ORIGINAL ARTICLE

# Adipose Tissue: All Parts Is Useful *In Vitro* Study About Adipose-Derived Mesenchymal Stem Cell

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**ABSTRACT**

**Introduction:** Adult-adipose tissue produce mesenchymal stem cells (MSCs), known as adipose-derived mesenchymal stem cells (AMSCs). Unlike other source of stem cell (SC), adipose tissue has abundant availability and easy to obtained without any conflicting ethical issues. The aim of the study is to demonstrate that each part of adipose tissue will generates SC with similar MSCs characteristic and specific markers thus no part of it will be waste. **Methods:** Using one-way experimental design, 20 grams of subcutaneous adipose tissue were cultured with different methods to obtained 25 samples that divided into five different group. Sterile gauze, a 100 μm and 70 μm strainer were used in filtration step of culture process. Cell morphology were observed by inverted microscope. Fluorescence microscope was used to analyze expression of CD34, CD45, CD90, and CD105. **Results:** At passage 1, every group adhered to plastic surface with fibroblastic-like morphology in 3rd day. CD34 and CD45 expressions were detected in control group, and gradually decreased among another groups whereas lack of CD45 expression were consistent on other remaining groups. The expression of CD90 and CD105 were visibly increased significantly from control group to fourth group. **Conclusion:** This study demonstrated that either SVF and/or supernatant generates fibroblastic-like cells and surface markers expression that similar to MSCs criteria. Although supernatant still produce better SC than SVF. Therefore, every parts of adipose tissue are an appealing alternative of stem cell source from any available culture methods.

**Keywords:** Adipose tissue, Mesenchymal stem cell, cell culture

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## INTRODUCTION

Heart failure (HF) has high morbidity and mortality rates. HF events may reflects either pathophysiological progression and/or ongoing myocardial injury. To date, most HF treatments focused on preserving heart function without really recognize the cardiomyocytes dysfunction. Although prior HF hospitalization predicts all-cause mortality, most patient improve symptomatically and survive until discharge, this suggest that such patients may be functioning near their respective cardiovascular reserve and prone to be more severe adverse outcome in further episode. Thus, regenerating dysfunctional cardiomyocytes became a new field of interest for treatment approach of HF (1–3).

Stem cells (SC) as part of cell-based therapy has been researched worldwide but the selection of appropriate source of SC for tissue regeneration remains challenging. Whilst the heart has its endogenous SC known as *cardiac progenitor cells* (CPC) with turn-over rate were deemed inadequate in progressive myocardial damage caused by ischemic episodes hence the need for exogenous SC arises (4,5). Adipose tissue has unique advantages in terms of abundant availability, ease of isolation,

high degree of homogeneity, with adequate ability to differentiate into multipotent cells. Those multipotent cells known adipose-derived mesenchymal stem cells (AMSCs) and/or dedifferentiated fat cell (DFAT) were proven to have multi-lineage ability to differentiate into specific cells across all three germinal layers (6–11).

Previously, most AMSCs were obtained from culture techniques with a colony-forming unit-fibroblast (CFU) approach where unpurified SVF are plated directly into petri dish or cell culture plates. These cells were proven to have multi-lineage potential. Further advancement of culture techniques introduced ceiling culture method in order to generates AMSCs with higher purity. Ceiling culture used "a thin, white, floating layer" and plastic culture flask as described by Sugihara et al (12) and Zuk et al (13,14) modified previous method, adding filtration step and used cellular pellet known as stromal vascular factor (SVF). Both methods generated AMSCs with relatively similar profile with different quantity of mesenchymal stem cell (MSCs) markers. We assume addition of filtration step contributed in increased levels of MSCs marker, in accordance to Jumabay, et al and Matsumoto, et al (3,15) studies that used double filtration step in the culture method to obtained AMSCs that later known as DFAT cells. DFAT cells significantly lacked expression of CD34 and CD45 which support the statement that DAFT has higher purity of MSCs. There is no consensus about the most optimal culture techniques or which adipose tissue parts as the best source to generates MSCs. Different culture techniques inherently used different part of adipose tissue thus rendered the opposite part useless. Therefore, based on aforementioned facts, we aim to demonstrate that different adipose tissue parts, SVF and/or supernatant able to give rise to AMSCs, despite different culture methods chosen. In hope that the results of this study can be an alternative to validate the use of every parts of adipose tissue as MSCs source.

## MATERIALS AND METHODS

This research is an *in vitro* experimental research using one-way experimental design. The experiments follow ethical guidelines from Faculty of Medicine Airlangga University, Indonesia No. 62/EC/KEPK/FKUA/2021. This research uses alpha minimum essential medium ($\alpha$MEM) and fetal bovine serum (FBS) purchased from Gibco. Collagenase type I provided from Sigma. CD31, CD34, CD45, CD90, CD105 with FITC label were purchased from Thermo Fisher and Sigma. All materials analytical grade and obtain commercially.

Subcutaneous adipose tissue obtained by abdominoplasty procedure from a single healthy 60 years old man, healthy status confirmed using laboratory blood test and echocardiography. The subject has no medical comorbidity such as diabetes mellitus, history of malignancy or autoimmune disease (Table I). Approximately 20 g of adipose tissue was excised, minced, and washed with phosphate buffer saline (PBS), before undergoing MSC's isolations with predefined culture techniques.

**Table I. Adipose Tissue Donor Baseline Characteristics**

| Gender | Male |
|---|---|
| Age | 60 |
| Blood works | Within normal limit |
| Echocardiography | Within normal limit |
| Diabetes Mellitus | None |
| Malignancy | None |
| Autoimmune diseases | None |
| Height/weight | 175 cm/75kg |
| BMI | 25 |

**Table I: Baseline Characteristics of subject.** Subject was 60 years old man no recorded medical comorbidity such as diabetes mellitus, history of malignancy and or autoimmune disease

Baseline Characteristics of subject. Subject was 60 years old man no recorded medical comorbidity such as diabetes mellitus, history of malignancy and or autoimmune disease.

Tissue sample incubated at 370C for 45 minutes in erlenmeyer flask filled with collagenase type 1, then the samples were given PBS and incubated for 15 minutes and divided into five different conical tube for further use. All sample was centrifuged at 3000rpm for 5 minutes and the control group filtered with sterile gauze to obtain supernatant. Supernatant then moved to 10 cm-petri disk filled with $\alpha$MEM and incubated in $CO_2$ incubator for 20 days, until reached 70-80% confluency. Culture medium replaced every three days.

First experiment group was filtered with sterile gauze. Cell pellet obtained after centrifugation later washed with PBS and added 10 ml $\alpha$MEM then filtered with 100 µm of cell strainer. Pellet then moved into a 10 cm-petri dish filled with $\alpha$MEM and incubated until reached 70-80% confluency, approximately 14 days. Second experiment group use cell pellet obtained from pervious filtration with a 100 µm cell strainer was filtered with 70 µm before moved into 10cm-petri dish filled with $\alpha$MEM. Cells were incubated in $CO_2$ incubator until reached 70-80% confluency, approximately 10-14 days and cultured medium replace every 3 days. Sample for the third experiment group collected into a 50mL conical tube, centrifuged at 3000 rpm for 5 minutes. Supernatant removed and filtered with 100 µm cell strainer. Cell collected after filtration then put into T25 flask. The flask was incubated invertedly for seven days. Flask then reverted into original position after cell colonies were observed before moved into multiwall plate M6 for characterization. Sample for fourth group uses supernatant obtained from previous centrifugation were double-filtered with a 100 µm and 7 0 m cell strainer. Cell collected after filtration then put into

T25 flask, incubated for seven days invertedly in $CO_2$ incubator. Later cells were processed with established insert culture protocol (3). Flask then reverted into original position after cell colonies were detected before moved into multiwall plate M6 for characterization.

After all cell groups reached confluency 70-80%. Culture medium was removed, and cells were washed with PBS and TrypLE Express (Thermo Fisher) before incubated in $CO_2$ incubator for 5 minutes. After confirmed that single cells were attached to the bottom of petri dish, complete culture of medium added and the cell resuspended before moved into conical tube and centrifuged at 2500 rpm for 5 minutes.

For immunocytochemistry analysis, single cell was obtained from each group samples and planted into 96-multiwell plate with estimated cell count 5000 cells/well. After cells were attached to the bottom of the well, fixation process carried out using methanol before incubated for 30 minutes at 40C with the FITC-conjugated antibodies against CD34, CD45, CD90, CD105.

## RESULTS

At passage 1, all cell groups adhered to plastic surface with fibroblastic-like morphology (Fig.1).

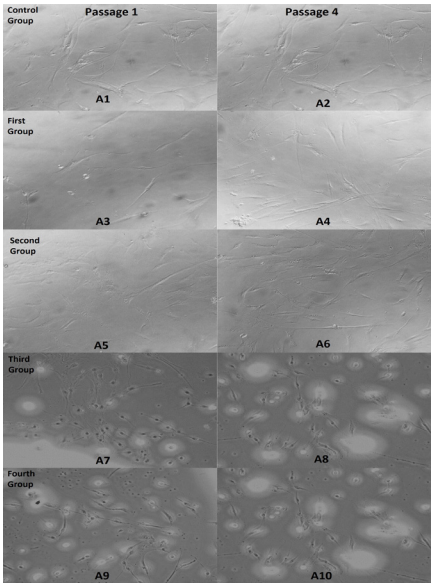


**Fig.1. Comparison of AMSCs and DFAT Morphology Observed by Inverted Microscope.**

(A1) shown fibroblastic-like cells corresponded with MSCs criteria by The International Society for Cellular Therapy at passage 1 and (A2) at passage 4. These cells were obtained from control group through filtering using sterile gauze. (A3) was AMSCs at passage 1 and (A4) at passage 4 were obtained from first group that used SVF as AMSCs source and 100 µm cell strainer on filtration step. A double-filtration method with a 100 µm and 70 µm subsequently was used for second group to obtained DFAT cells (A5) at passage 1 and (A6) at passage 4. Supernatants were source to obtained DFAT cells (A7) at passage 1 and (A8) at passage 4 with single filtration step using 100 µm and a double-filtration method with a 100 µm and 70 µm generated (A9) at passage 1 and (A10) at passage 4.

### Immunocytochemistry of Mesenchymal Stem Cell Marker

In this study, we used four surface markers to characterize MSCs in all five groups. Immunocytochemistry results were shown on Fig.2 for hematopoietic lineage markers, CD34 and CD45; and Fig. 3 for MSCs markers, CD90 and CD105.

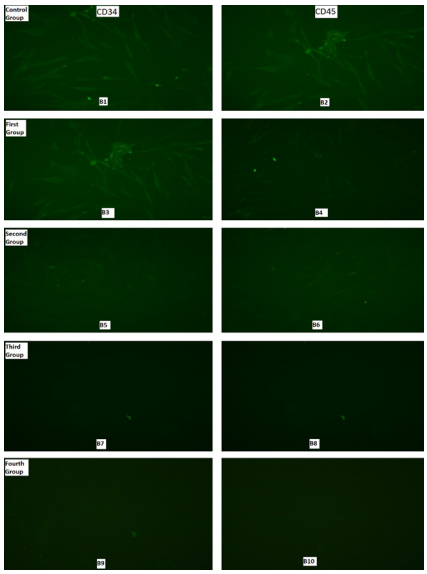


**Fig.2. Immunocytochemistry Images Comparison of CD34 and CD45 Expressions.**

The expressions of hematopoietic markers CD34 (B1) and CD45 (B2) were detected on control group. The first group (B3) shown positive expression of CD34. The lack expressions of CD34 was detected on the second group

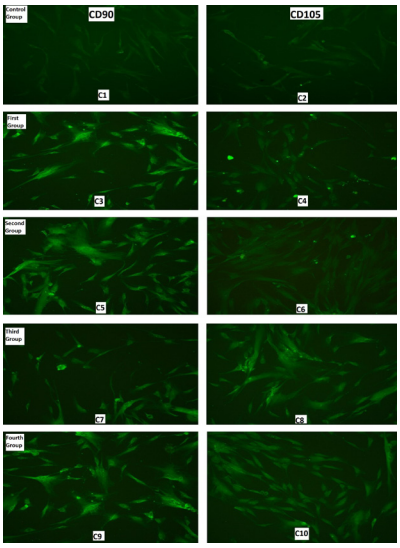


**Fig.3. Immunocytochemistry Images Comparison of CD90 and CD105 Expressions.**

(B5). The CD45 expression was visibly decreased further on first group (B4) and second group (B6) subsequently, whereas on third and fourth group, no CD34 (B7 and B9) and CD45 (B8 and B10) markers expression were detected.

The MSCs markers CD90 (C1) and CD105 (C2) were detected on control group. AMSCs generated from SVF and single filtration shown visibly higher expression of CD90 (C3) and CD105 (C4). A higher value of CD90 (C5) and CD105 (C6) and simultaneously increase cell counts were detected on second group. Both third and fourth group used supernatant as MSCs source. A single filtration method generates significant expression of CD45 (C7) and CD105 (C8) and a double-filtration method shown a visibly significant increase of expression and cell counts of CD45 (C9) and CD105 (C10).

## DISCUSSION

Based on International Society Cell and Gene Therapy (ISCT) criteria, multipotent mesenchymal stromal cells (MSCs) are reported to express CD105 (SH2), CD73 (SH3/SH4), CD29, CD44, CD90, CD71, CD106, STRO-1, GD2, and CD146, and lacks of expression of CD45, CD34, CD14, CD11b, CD79a, CD19, and class II histocompatibility complex antigens (human leukocyte antigens class II). MSCs derived from tissue, such as adipose and/or peripheral blood may express several hematopoietic markers such as CD31 and CD34, but consistently lack the expression of CD45 (16).

DFAT cells in passage 0 will show positive expression of the markers CD13 (aminopeptidase N), CD73 (5′-nucleotidase), CD90 (Thy-I), and CD105 (endoglin), and negative of the CD14 marker (myelomonocytic differentiation antigen). Meanwhile, there is still controversy regarding the expression of the markers CD34 (hematopoietic progenitor cell antigen) and CD45 (protein tyrosine phosphatase, receptor C), which will be expressed in amounts less than 1%. AMSCs surface antigen profile expression at passage 0 found similar with DFAT cells (Table II) (17).

**Table II. Comparison of Surface Markers Expression Between AMSCs and DFAT Cells according to Immunocytochemistry Analysis.**

| Antigen | AMSCs | DFAT |
|---|---|---|
| | Percentage | Percentage |
| α SMA | >2% | <2% |
| CD14 | 0% | 0% |
| CD31 | 8% | 1% |
| CD34 | <1% | 0% |
| CD44 | 88% | 89% |
| CD45 | 2% | <1% |
| CD13 | 99% | 100% |
| CD73 | 98% | 95% |
| CD90 | 99% | 99% |
| CD105 | 99% | 100% |
| CD106 | 1% | 0% |
| CD117 | <1% | <1% |

Table II. Comparison of Surface Markers Expression Between AMSCs and DFAT Cells according to Immunocytochemistry Analysis. (Modified from (17–19))

Culture technique that generates DFAT cells start with filtration through cell strainer (core size 100 µm) and centrifugation. The floating top layer of adipocytes mixture was collected and washed repeatedly, then floating adipocytes used for generated DFAT cell filtered with 70 µm-filters and incubated for 5 days. The adipocytes that sink through the filters and attach to bottom of the dishes called DFAT (3,7). This study using cell strainer (70 and 100 µm) for filtration of single cells and isolation cellular aggregates (11,18). DFAT will produce cell types that have a similar morphology to AMSCs and lacked expression of CD34 and CD45 (17,19). This cells have active proliferative capabilities and able to differentiate into osteoblasts, chondrocytes, cardiomyocytes, or re-differentiate back into adipocytes under the influence of the appropriate culture (20–22).

In this study (see Table III), we demonstrated that each part of adipose tissue, whether SVF or supernatant layer exhibit similar AMSCs characteristic and specific markers. Control group, in which adipose tissue filtered with sterile gauze to obtained supernatant, showed CD105 (+) and CD90 (+), whereas CD34 (+) and CD 45 slightly negative (±). It seems control group contains mixture of hemopoietic stem cell and mesenchymal stem cell. The first group using 100 µm to filtered SVF pellet and moved into petri dish (12) and generate cells that expressed MSCs characteristic almost same as control group that expressed MSCs marker CD34 slightly negative (±) and negative of CD45 (-).

**Table III. Summary of Mesenchymal Stem Cell Immunocytochemistry Results**

| | CD45 | CD34 | CD90 | CD105 |
|---|---|---|---|---|
| Control | ± | + | + | + |
| First group | - | ± | + | + |
| Second group | - | - | + | ++ |
| Third group | - | - | ++ | ++ |
| Fourth group | - | - | ++ | ++ |

**Table III**: Summary of Mesenchymal Stem Cell Immunocytochemistry Results. Control group still expressed hemopoietic markers CD34 (+) and CD45 (±). First group slightly expressed CD34 (±) only as a hemopoietic markers. But second, third and fourth group showed negative expression of hemopoietic markers CD34 (-) and CD45 (-).

Control group still expressed hemopoietic markers CD34 (+) and CD45 (±). First group slightly expressed CD34 (±) only as a hemopoietic markers. But second, third and fourth group showed negative expression of hemopoietic markers CD34 (-) and CD45 (-).

The second group, SVF pellet undergo double-filtration

step using a 100 µm and 70 µm (13). This process different from ceiling culture technique to generate DFAT cells. The result showed CD105 slightly higher (++) and CD90 (+) were positive, whereas CD45 and CD34 were found negative (-). Based on the result of specific markers, cell that generate from this procedure were characteristically similars to AMSCs. The third and fourth groups, which generated DFAT from supernatant layer, showed visibly significant expression increment of CD105 (++) and CD90 (++) whilst expression of CD34 (-) and CD45 (-) were consistent. This study showed that cells obtain from supernatant (third group and fourth group) can generate stem cell with characteristic of Mesenchymal Stem Cells better than cells derived from pellet (first group and second group). This study also showed that one step filtering of supernatant with 100 µm strainer as good as two step filtering with 100 µm and 70 µm strainer to generate cell with specific markers of DFAT cells.

In this study, we only demonstrated that AMSCs and DFAT cells produced from highly purified adipocytes had the same characteristics as MSCs criteria by ISCT, all data were reported descriptively therefore we weren't able to analyze the association between different culture techniques and MSCs marker expression. Immunocytochemistry was not a golden standard method to characterized MSCs therefore other method such as flow cytometry and/or reverse transcriptase polymerase chain reaction (RT-PCR) were needed to validate and further establish multipotency level of AMSCs and/or DFAT cells. Further study to analyze the correlation between MSCs marker expressions and multipotency levels and differentiation ability into other specific cells are also encouraged.

## CONCLUSION

Immunocytochemistry analysis of MSCs markers showed that AMSCs can be obtained both from SVF pellets and supernatant parts of adult adipose tissue regardless of the culture technique used. SCs that obtained from supernatant are better than pellet. A double filtration method both on SVF and supernatant visibly increase expressions quality of CD45 and CD105 as well as significantly lowered the expressions of CD34 and CD45. Although single filtration also can be used as a source of AMSC. Our results should promote further clinical and laboratory study and application of AMSCs and DFAT cells.

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