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Protocol for obtaining and characterizing the stromal vascular fraction of human adipose tissue by mechanical dissociation: an original article

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Abstract

The present technical study presented the mechanical dissociation protocol of adipose tissue from manual liposuction, as well as showed the immunobiological characterization (immunophenotyping), cell viability of the stromal vascular fraction of human adipose tissue, and cell concentration per milliliter. It was observed that the technique of the present study obtained a characterization yield of the stromal vascular fraction of the adipose tissue in terms of cell viability, cell concentration per milliliter, and immunophenotyping comparable and superior to most published studies with good scientific evidence.

Keywords: Adipose tissue. Stromal vascular fraction. Mechanical technique. Optimization.

Introduction

In the regenerative medicine scenario, the identification of cells from the stromal vascular fraction (SVF) of human adipose tissue has opened a promising new field [1,2]. After decades of dealing with liposuction and fat grafting, it is possible to harvest adipose tissue in almost any desirable volume, quickly and easily and with relatively low morbidity, representing an unprecedented source of adipose-derived stem cell (ADSC) [3-10].

In this sense, cell-based therapies using liposuction are gaining popularity due to their regenerative potential [1,2,11]. Various liposuction processing devices/systems have become available to isolate cells for therapeutic use, with published evidence reporting their clinical relevance. However, few studies have parallel analyzed the composition of their minimally manipulated cellular products, vital information to understand the mechanisms by which these therapies can be effective [12].

In this context, the practical applications of cell therapy are still limited, but this new perspective of potential applications introduces a concern with the quality of disposable fat as a source of donor cells and liposuction procedures must be properly used and must be optimized in this sense. Liposuction is the method of choice for ADSC extraction, with variations in protocols using all the fat obtained or even just the saline/blood fraction of liposuctions, but tissue harvesting procedures are often underestimated as a factor to impact results. The laboratory protocols for extracting ADSC from the material obtained should also be duly evaluated, from simple cell counts to its characterization as mesenchymal tissue and its biological properties *in vitro* [11,12].

Therefore, the present technical study presented the mechanical dissociation protocol of adipose tissue from manual liposuction, as well as showed the immunobiological characterization (immunophenotyping), cell viability of the stromal vascular fraction of human adipose tissue, and cell concentration per milliliter.

Methods

Mechanical Dissociation Protocol

After the liposuction process in the operating room, 30.0 mL of adipose tissue was voluntarily donated by the patient for the characterization of the present

research protocol and was placed in sterile 15 mL falcon tubes for sample homogenization. 10 samples were established from the 30 mL of liposuction. After that, the adipose tissue was aspirated in a 3 mL needleless syringe. The adipose tissue was subjected to mechanical dissociation using two syringes connected to pass the sample in both directions until obtaining a highly liquefied sample, with the disaggregation and separation of cells from the stromal vascular fraction (cellular solution). The solution containing the dissociated cells was placed in syringes that remained in an upright position for ten minutes. Then, the cell solution was centrifuged at 800xg to separate cell debris and mature adipocytes. The collected material was again centrifuged for 10 minutes at 800xg. The precipitate (pellet) obtained was resuspended in 1.0 mL of sterile hospital saline solution, and 10 μL was removed for counting and determination of cell viability and flow cytometry analysis (immunophenotyping).

Cell count and viability

Trypan blue is a "vital dye", that is, it crosses the membrane of dead cells, but not living cells. Thus, by resuspending the cells in a trypan blue solution, it is possible to assess the viability of the suspension. Counting the number of cells in a suspension, using the Neubauer chamber, is based on counting the number of cells in a sample of a defined volume, which is then converted into the number of cells per mL or the total number of cells in the sample, with the appropriate formulas. According to Dominici et al. 2006 [8], 10.0 uL of cell solution was placed in the Neubauer chamber for quantification of live and dead cells, with replicates of n=3.0. The Neubauer chamber has its central strip in a millimetrically determined position below the sides, so that the placement of a coverslip, well adhered to the edges of the depression, originates a chamber with a well-determined volume. After placing a sample of the cell suspension in this chamber, so that it is filled, but without extravasation, the viable (unstained) cells are counted under the microscope. The chamber has four quadrants (Q1, Q2, Q3, and Q4) with an area of $0.1mm^2$ each. By integrating a glass coverslip into the Neubauer Chamber, a height of 0.1 mm is obtained.

- Calculation of cell viability:

Cell viability (%) = number of live cells / number of total cells (live + dead) (Equation 02) [8].

Immunophenotyping Assay

After removing the stromal vascular fraction from the falcon tubes, they were suspended in 1.0 mL of PBS supplemented with 3.0% human albumin (Human Albumin Grifols® 20.0%, PBS/A). The resulting suspension was subjected to the determination of the number and proportion of non-viable cells by the Trypan blue staining method, followed by adjustment of the viable cell density to 106 cells mL^{-1}. The immunophenotypic evaluation of these cells was performed using the immunophenotyping technique by flow cytometry in a FACSCanto II® cytometer (BD Biosciences), using monoclonal antibodies conjugated with fluorochromes, and their respective isotypic controls. Fresh FEV cells homogenized in PBS/A were aliquoted in 100.0 μL and dispensed into 5.0 mL tubes for flow cytometry. Afterward, 5.0 μL of each antibody was added to the tubes and homogenized by vortexing. The suspensions were then incubated for 15.0 min at room temperature, in the dark. Then, 400.0 μL of PBS/A was added to each tube, followed by homogenization by shaking and centrifugation at 400xg for 10.0 min. The supernatant was discarded and the pellet was homogenized in 500.0 μL of PBS/A. To tube 3 of each sample, 5.0 μL of 7-AAD was added moments before acquisition in the cytometer. The CTMA was then acquired in a cytometer coupled to the analysis software BD FACSDiva II® (BD Biosciences) and subsequently analyzed using the Infinicyt® software (Cytognos) for the binding/marking of each antibody (marker expression) and 7-AAD (non-viable cells) [8].

Ethical Approval

The present study was approved by the Research Ethics Committee of the Hospital Beneficência Portuguesa de São José do Rio Preto/SP, under approval process number 030427/2015. The term of consent was applied before the analysis of *in vitro* cell samples from adipose tissue.

Results and Discussion

Table 1 presents the results of cell concentration (cells/mL) and cell viability (%) of the 10 samples obtained from the stromal vascular fraction of adipose tissue through mechanical dissociation. Analyzes were performed immediately after liposuction collection.

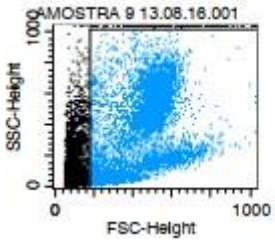
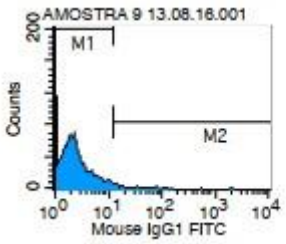
Table 2 presents the results of immunophenotyping of the stromal vascular fraction of a representative sample of ten samples. The CD44+ surface marker is highly specific for adipose mesenchymal stem cells. Analyzes were performed immediately after liposuction was collected through mechanical dissociation.

Table 1. Cell quantification values after mechanical dissociation and sample viability values, n=10.

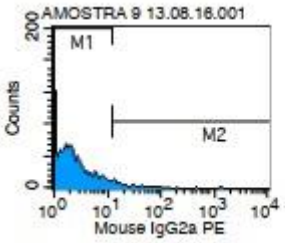
Samples (n=10)	Cell Quantification (x10^7) /mL	Cellular Viability (%)
1.	1.2	99.8
2.	1.4	99.5
3.	1.5	99.7
4.	1.0	99.6
5.	1.0	99.4
6.	1.3	98.6
7.	1.4	99.6
8.	1.2	99.4
9.	1.6	99.5
10.	1.7	99.6
Média (±SD)	**1.3 (± 0.2)**	**99.5 (± 0.3)**

Source: Own authorship.

Table 2. Immunophenotyping results of the stromal vascular fraction of a representative sample of ten samples. The CD44+ surface marker is highly specific for adipose mesenchymal stem cells (n=10).

ADSC surface markers	Graphics-Immunophenotyping-ADSC
SSC vs. FSC	 File: AMOSTRA 9 13.08.16.001 Sample ID: Gate: No Gate Gated Events: 10000 Total Events: 10000 Region Events % Gated % Total X Mean Y Mean PxPy R1 7207 72.07 72.07 446.64 374.87 1, 2
Control 1	 File: AMOSTRA 9 13.08.16.001 Sample ID: Patient ID: Gate: G1 Gated Events: 7207 Total Events: 10000 Marker Left, Right Events % Gated % Total Mean CV Median All 1, 9910 7207 100.00 72.07 3.79 784.90 2.09 M1 1, 12 7055 97.89 70.55 2.70 72.47 2.07 M2 12, 9910 153 2.12 1.53 53.93 386.65 17.78

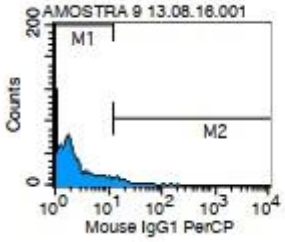
Control 2



File: AMOSTRA 9 13.08.16.001 Sample ID:
Gate: G1 Gated Events: 7207
Total Events: 10000

Marker	Left, Right	Events	% Gated	% Total	Mean	CV	Median
All	1, 9910	7207	100.00	72.07	3.75	535.61	1.88
M1	1, 12	6953	96.48	69.53	2.61	83.14	1.83
M2	12, 9910	262	3.64	2.62	34.41	291.22	17.08

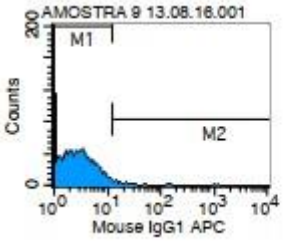
Control 3



File: AMOSTRA 9 13.08.16.001 Log Data Units: Linear Values
Sample ID: Gate: G1

Marker	Left, Right	Events	% Gated	% Total	Mean	CV	Median
All	1, 9910	7207	100.00	72.07	2.99	189.80	1.63
M1	1, 12	6972	96.74	69.72	2.26	87.81	1.60
M2	12, 9910	238	3.30	2.38	24.36	81.02	17.78

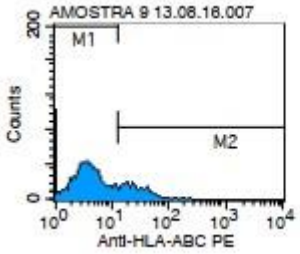
Control 4



File: AMOSTRA 9 13.08.16.001 Log Data Units: Linear Values
Gate: G1

Marker	Left, Right	Events	% Gated	% Total	Mean	CV	Median
All	1, 9910	7207	100.00	72.07	3.63	447.35	2.41
M1	1, 12	7093	98.42	70.93	3.08	72.66	2.39
M2	12, 9910	119	1.65	1.19	36.88	328.18	14.99

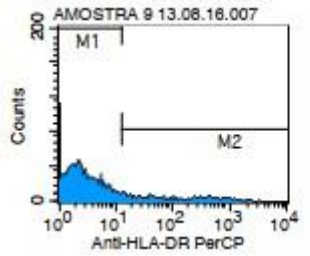
Control 5



File: AMOSTRA 9 13.08.16.007 Log Data Units: Linear Values
Gate: G1

Marker	Left, Right	Events	% Gated	% Total	Mean	CV	Median
All	1, 9910	7260	100.00	72.60	8.49	155.46	3.89
M1	1, 12	5866	80.58	58.66	3.72	67.44	3.25
M2	12, 9910	1422	19.53	14.22	28.16	69.68	22.47

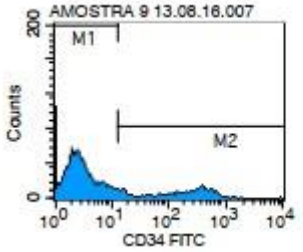
Control 6



File: AMOSTRA 9 13.08.16.007 Log Data Units: Linear Values
Gate: G1

Marker	Left, Right	Events	% Gated	% Total	Mean	CV	Median
All	1, 9910	7280	100.00	72.80	31.75	421.29	2.54
M1	1, 12	6347	87.18	63.47	3.10	76.00	2.25
M2	12, 9910	937	12.87	9.37	225.75	137.15	64.36

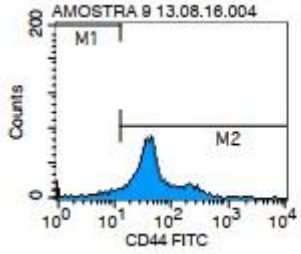
CD34



File: AMOSTRA 9 13.08.16.007 Log Data Units: Linear Values
Gate: G1

Marker	Left, Right	Events	% Gated	% Total	Mean	CV	Median
All	1, 9910	7280	100.00	72.80	55.62	299.16	3.05
M1	1, 12	5806	79.75	58.06	3.29	67.02	2.57
M2	12, 9910	1477	20.29	14.77	261.24	110.57	237.14

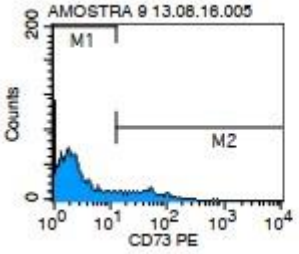
CD44



File: AMOSTRA 9 13.08.16.004 Log Data Units: Linear Values
Sample ID: Gate: G1

Marker	Left, Right	Events	% Gated	% Total	Mean	CV	Median
All	1, 9910	7558	100.00	75.58	71.06	218.10	39.60
M1	1, 12	548	7.25	5.48	6.93	51.01	6.98
M2	12, 9910	7020	92.88	70.20	75.98	210.24	41.05

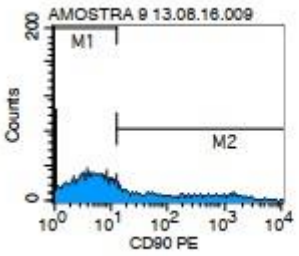
CD73



File: AMOSTRA 9 13.08.16.005 Log Data Units: Linear Values
Gate: G1

Marker	Left, Right	Events	% Gated	% Total	Mean	CV	Median
All	1, 9910	7838	100.00	78.38	9.87	253.03	2.19
M1	1, 12	6602	84.23	66.02	2.54	78.69	1.92
M2	12, 9910	1242	15.85	12.42	48.85	94.09	36.19

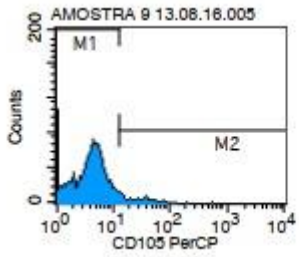
CD90



File: AMOSTRA 9 13.08.16.009 Log Data Units: Linear Values
Gate: G1

Marker	Left, Right	Events	% Gated	% Total	Mean	CV	Median
All	1, 9910	7718	100.00	77.18	152.13	379.96	5.99
M1	1, 12	5385	69.77	53.85	4.56	64.97	3.85
M2	12, 9910	2347	30.41	23.47	489.87	197.39	86.60

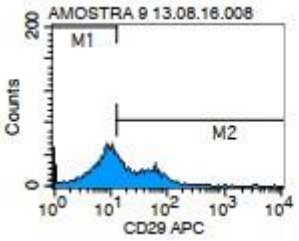
CD105



File: AMOSTRA 9 13.08.16.005 Log Data Units: Linear Values
Gate: G1

Marker	Left, Right	Events	% Gated	% Total	Mean	CV	Median
All	1, 9910	7838	100.00	78.38	5.70	158.70	4.26
M1	1, 12	7411	94.55	74.11	4.28	54.32	4.10
M2	12, 9910	438	5.59	4.38	29.74	92.75	24.47

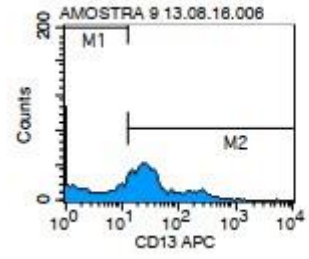
CD29



File: AMOSTRA 9 13.08.16.008 Log Data Units: Linear Values
Gate: G1

Marker	Left, Right	Events	% Gated	% Total	Mean	CV	Median
All	1, 9910	7497	100.00	74.97	28.10	676.19	10.94
M1	1, 12	4165	55.56	41.65	7.13	40.49	7.37
M2	12, 9910	3361	44.83	33.61	53.96	522.00	32.78

CD13



File: AMOSTRA 9 13.08.16.006 Log Data Units: Linear Values
Gate: G1

Marker	Left, Right	Events	% Gated	% Total	Mean	CV	Median
All	1, 9910	7602	100.00	76.02	42.38	223.55	19.11
M1	1, 12	2602	34.23	26.02	4.15	87.76	2.25
M2	12, 9910	5018	66.01	50.18	62.09	179.69	27.63

Source: Own authorship.

Table 3 shows the results of the main surface markers of mesenchymal stem cells from adipose tissue, values per sample, and the overall average of each surface marker, with the CD44+ surface marker showing a yield of 75.1 ± 12.6%. This result corroborates that the technique of this study optimized the obtaining of the stromal vascular fraction through the proposed mechanical dissociation technique.

Table 3. Results of the main surface markers of mesenchymal stem cells from adipose tissue, values per sample, and general mean of each surface marker. Note: The CD44+ surface marker is highly specific for mesenchymal stem cells.

Samples (n=10) / ADSC surface markers	CD 44 (%)	CD 73 (%)	CD 90 (%)	CD 105 (%)
1.	70.2	14.5	42.4	13.2
2.	71.3	12.5	23.47	4.38
3.	70.3	11.5	24.65	3.48
4.	71.5	11.2	23.48	4.31
5.	84.3	10.5	23.6	4.46
6.	77.4	12.1	31	4.8
7.	95.7	12.3	24.4	4.51
8.	65.6	12.6	23.15	4.63
9.	75.6	11.9	24	5.7
10.	68.8	12.7	42.4	13.2
Média (±SD)	**75.1 (±12.6)**	**12.2 (±2.1)**	**28.25 (±19.5)**	**6.3 (±8.2)**

Source: Own authorship.

The present study presented results comparable to 15 studies that were published on techniques of mechanical dissociation of adipose tissue to obtain the stromal vascular fraction. The 15 studies were analyzed and published by the authors Liu et al. 2022 through a systematic review [12]. All studies reported the cell concentration (number of cells regardless of phenotype per milliliter of liposuction) for their devices/systems (range 0.005-21×106) [12]. The present study was able to obtain an average of 1.3 ± 0.2 x107 cells/mL, that is, about one thousand more cells per mL. Also in the study by Liu et al. 2022, ten articles reported cell viability (the measure of live cells ranging from 60 to 98%) [12]. In the present study, cell viability was 99.5± 0.3%, which is higher than the average of published articles. Furthermore, in the study by Liu et al. 2022, 11 articles performed immunophenotypic analyzes of cell subtypes [12]. In the present study, a yield of 75.1 ±12.6% was obtained for the CD44+ cell surface marker, which is highly specific for mesenchymal stem cells.

Conclusion

It was concluded that the technique of the present study obtained a characterization yield of the stromal vascular fraction of adipose tissue in terms of cell viability, cell concentration per milliliter, and immunophenotyping comparable and superior to most published studies with good scientific evidence.

Acknowledgement

Not applicable.

Funding

Not applicable.

Ethics approval

The present study was approved by the Research Ethics Committee of the Hospital Beneficência Portuguesa de São José do Rio Preto/SP, under approval process number 030427/2015. The term of consent was applied before the analysis of *in vitro* cell samples from adipose tissue.

Informed consent

The term was applied.

Data sharing statement

No additional data are available.

Conflict of interest

The authors declare no conflict of interest.

Similarity check

It was applied by Ithenticate@.

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