5, 14, 15]

1- Fresh adipose tissue samples obtained from abdominoplasty and breast reduction were washed 3 times with sterile PBS to remove contaminating debris and erythrocytes.
2- The adipose tissues were then cut into small fragments and digested with 0.075% working collagenase solution for 30 minutes at 37^0 C with gentle agitation.
3- The collagenase was then inactivated with an equal volume of low glucose DMEM with 10% FBS.
4- The filtrate was centrifuged at 1200 rpm for 10 minutes and the pellet, which represents an initial stromal vascular fraction (SVF), was re-suspended in 2 ml low glucose DMEM with 10% FBS.
5- 2 ml of SVF cells suspended in low glucose DMEM with 10% FBS of an average count of 2.5×10^5 cells/ml were plated in T-25 cm^2 Nunc culture flasks.
6- 2 ml of pre-warmed low glucose DMEM with 10% FBS to 37^0C were added.
7- Culture flasks were incubated in a humidified atmosphere containing 5% CO_2 at 37 °C.
8- They were examined after two days under inverted light microscope for cell growth.

III. Chemical protocol for isolation of MSCs from adipose tissue, followed by their expansion using a FBS-free culture media:

- ***Protocol IIIa:***

1- Preparation of platelet-rich-plasma (PRP):

A- Fresh platelet-rich-plasma: (PRP)

Pure ***autologous*** PRP was prepared by:

- 6 ml venous blood sample was divided into three vacutainer tubes containing sodium citrate anti-coagulant (3.2%) under complete sterile conditions.
- Blood was then centrifuged at a speed of 1290 rpm for 5 minutes at 20 ^{0} C.
- 1 ml per tube of platelet-rich supernatant on the red blood cell pellet was then collected under sterile condition in a 15 ml falcon with care taken to avoid leucocyte harvesting.

B- Thawed platelet-rich plasma (tPRP):

- Pooling of PRP prepared in same protocol as explained above from 10 different allogenic venous blood samples collected in sodium citrate vacutainer tubes (3.2%) was done and heparin was added.
- Platelets suspended in plasma were then frozen to -20^{0}C and then thawed to 37 ^{0}C and heparin was added before using it in cell culture.

2- Initial SVF pellet obtained using chemical isolation as previously explained in ***protocol IIa*** *(methods, page 19)* was then re-suspended in 2 ml high glucose DMEM with L-glutamine. Cells suspended in high glucose DMEM with L-glutamine were then plated in T-25 cm^2 culture flasks with an initial plating density of 4 x 10 ^{5} cells/ml using 10%, 50% and 80% PRP.

3- Culture flasks were incubated in a humidified atmosphere containing 5% CO_2 at 37 °C.

4- They were examined after two days under inverted light microscope for cell growth.

- ***Protocol IIIb:***

1- Preparation of platelet lysate: [99]

- Pooling of PRP prepared in same protocol as explained above from 10 different allogenic venous blood samples collected in sodium citrate vacutainer tubes (3.2%) was done.
- Total number of platelets was 8 x 10^9 platelets suspended in 10 ml plasma.
- Platelets suspended in plasma were frozen to -80 ^{0}C for 12 hours then thawed to 37^{0}C.
- Two freeze- thaw cycles were done for complete disruption of platelets, then centrifugation at 2600 x g for 30 min was done to remove debris of platelet bodies, heparin was then added before addition to culture medium.

2- Initial SVF pellet obtained using chemical isolation as previously explained in ***protocol IIa*** *(methods, page 19)* was then re-suspended in 2 ml high glucose DMEM with L-glutamine. Cells suspended in high glucose DMEM with L-glutamine were

then plated in T-25 cm^2 culture flasks with an initial plating density of 4 x 10^5 cells/ml using 10%, 50% and 80% platelet lysate.

3- Culture flasks were incubated in a humidified atmosphere containing 5% CO_2 at 37 °C.
4- They were examined after two days under inverted light microscope for cell growth.

Immunophenotyping of isolated cells:

1- Initial stromal vascular fraction (SVF) obtained by chemical isolation using collagenase enzyme and Fibroblast-like adherent cells were harvested at Day 7, 14, 21 and 28 using 0.05% trypsin /1.0mM EDTA at 37 °C for 10 min.

2- Then, washed once with and re-suspended in sterile phosphate buffer saline (PBS).

3- Cells were then counted;
- **Initial SVF i.e. day 0** was: 10 x 10^6/ul.
- **Day 7** was: 15 x 10^6/ul.
- **Day 14** was: 20 x 10^6/ul.
- **Day 21** was: 5 x 10^6/ul.
- **Day 28** was: 5 x 10^6/ul.

4- Cells were treated at room temperature as; 100 ul of cell suspension + 5 ul of antibodies, with the following specific anti-human antibodies:
- CD45 –FITC.
- CD34-PE
- CD90- FITC.
- CD73-FITC.
- CD105-FITC
- CD 49d-FITC.
- CD14-FITC.
- CD19-FITC/PE.
- CD3- FITC.
- HLA-DR-FITC.

5- Tubes were then incubated in the dark for 30 minutes.

6- Cells were washed with 500 ul PBS and centrifuged at 2000 rpm for 5 minutes and then re-suspended in 200 ul PBS.

7- The specific fluorescent labeling was analyzed by BD FACSCalibur flow cytometer (BD biosciences, California, USA) using the Cell Quest software.

RESULTS

- The present study was carried out on adipose tissue obtained from 12 subjects; six were obtained from subjects undergoing abdominoplasty, three obtained from subjects undergoing breast reduction and three obtained from subjects undergoing liposuction. *(table 1)*

Table (1): Number of cases, sources from which adipose tissue was obtained and type of protocols applied

Number of Cases	Source	Protocol applied
Four cases	**Three** from abdominoplasty and **one** from breast reduction.	Protocol I *(methods, page 18)*
Five cases	**Three** from liposuction , **one** from abdominoplasty and **one** from breast reduction	Protocol IIa *(methods , page 19)* and protocol IIIa and IIIb *(methods, page 22)*
Three cases	**Two** from abdominoplasty, **one** from breast reduction.	Protocol IIb *(methods, page 21)*

- Success of each protocol used for isolation and expansion of MSCs in the present study was assessed by observing cell growth and proliferation using inverted microscope (Olympus).

- Examining the morphology of cells cultured using different protocols by inverted microscope , we found the following: *(table 2)*

Table (2): Applied protocols and morphology of cultured cells at day 0, day 2, day 7, day 15 and day 18 of initial culture

Protocol	Day 0	Day 2	Day 7	Day 15	Day 18
Protocol I *(methods, page 18)*	Not applicable.	Fibroblast-like adherent cells were seen. *(figure 6)*	Not available.	Not available.	Not available.
Protocol IIa *(methods, page 19)*	Numerous rounded non-adherent cells were seen. *(figure 7a)*	Fibroblast-like adherent cells were seen. *(figure 7b)*	Numerous early 15-20 colonies of fibroblast-like adherent cells. *(figure 7c)*	70% confluence. *(figure 7d)*	100% confluence. *(figure 7e)*
Protocol IIb *(methods, page 21)*	Numerous rounded non-adherent cells were seen.*(figure 7a)*	Fibroblast-like adherent cells were seen. *(figure 7b)*	Not available.	Not available.	Not available.
Protocol IIIa *(methods, page 22)*	Numerous rounded non-adherent cells were seen. *(figure 7a)*	Fibroblast-like adherent cells were seen. *(figure 7b)*	Not available.	Not available.	Not available.
Protocol IIIb *(methods , page 22)*	Numerous rounded non-adherent cells were seen.*(figure 7a)*	Fibroblast-like adherent cells were seen. *(figure 7b)*	Not available.	Not available.	Not available.

The following figures *(figure 6 and 7)* demonstrate how the cultured cells look like under the inverted microscope (Olympus).

Figure (6): Fibroblast-like adherent cells *(marked by an arrow)* at day 2 of initial culture using protocol I.

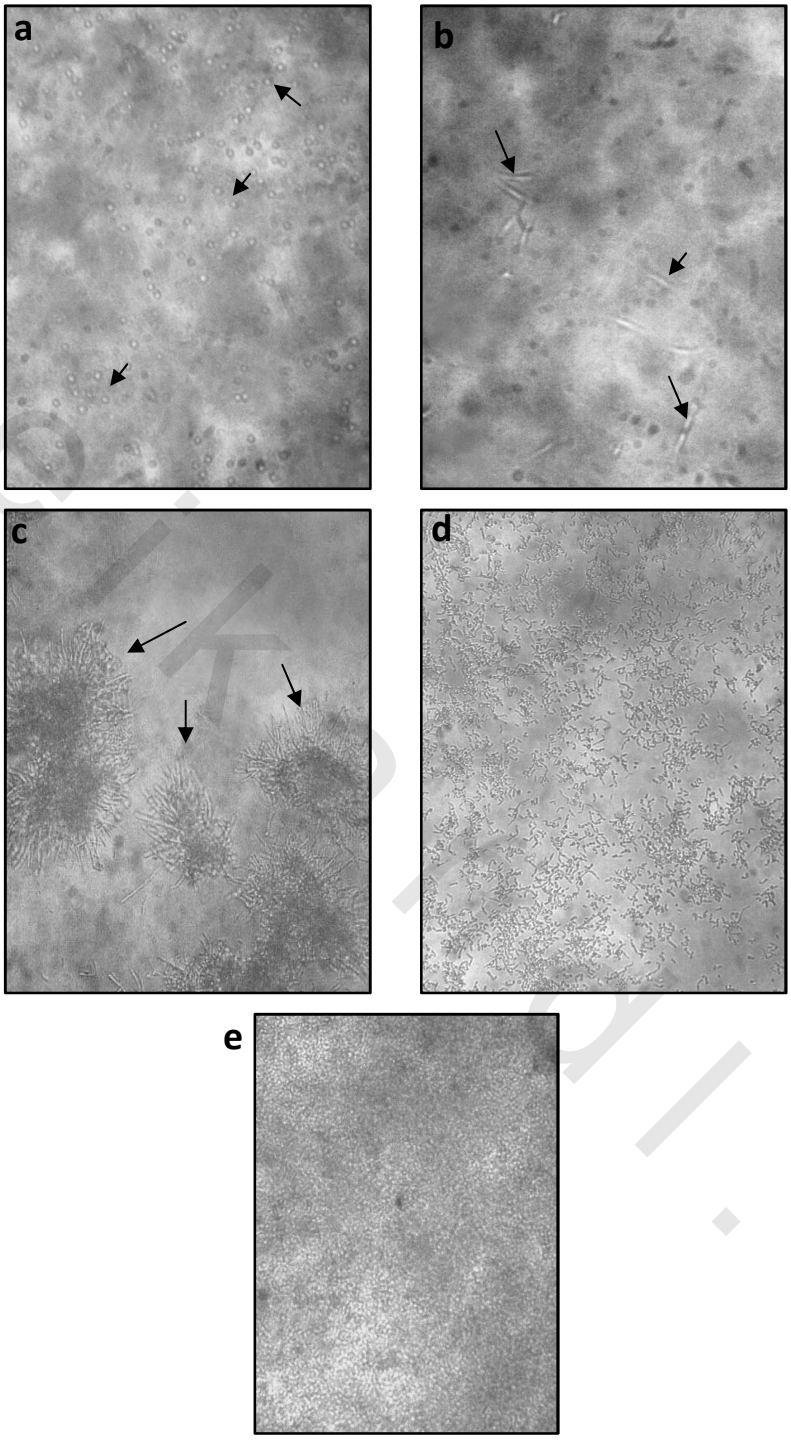


Figure (7): (a) ***Day 0*** of initial culture *using protocol IIa, IIb, IIIa& IIIb:* showing numerous rounded non-adherent cells.*(marked by an arrow)* (b) ***Day 2*** of initial culture *using protocol IIa, IIb, IIIa& IIIb*: showing initial fibroblast-like adherent cells.*(marked by an arrow*,) (c) ***Day 7*** of initial culture *using protocol IIa*: showing numerous early 15-20 colonies of fibroblast-like adherent cells.*(marked by an arrow)* (d) ***Day 15*** of initial culture *using protocol IIa:* showing 70% confluence. (e) ***Day 18*** of initial culture *using protocol IIa*: showing 100% confluence.

- Protocol I *(methods, page 18)*, protocol IIb *(methods, page 21)*, protocol IIIa *(methods, page 22)* and protocol IIIb *(methods, page 22)* **failed**. The resulting population was too low to be tested by flow cytometery. The cells disintegrated and died within 5-7 days of initial culture.
- Protocol IIa *(methods, page 19)* **succeeded** as; by the 7^{th} day of initial culture, numerous early 15-20 colonies of fibroblast-like adherent cells were seen. *(figure 8c)* . On the 15^{th} day of initial culture; a homogenous layer of fibroblast-like adherent cells of almost 70% confluence occupied the whole plastic surface of culture flasks. *(Figure 8d)*, which reached 100% confluence by the day 18 th of initial culture. The cultured cells retained their fibroblast-like morphology and were propagated in vitro by repeated passaging for 40 days.

- **Cell count and viability:**

Cell count and viability was assessed; after collagenase digestion of adipose tissue to assess initial stromal vascular fraction (SVF) pellet count and viability and with every passage to asses cell growth and proliferation.

- **Cell count:**

Cells per ml = average number of viable cells (unstained) per one square x dilution factor $x10^4$

$= 64/4 \times 2 \times 10^4 = 3.2 \times 10^5$ cells/ml.

Total number of cells = number of cells per ml x original volume of fluid from which cells were taken= $3.2 \times 10^5 \times 2 = 6.4 \times 10^5$ cells/ml.

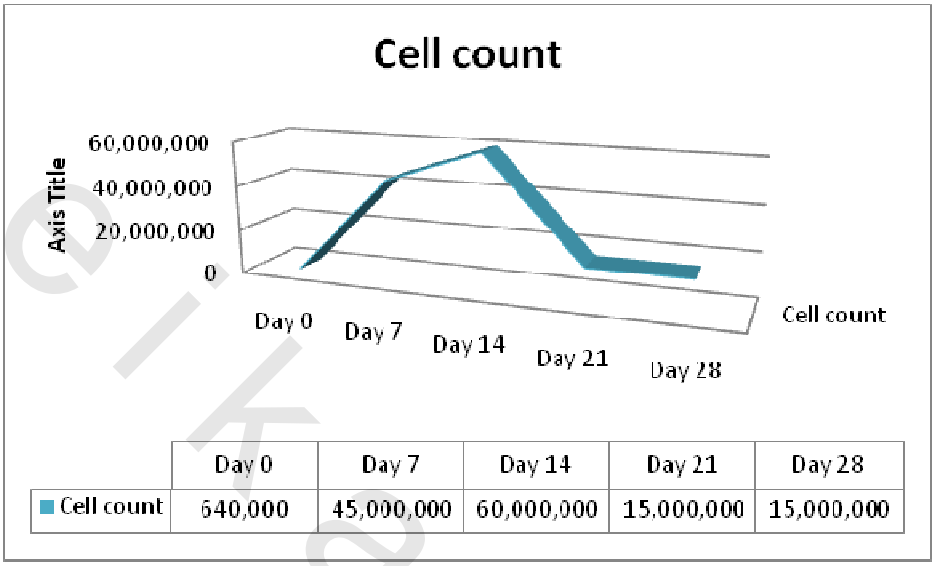
- **Cell viability (%):**

Total viable cells (unstained) / total cells (stained and unstained) x 100

$= 6.4 \times 10^5 / 8 \times 10^5 \times 100 = 80\%$.

- **Cell proliferation:**

By observing the behavior of isolated cells using ***protocol IIa*** *(methods ,page 19)* , we noticed that ***proliferation*** of isolated cells increases from ***day 0*** to reach maximum by ***day 14*** and then starts to decrease and becomes plateau as the cells senesce and die. *(Figure 8)*



	Day 0	Day 7	Day 14	Day 21	Day 28
Cell count	640,000	45,000,000	60,000,000	15,000,000	15,000,000

Figure (8): Graphic representation of proliferation of isolated cells; initial plating density i.e. day 0 (0.64 x 10^6 cells/ul), day 7 (45 x 10^6 cells/ul), day 14 (60 x 10^6 cells/ul), day 21 (15 x10^6 cells/ul) and day 28 (15 x 10^6 cells/ul).

- Difference in the behavior of cells isolated and cultured from adipose tissue was noticed. Cells isolated and cultured from lipoaspirate material showed better proliferation rate than those isolated and cultured from abdominoplasty and breast reduction. The results were summarized in *table 3.*

Table (3) Rate of proliferation of isolated cells from different sources of adipose tissue

Source	Rate of proliferation
Abdominoplasty	+
Breast reduction	++
Liposuction	+++

+: good, ++: very good, +++: excellent.

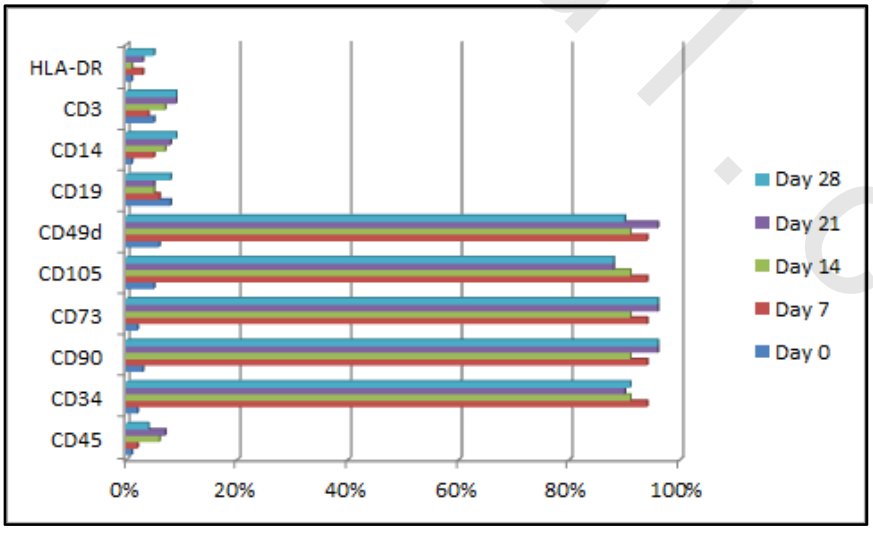
- **To confirm that ; the fibroblast-like adherent cells isolated from 5 cases using protocol IIa *(methods, page 19)* were MSCs, we performed immunophenotyping:**

Using BD FACSCalibur (BD biosciences, California, USA) and the Cell Quest software we found the following;

- **Day 0:** Our isolated were ***negative*** for CD45, CD34, CD90, CD73, CD105, CD49d, CD19, CD14, CD3 and HLA-DR.
- By **day 7**, the isolated cells were ***positive*** for CD34, CD 90, CD73, CD105, CD49d *(figure 11& 12)* and remained ***negative*** for CD45, CD19, CD14, CD3 and HLA-DR. The same results were also noticed at **day 14**, **day 21** and **day 28**. *(table 4)* and *(figure 10)*

Table (4): CD expression and mean percentage (%) of gated cells out of control

CD	Day 0	Day 7	Day 14	Day 21	Day 28
CD45	1%	2%	6%	7%	4%
CD34	**2%**	**94%**	**91%**	**90%**	**91%**
CD90	**3%**	**94%**	**91%**	**96%**	**96%**
CD73	**2%**	**94%**	**91%**	**96%**	**96%**
CD105	**5%**	**94%**	**91%**	**88%**	**88%**
CD49d	**6%**	**94%**	**91%**	**96%**	**90%**
CD19	8%	6%	5%	5%	8%
CD14	1%	5%	7%	8%	9%
CD3	5%	4%	7%	9%	9%
HLA-DR	1%	3%	1%	3%	5%



Figure(9): Bar chart showing CD45, CD34, CD90, CD73, CD105, CD49d, CD19,CD14, CD3 and HLA-DR and mean percentage (%) of gated cells out of control **positive** for CD 34, CD90, CD73,CD105, CD49d and **negative** for CD19, CD14,CD3 and HLA-DR at day 0, 7, 14, 21 and 28.

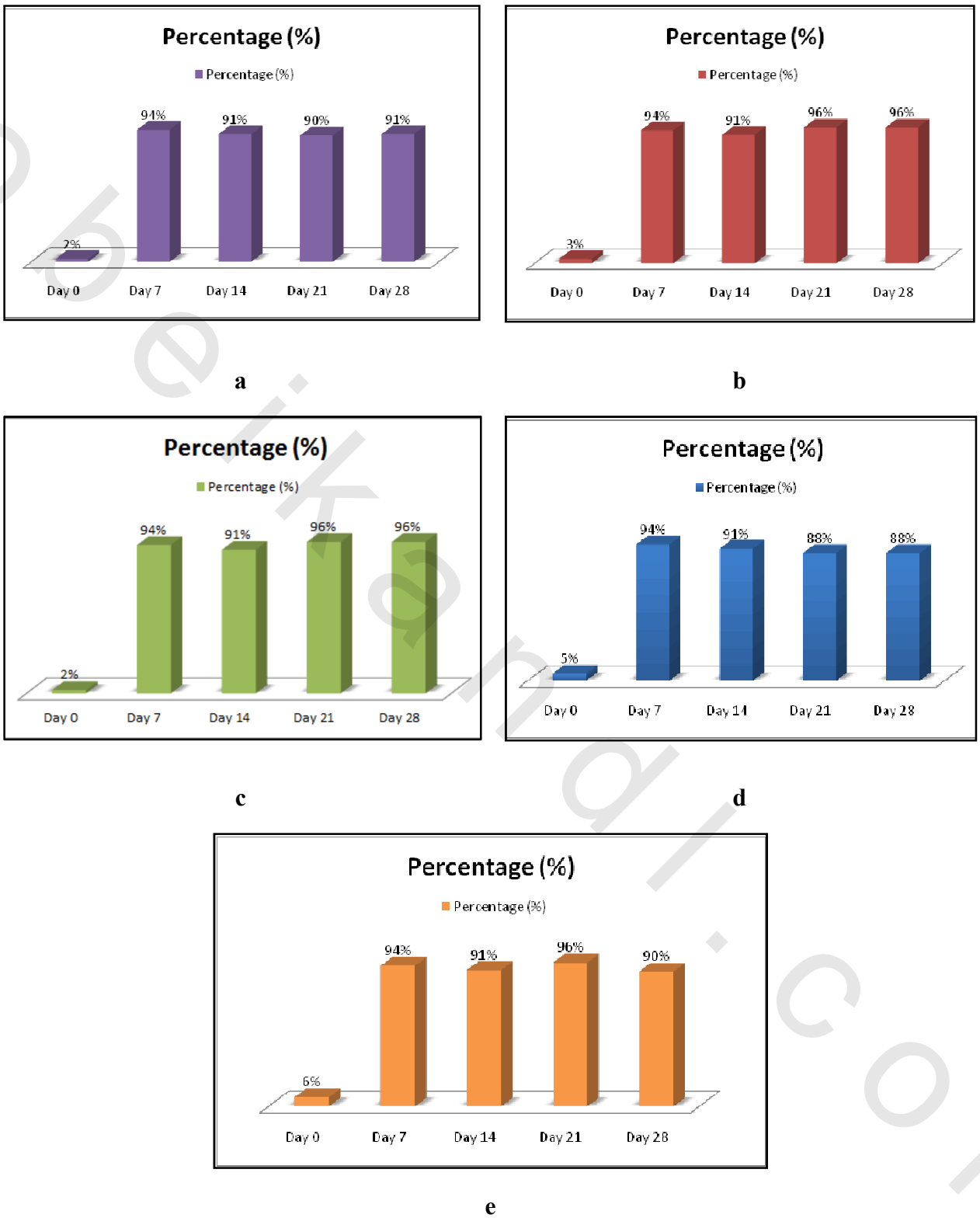


Figure (10): Graph representation of mean percentage (%) of gated cells out of control **positive** for (a) CD 34, (b) CD 90, (c) CD 73, (d) CD 105 and (e) CD49d at day 0,7,14,21 and 28.

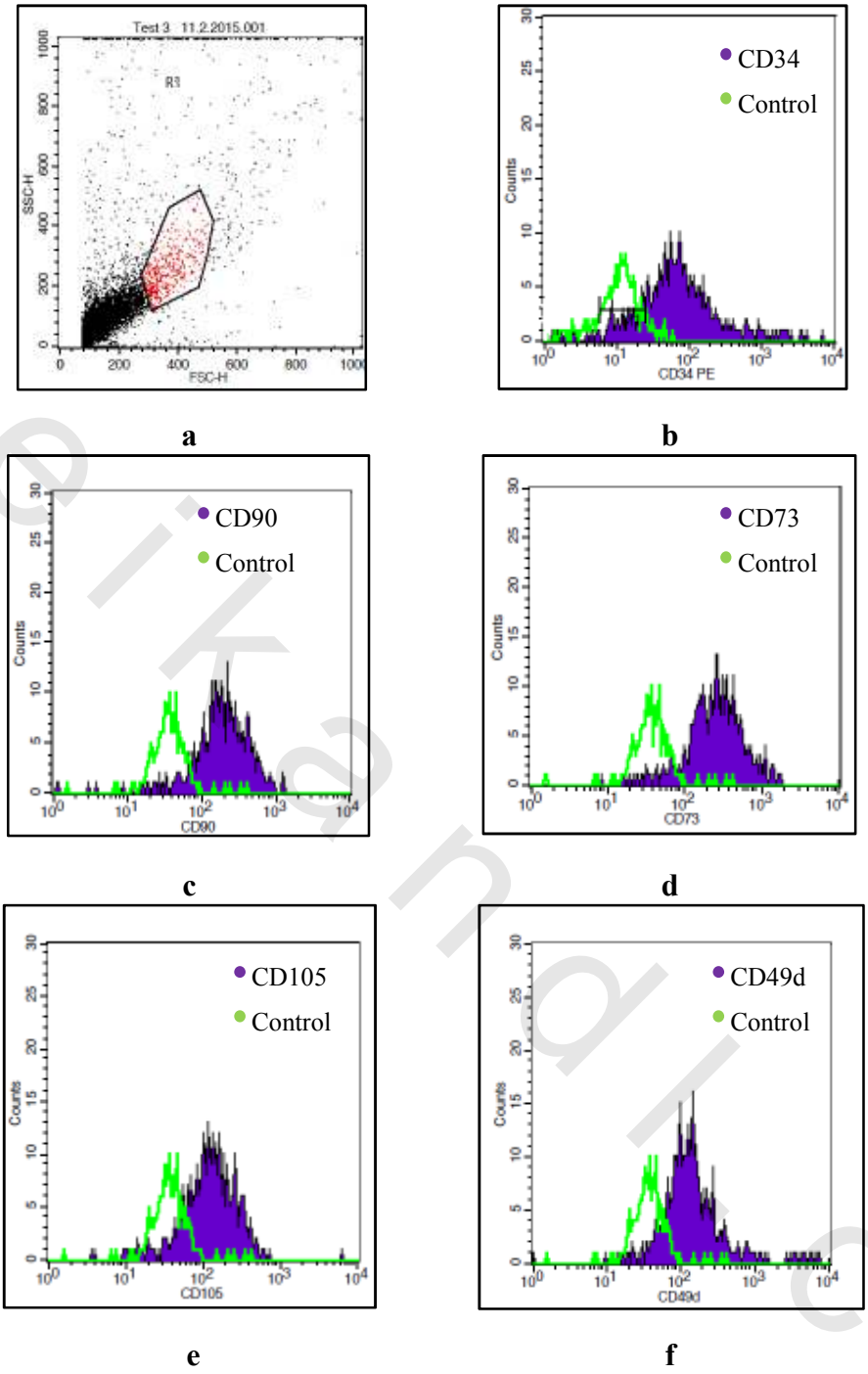


Figure (11): Histogram plots of cultured cells at **day 7** showing: (a): gating of cultured cells. (b) Cells are CD34 positive. (c) Cells are CD90 positive. (d) Cells are CD73 positive. (e) Cells are CD105 positive and (f) cells are CD49d positive. The same results were noticed at day 14, 21 and 28.