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Technique for Obtaining Mesenchymal Stem Cell from Adipose Tissue and Stromal Vascular Fraction Characterization in Long-Term Cryopreservation

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TITLE:

Technique for Obtaining Mesenchymal Stem Cell from Adipose Tissue and Stromal Vascular Fraction Characterization in Long-Term Cryopreservation

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KEYWORDS:

Mesenchymal stem cell; adipose tissue; stem cells; abdominal liposuction; cell therapy; protocol.

SUMMARY:

The present protocol describes an improved methodology for ADSC isolation resulting in a tremendous cellular yield with time gain compared to the literature. This study also provides a straightforward method for obtaining a relatively large number of viable cells after long-term cryopreservation.

ABSTRACT:

Human mesenchymal stem cells derived from adipose tissue have become increasingly attractive as they show appropriate features and are an accessible source for regenerative clinical applications. Different protocols have been used to obtain adipose-derived stem cells. This article describes different steps of an improved time-saving protocol to obtain a more significant amount of ADSC, showing how to cryopreserve and thaw ADSC to obtain viable cells for culture expansion. One hundred milliliters of lipoaspirate were collected, using a 26 cm three-hole and 3 mm caliber syringe liposuction, from the abdominal area of nine patients who subsequently underwent elective abdominoplasty. The stem cells isolation was carried out with a series of

washes with Dulbecco's Phosphate Buffered Saline (DPBS) solution supplemented with calcium and the use of collagenase. Stromal Vascular Fraction (SVF) cells were cryopreserved, and their viability was checked by immunophenotyping. The SVF cellular yield was 15.7 x 10^5 cells/mL, ranging between 6.1–26.2 cells/mL. Adherent SVF cells reached confluence after an average of 7.5 (±4.5) days, with an average cellular yield of 12.3 (± 5.7) x 10^5 cells/mL. The viability of thawed SVF after 8 months, 1 year, and 2 years ranged between 23.06%–72.34% with an average of 47.7% (±24.64) with the lowest viability correlating with cases of two-year freezing. The use of DPBS solution supplemented with calcium and bag resting times for fat precipitation with a shorter time of collagenase digestion resulted in an increased stem cell final cellular yield. The detailed procedure for obtaining high yields of viable stem cells was more efficient regarding time and cellular yield than the techniques from previous studies. Even after a long period of cryopreservation, viable ADSC cells were found in the SVF.

INTRODUCTION:

Human mesenchymal stem cells are advantageous in both basic and applied research. The use of this adult cell type overpasses ethical issues—compared to the use of embryonic or other cells—being one of the most promising areas of study in autologous tissue regeneration engineering and cell therapy[1], such as the neoplastic area, the treatment of degenerative diseases, and therapeutic applications in the reconstructive surgery area[2–5]. It has been previously reported that there is an abundant source of mesenchymal multipotent and pluripotent stem cells in the stromal vascular cell fraction of adipose tissue[6,7]. These ADSC are considered great candidates for use in cell therapy and transplantation/infusion since a considerable number of cells with a strong capacity for expansion *ex vivo* can be easily obtained with a high yield from a minimal invasive procedure[5,8].

It was also demonstrated that adipose tissue presents a greater capacity to provide mesenchymal stem cells than two other sources (bone marrow and umbilical cord tissue)[9]. Besides being poorly immunogenic and having a high ability to integrate into the host tissue and to interact with the surrounding tissues[4,10], ADSC has a multipotent capacity of differentiation into cell lines, with reports of chondrogenic, osteogenic, and myogenic differentiation under appropriate culture conditions[11–13], and into cells, such as pancreatic, hepatocytes, and neurogenic cells[14–16].

The scientific community agrees that the mesenchymal stem cells' immunomodulatory effect is a more relevant mechanism of action for cell therapy[17–19] than their differentiation property. One of the most significant merits of the ADSC use is the possibility of autologous infusion or grafting, becoming an alternative treatment for several diseases. For regenerative medicine, ADSC have already been used in cases of liver damage, reconstruction of cardiac muscle, regeneration of nervous tissue, improvement of skeletal muscle function, bone regeneration, cancer therapy, and diabetes treatment[20,21].

To this date, there are 263 registered clinical trials for the evaluation of ADSC's potential, listed on the website of the United States National Institutes of Health[22]. Different protocols to harvest adipose tissue have been established, but there is no consensus in the literature about a standardized method to isolate ADSC for clinical use[23,24]. Lipoaspirate processing methods during

and after surgery can directly affect cell viability, the final cellular yield[25], and the quality of the ADSC population[20]. Regarding the surgical pre-treatment, it is not well established which surgical pre-treatment technique yields a more significant number of viable cells after isolation or whether the anesthetic solution injected into adipose tissue affects cell yield and its functions[26]. Similarly, the difference between techniques for obtaining adipose cells can lead to as much as a 70% decrease in the number of viable ADSC[20]. According to the literature, mechanical treatments to obtain cell populations with high viability—including ultrasound—should be avoided, for they can break down the adipose tissue[20]. However, the manual fat aspiration method with syringes is less harmful, causing less cell destruction, with tumescent liposuction yielding a significant number of cells with the best quality[26].

This technique uses a saline solution with lidocaine and epinephrine that is injected into the liposuction area. For each 3 mL volume of solution injected, 1 mL is aspirated. In this study, the wet liposuction technique was performed, in which for each 1 mL of adrenaline and saline solution injected, 0.2 mL of adipose tissue is aspirated. The use of digestive enzymes, especially collagenase, is common for the process of isolating ADSC.

After the first isolation step in the laboratory, the final pellet is called stromal vascular fraction (SVF). It contains different cell types[27], including endothelial precursor cells, endothelial cells, macrophages, smooth muscle cells, lymphocytes, pericytes, pre-adipocytes, and ADSCs, which are capable of adhesion. Once the final isolation is concluded from *in vitro* cultures, cells that did not adhere to the plastic are eliminated in medium exchanges. After eight weeks of expansion, medium changes, and passages, ADSCs represent most of the cell population in the flasks[20]. One of the most significant advantages of using isolated adipose-derived stem cells for a possible future therapy is the possibility of cryopreservation. It was demonstrated that cryopreserved lipoaspirate is a potential source of SVF cells even after 6 weeks of freezing[28], with biological activity even after 2 years of cryopreservation[29], and full capability to grow and differentiate in culture[30]. However, during the thawing process, a considerable percentage of cells is usually lost[31]. Therefore, the lipoaspirate removal process and the following methods of cell isolation must ensure the highest cell yield.

This study describes a faster methodology for collecting and isolating ADSC, demonstrating high cellular yield and viability for better efficiency of cellular therapeutics. Furthermore, the effect of this improved technique after long-term SVF cryopreservation was evaluated.

PROTOCOL:

The present study is approved by the Ethics Committee of the UNIFESP (protocol number: 0029/2015 CAAE: 40846215.0.0000.5505), performed after obtaining written informed consent from the patients according to the Declaration of Helsinki (2004). The sample of the present study is composed of nine female patients, aged 33–50 years (average age 41.5) and average initial body mass index (BMI) of 24.54 (ranging between 22.32–26.77) (**Table 1**) who underwent aesthetic abdominoplasty due to excess of skin after pregnancies, at the Division of Plastic Surgery of the Universidade Federal de São Paulo (UNIFESP), Brazil. To reduce bias, the patients were selected as a homogeneous group considering sex, age, and BMI. The datasets used and/or

analyzed during this study are available from the corresponding author upon reasonable request.

1. Collection of lipoaspirate

NOTE: This step needs to be performed in the surgery center.

1.1. Use 4% chlorhexidine gluconate (see **Table of Materials**) for skin preparation and asepsis.

1.1.1. Perform a 2 mm subcutaneous skin incision (between the sub-dermis and aponeurosis). Insert a Klein cannula of 26 mm 3 G three-hole and 3 mm caliber and a syringe to inject a total volume of 500 mL of an adrenaline solution (1 mg/mL) (see **Table of Materials**) diluted in saline (1:1,000,000) in the infraumbilical area.

1.2. Connect a 60 mL syringe to a 26 mm 3 G three-hole and 3 mm caliber liposuction cannula and insert it through the skin incision, locking the plunger to create a vacuum.

1.2.1. Make pushing and pulling movements so that, with the vacuum created, the lipoaspirate remains in the 60 mL syringe.

1.3. Using a sterile connector with a valve, transfer the 100 mL of the collected lipoaspirate to a 150 mL polyvinyl chloride transfer bag (see **Table of Materials**).

1.3.1. Pack the transfer bag in a polystyrene box at room temperature (~25 °C) and take it immediately to the laboratory. Do not take longer than 30 min to start the tissue processing.

2. Processing of lipoaspirate

NOTE: This step is to be performed in the laboratory.

2.1. First, weigh the bag, gauge the temperature with a digital non-contact infrared clinical thermometer, and leave the bag resting for 5 min inside the laminar flow chamber for precipitation of the greasier layers (bubbles) and tissue separation containing the cells of interest.

2.1.1. Perform a series of tissue washes. First wash: inject 100 mL of DPBS with calcium (1x) into the transfer bag and mix it with the hands.

2.1.2. Let it stand for 5 min and remove most of the basal liquid that precipitates.

2.1.3. Discard the basal liquid with a 60 mL syringe attached to the bag adapter. This process must be repeated twice.

2.2. Add 100 mL of digestion solution to the bag (93 mL of calcium-free DPBS + 60 µL of calcium chloride (1 g/L) + 7 mL of 0.075% sterile collagenase, see **Table of Materials**) and leave at 37 °C for 30 min under slow stirring.

2.3. Transfer all the bag's content to four conical tubes of 50 mL and centrifuge them at 400 x *g* at 22 °C for 10 min.

2.3.1. Remove and discard the supernatant and add 5 mL of Dulbecco's modified Eagle's medium (DMEM) low glucose supplemented with 20% FBS (Fetal bovine serum) to the cell pellet (**Figure 1**).

3. Counting of the SVF cells

3.1. Mix a fresh solution of 10 µL of trypan blue at 0.05% in distilled water with 10 µL of cellular suspension for 5 min.

3.2. Count viable cells in a Neubauer cell counting chamber[32] using an inverted light microscope (see **Table of Materials)** at 20x magnification.

3.3. Resuspend the cell pellet in a cryoprotective medium (5 mL of FBS + 10% of Dimethyl Sulfoxide - DMSO) at a concentration of 1 x 10^6 cells/mL.

3.4. Place 1 mL of this mix in cryovials. Use a freezing container (see **Table of Materials)** with a cooling rate of (1 °C/min to -80 °C).

3.4.1. Store at -80 °C for 1 year.

3.4.2. After this time, store in standard cassette boxes immersed in the liquid nitrogen vapor phase (-165 °C).

4. Thawing process of the cells

4.1. Remove the vials from liquid nitrogen and place them immediately in the 37 °C water bath for 1 min.

4.2. Place the SVF cells in a conical tube with 4 mL of DMEM (low glucose supplemented with 20% FBS) preheated at 37 °C.

4.3. Centrifuge at 400 x *g* at 22 °C for 5 min.

4.4. Remove the supernatant and add 1 mL of DMEM (low glucose) + 10% FBS. Perform immunophenotyping following the steps below.

5. Flow cytometry technique (immunophenotype multiple labeling)

5.1. Place 1 mL of cell pellet (concentration of 1,000 cells/µL) in five cytometry tubes (200 µL each).

5.2. Centrifuge at 400 x *g* at 22 °C for 5 min and discard the supernatant with a pipette.

5.3. Add 300 µL of Phosphate-Buffered Saline (PBS) (10x), centrifuge at 400 x *g* at 22 °C and discard the supernatant with a pipette.

5.4. Prepare five tubes for different marker combinations as follows: 5 µL of CD11B/5 µL of CD19/20 µL of CD45; 5 µL of CD73/20 µL of CD90/5 µL of CD105/20 µL of CD45; 20 µL of CD34/5 µL of HLA-DR/20 µL of CD45; Cell viability assay—5 µL of fluorescent reactive dye. (see **Table of Materials**) and a tube with unstained cells and PBS as the negative control. Homogenize in a vortex and incubate at 4 °C for 30 min.

5.4.1. Centrifuge at 400 x *g* at 22 °C for 5 min, discard the supernatant with a pipette, add 500 µL of PBS (10x), and proceed with cell sorting.

NOTE: Five thousand events are acquired per antibody set in the Flow Cytometer of four colors and five parameters and analyzed with CellQuest software.

6. Seeding of passage 1 (P1)

6.1. Seed 2 x 10^5 cells in a 75 cm^2 culture flask.

6.2. Add 12 mL of DMEM low glucose + 20% of FBS + 10% antibiotic/antimycotic (with 10,000 units penicillin, 10 mg streptomycin, and 25 µg amphotericin B per mL, 0.1 µm).

6.3. When the cells reach between 80%–90% confluence, perform trypsinization of adherent cells with 2 mL of 0.25% EDTA-trypsin for 3 min.

6.4. Count cells again (as mentioned in step 3).

6.5. Perform immunophenotyping again (as mentioned in step 5).

7. Statistical analysis

7.1. Use Spearman's Rho Calculator[33] to measure the strength of association between the following variables with $P < 0.05$, as mentioned below.

7.1.1. Select SVF cellular yield and the number of days SVF stays in culture in the first passage (P1) until 80%–90% confluence (days to P1).

7.1.2. Select SVF cellular yield before and after going to P1.

7.1.3. Consider days to P1 and cellular yield before going to P1.

7.1.4. Select SVF cellular yield with the average percentage of confirmed ADSC.

7.1.5. Calculate the percentage of confirmed ADSC and the cellular yield before going to P1.

7.1.6. Determine the BMI and SVF cellular yield.

8. Differentiation assay

8.1 Perform the differentiation assay following a differentiation kit protocol (see **Table of materials**). **Figure 4** demonstrates the results for Case 1.

REPRESENTATIVE RESULTS:

The characterization of the nine individuals studied, including their age, weight, height, and BMI, are shown in **Table 1**.

According to the cellular yield initially presented, the cell volume inoculated in culture was calculated to be as close as possible to the capacity of the 75 cm^2 culture flask. The sample volume seeded in each case is described in **Table 2**. Then, according to the initial cellular yield, a variable volume of cells for each sample was determined: 1 mL for samples with higher cellular yield, 1.1 mL for samples with intermediate cellular yield, and 2 mL for samples with lower cellular yield so as to perform more similar cell seeding between cases. When the culture reached about 80%–90% confluence (**Figure 2A**) (about 7.5 ± 4.5 days), trypsinization of adherent cells was carried out (**Table 2** and **Figure 2B**).

The cellular yield before passage 1 broadly varied even when the same confluence before trypsinization was observed (**Table 2**). This can be explained by the fact that cells may have grown in layers. Different parameters from the patients' ADSC were also assessed at different periods, as demonstrated in **Table 2**.

Some samples (Case 1, Case 2, Case 7) could not be evaluated regarding the percentage of confirmed ADSC and the estimated number of ADSC in culture due to bacteria contamination and lack of available cells to perform cryopreserved SVF immunophenotyping. According to the Spearman's Rho Calculator[33], no statistical differences were found between SVF cellular yield and days to P1 (r = 0.37816, p = 0.31561), between SVF cellular yield before and after going to P1 (r = -0.33333, p = 0.38071), and between days to P1 and cellular yield before going to P1 (r = -0.53783, p = 0.13529). Furthermore, no significant differences were observed when correlating the SVF cellular yield with the average percentage of confirmed ADSC (r = -0.02857, p = 0.95716) and between the average percentage of confirmed ADSC and the cellular yield before going to P1 (r = 0.42857, p = 0.3965). Also, the correlation between BMI and SVF cellular yield could not be considered statistically significant (r = -0.46667, p = 0.20539). **Table 3** shows flow cytometric data performed on SVF cells cryopreserved. The initial SVF cells contained a subset of positive cells for hematopoietic markers (CD45, CD11b, CD19, HLA-DR)[34]. From the initial SVF cell population, a particular subgroup expressed CD11b[34] and CD19[34] stromal cell-associated markers. The levels of CD73[34], CD90[34], and CD105[34] were intermediate between these values. The initial SVF contained

a subpopulation of cells positive for stem cell-associated markers (**Figure 3**). A mean of 79% of SVFs expressed the HSC-associated marker CD34[34].

In total, 21 min were necessary for the three washes, 30 min for collagenase digestion, 10 min for centrifugation, and 5 min for cell counting and plating.

FIGURE AND TABLE LEGENDS:

Figure 1: Steps from the protocol adipose-derived stem cells isolation. (**A**) Bag for lipoaspirate transport in a closed system. (**B**) The step of resting bag repeated three times, after washing. (**C**) Lipoaspirate after three washes with DPBS. (**D**) Lipoaspirate after collagenase digestion. (**E**) Lipoaspirate after digestion is distributed in a 50 mL tube. (**F**) Digested lipoaspirate after centrifugation. (**G**) Final process isolation with the pellet with the stromal vascular fraction (SVF).

Figure 2: Morphology and viability of ADSCs. (**A**) Plastic adherent mesenchymal adipose-derived stem cells at the first passage after isolation at light microscopy. The cells show adhesion to the plastic and fibroblast-like morphology. (**B**) Trypan blue assay showing viable cells counted in Neubauer chamber using a light microscope.

Figure 3: Subpopulation of cells positive for stem cell-associated markers in SVF of Case 9 after 8 months of cryopreservation. R1: Total cellular region analyzed in FSC (Forward Scatter) x SSC (Side Scatter) (Size x Complexity); R2: CD45 negative region, whose populations CD73, CD90, and CD105 are positive in this region.

Figure 4: Differentiation assay. (**A**) ADSC differentiation in chondrocytes. (**B**) ADSC differentiation in osteocytes. (**C**) ADSC differentiation in adipocytes.

Table 1: Data from the samples of the individuals studied. *BMI: body mass index.

Table 2: Data from different steps of the procedure from the nine patients analyzed. SVF: stromal vascular fraction; ADSC: adipose-derived stem cell; P1: passage 1; na: data not available.

Table 3: Flow cytometry data from six of the patients. (*) From these CD45- cells, % of ADSC with different combinations of stem cell markers was determined. ADSC: adipose-derived stem cell; SVF: stromal vascular fraction; (*) From these CD45- cells, % of ADSC with different combinations of stem cell markers was determined.

DISCUSSION:

Isolation yield

It is well established that the cryopreservation process, frequently required in cellular therapy, results in significant cell loss, sometimes greater than 50%[29,30,35]. Thus, a technical improvement for obtaining high initial cellular yield in isolation is fundamental. The collecting method of lipoaspirate and the isolation method of the cells must focus on preserving a greater number of

cells, maintaining high viability, and extracting the maximum number of cells from the initial material while accounting for the long-term culture and manipulation of the cells. Therefore, straight culture maintenance is required to keep the cells away from apoptosis, senescence, or genetic instability, since cell therapy is likely to be effective and safe for patients.

To the best of our knowledge, there is no previous article using this set of steps for the isolation of mesenchymal stem cells derived from lipoaspirate, which results in a time-saving and cost-benefit technique. In this study, each methodological step was reasoned according to the literature that showed the highest final cell yield in cellular isolation. The novelty of the technique performed in this study was the use of manual aspiration associated with a series of lipoaspirate washes, with the subsequent bag resting. The collection bag used to transport and process lipoaspirate allowed undigested tissue fragments to not participate in collagenase digestion. The most critical step is adding calcium chloride to the fresh digestion solution as it potentiates the action of collagenase. The time gain is not yet reported in the literature with a cell thawing method that allows viable cells even after long cryopreservation time. The SVF cellular yield found in this study varied broadly from 6.15 to 26.2 x 10^5 cells/mL with an average of 15.7 x 10^5 cells/mL. This may be due to the presence of a more significant amount of adrenaline solution suctioned, which may have been higher or lower according to the stage of the surgical procedure and to the number of other known cell types generally found in the SVF. Although some studies have found negative correlations between BMI and ADSC yield[36,37], this study found no significant correlation, like the other two studies[38,39], decreasing the possibility of that being the cause of the incredible variety of SVF cellular yield found in this study. These data show that the lowest SVF cellular yield obtained was 6.15 x 10^5 cells/mL. Some studies had already measured the efficiency of ADSC isolation according to the surgical technique for obtaining lipoaspirate. One study obtained 0.087 x 10^5 cells/mL in freshly isolated SVF for the liposuction technique using adrenaline solution (as used in this study) and 0.143 x 10^5 cells/mL without it[26]. This work highlighted the significance of the adrenaline solution injection due to the vasoconstrictive effect that decreases intraoperative bleeding and bruising, as the majority of surgeons choose to perform in clinical practice. Another study demonstrated that live ADSCs isolated ranged from 0 to 0.59 x 10^5 cells/g lipoaspirate harvested, with an average of 0.295 (±0.25) x 10^5 cells/g tissue[31]. Some studies have tested different ways to achieve higher ADSC yield. One of these studies achieved about 350 x 10^5 for the method that presented various constituents in collagenase digestion buffer and the use of an orbital shaker[40]. Another study showed 29.7 (±0.2) x 10^5 cells/mL as the total number of SVF cells from the abdominal area[41]. The abdominal area chosen in this study is still the reference area for the best availability and accessibility of lipoaspirate[42]. The body region for lipoaspirate collection, among other factors as donor age and method of the collection chosen, is a strong determinant of the quality of the ADSC yields.

The possibility of microorganisms' contamination causing the unavailability of cells to continue the experiments was the only execution problem that limited the completion of this study. Even using antibiotics and Good Manufacturing Practice requirements, contamination can occur due to the lack of a total aseptic environment to perform the aspiration of fat.

SVF immunophenotyping

According to The Mesenchymal and Tissue Stem Cell Committee of the International Society for Cellular Therapy[34], one of the three minimal criteria to define human mesenchymal stem cells is that the cells must express CD105[34], CD73[34], and CD90[34] and should not express CD45[34], CD34[34], CD14[34], or CD11b[34], CD79a[34] or CD19[34], and HLA-DR[34] surface membrane molecules. Mitchell[43] tested fresh SVF cells by immunophenotyping and found a maximum of 54% of cells with ADSC surface markers. In this study, the immunophenotyping revealed a higher percentage of confirmed ADSC (non-hematopoietic cells) of up to 78.91% in the SVF after long-term cryopreservation (ranging from 37.95%–78.91% with a mean of 53.68%). Evidence shows that the progenitors of a stem cell population not yet committed do not express the CD34 marker[34,44,45]. Depending on the stage of differentiation, the CD34[34] negative stem cells can generate not only hematopoietic progenitors but also more specific mesenchymal precursors, such as osteoclasts, chondrocytes, myocytes, adipocytes, and others. Some studies demonstrated the striking plasticity of the primitive stem cell population, composed of cells with stromal cell function and hematopoietic and mesenchymal progenitors[45]. According to the literature, the complete CD34[34] functional role in the tissue formation in SVF cells is still unknown[46]. Mitchell[43] showed a mean of 60% cells of SVF expressing CD34[34] marker, whereas, in this study, the mean was 78.81%. It is known that the expression of CD34[34] surface marker decreases along passages.

Adherent cells and differentiation assay

Depending on the number of cells obtained after isolation, the number of cells seeded in the first culture varied. The first culture time for reaching 80%–90% confluence in 75 cm^2 flasks took an average of 8.4 days and a standard deviation of 7.5 (±4.5) days ranging from 6.6 to 16.1 x 10^5 cells/mL. It is to be noted that even for the cases with lower cellular yield, the first culture time led to high cellular yield indexes compared to the literature, probably due to the best availability of viable cells maintained during the entire collection and isolation process. One study obtained a cellular yield of 3.75 (±1.42) x 10^5 ADSC per mL of lipoaspirate within a 4.1 (±0.7) day culture period[47]. Another study demonstrated a yield of nucleated SVF cells of 3.08 (±1.40) x 10^5 per millimeter with a mean of 6.0 (±2.4) days in the first culture period[43]. In this study, by estimating the number of ADSC seeded in culture, which varied as a function of the number of cells observed in the SVF from the collection of the same volume of 100 mL of lipoaspirate, it was verified that the number of days to reach P1 had no relation to cell volume. For example, for Case 9, in which 12.2 x 10^5 cells were incubated, 6 days were required to reach 80%–90% confluence. For Case 6, in which 14.6 x 10^5 cells were seeded in culture, a more extended period was necessary (10 days) to reach up to the same level of confluence. Perhaps, a minimum ADSC number is enough for the first adhesion period. There may be significant interindividual variation, such as comorbidities, age, and general health status. Some studies in the literature questioned the importance of considering the patients' inter-individual variability for SVF cell yield[48,49].

According to The Mesenchymal and Tissue Stem Cell Committee of the International Society for Cellular Therapy[34], mesenchymal cell must have the ability to differentiate into three different cell types as osteogenic, adipogenic and chondrogenic lineages as it was demonstrated in **Figure 4**.

Cell viability, cryopreservation, and genetic instability

Different methods have been used to determine viability loss, defined as plasma membrane integrity damage[50]. However, the cultures can also present early apoptotic cells that these approaches can ignore because they maintain an intact plasma membrane, but they are nonviable. The results reported here show a great range in viability marker, from 23.06%–72.34%, with a mean of 47.6% after long-term cryopreservation. Considering that cryopreservation is a significant step concerning cell therapy, recovery of the maximum number of viable and functional stem cells after thawing is one of the priority issues for the success of cell therapy. The literature has shown that at least 50% of cell viability is lost from 1–4 months after cryopreservation[43]. Notably, the lowest indexes presented in this study are from Case 5, Case 4, and Case 2, which are the oldest cryopreserved samples (about 2 years). Although they presented the lowest viability indexes, they demonstrated high cellular yield in trypan blue dye exclusion assay in fresh SVF. Although the literature supports causes of viability loss, these rates are lower than expected. Temperature fluctuations in cell storage due to technical reasons can cause the increase and accumulation of stress and favor the accumulation of aqueous portions, generating crystals that damage the plasmatic membrane during long-term cryopreservation[51]. The literature shows more than 70% viability for samples with the same or longer freezing time. However, the viability check was performed with a different technique than that carried out in this study[52]. Another study showed that longer cryopreservation negatively affects cellular viability[31], which can be explained by temperature variations in the -80 °C freezer. Hence, cells often need to stay too long in culture, which increases cell cycle stress, bringing risks for genetic instability and consequently compromising cellular therapy. There is also a consensus in the literature indicating some stress factors and how they affect cell cytogenetic stability, which is essential to maintain the prolonged stem cell cultivation required for cell therapy[35,53].

Methodology benefit

To date, literature shows no standardized protocol to isolate ADSC aiming for clinical applications. Most of the studies demonstrate complex, time-consuming protocols[24]. In this study, the efficiency of the method versus the time demanded to complete the initial cellular yield must be emphasized: about 1.5 h. According to literature, isolation of adipose-derived stem cells can take about 3 h to 8 h[54,55]. Thus, the gain of time allied to the high cellular income is critical for regenerative medicine therapeutics advancement. More cell viability assessments should be carried out parallel to those performed in this work to improve this method. Further randomized controlled trials incorporating a more extensive sample using this methodology are required to countersign these results.

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DISCLOSURES:

The authors declare no competing financial interests.

AUTHORS' CONTRIBUTIONS:

LP: study design, experiment/analysis performance, protocol optimization, data analysis, manuscript writing and reviewing; FN^1: lipoaspirate collection, manuscript reviewing; FN^2: experiment/analysis performance, protocol optimization; AMM: study design, manuscript revision; TRS: experiment/analysis performance, protocol optimization; AA, LMF: study design, manuscript reviewing; MIM: scientific coordination, study design, manuscript review, and approval. All the authors read and approved the final manuscript.

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Figure 1

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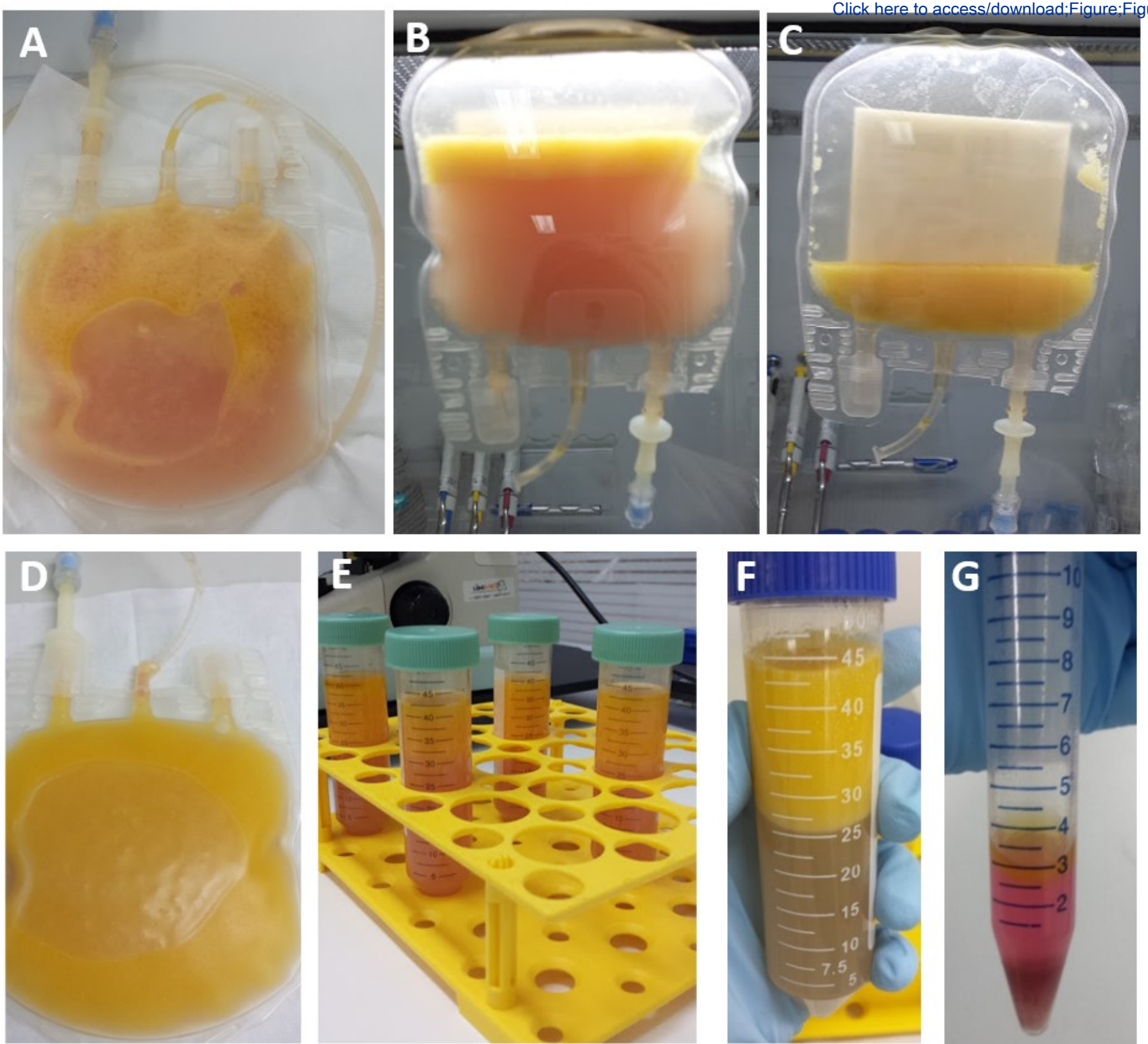


Figure 2

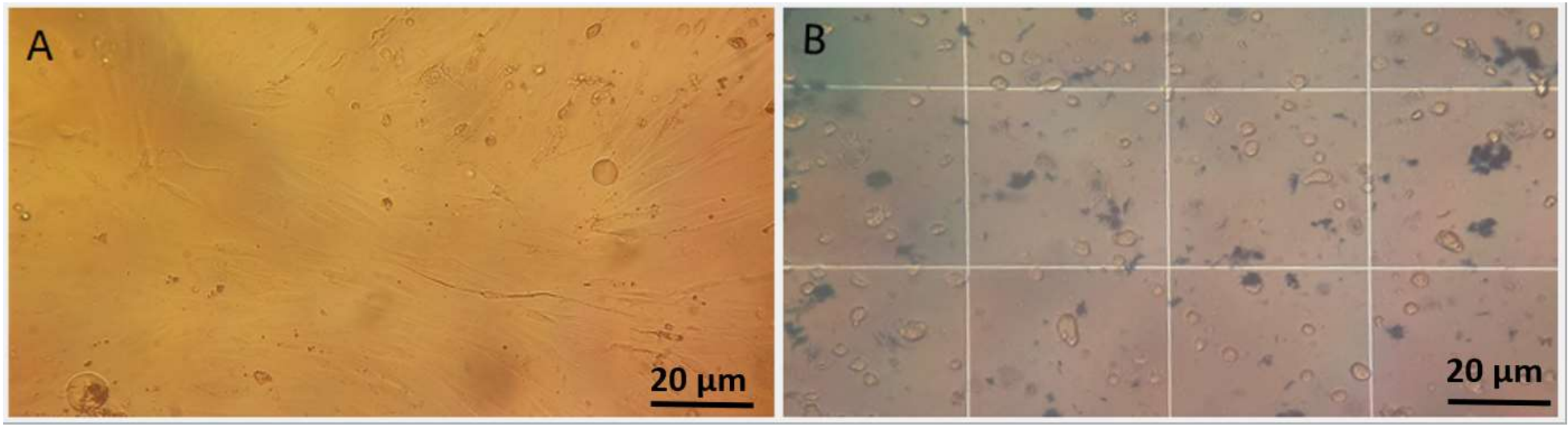
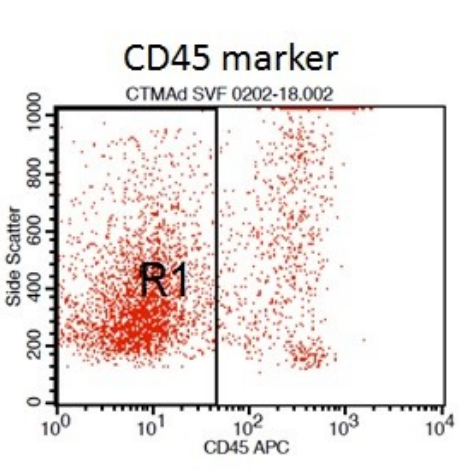
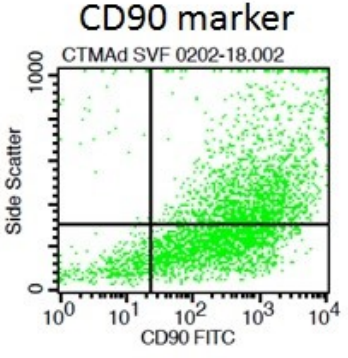
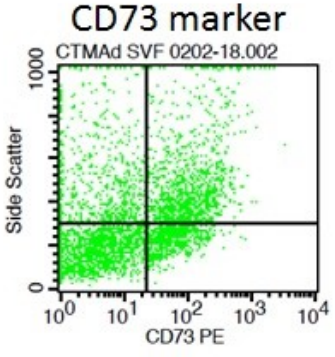


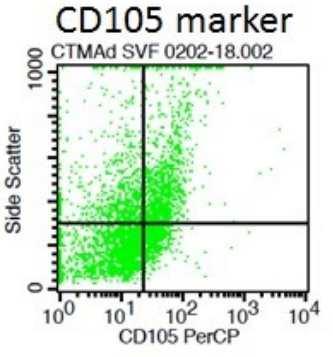
Figure 3

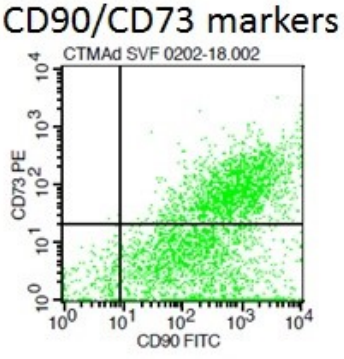


Region	Events	% Gated	% Total
R1	2807	76.90	56.14

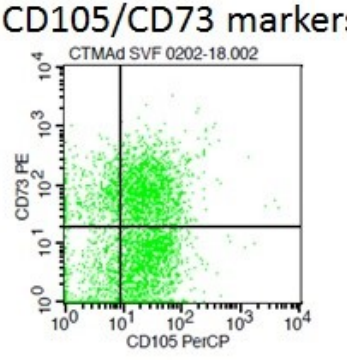




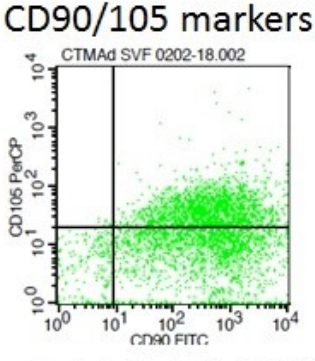




Quad	Events	% Gated	% Total
UL	3	0.08	0.06
UR	1736	47.52	34.72
LL	183	5.01	3.66
LR	1731	47.39	34.62



Quad	Events	% Gated	% Total
UL	413	11.31	8.26
UR	1326	36.30	26.52
LL	494	13.52	9.88
LR	1420	38.87	28.40



Quad	Events	% Gated	% Total
UL	9	0.25	0.18
UR	1742	47.69	34.84
LL	177	4.85	3.54
LR	1725	47.22	34.50

Figure 4

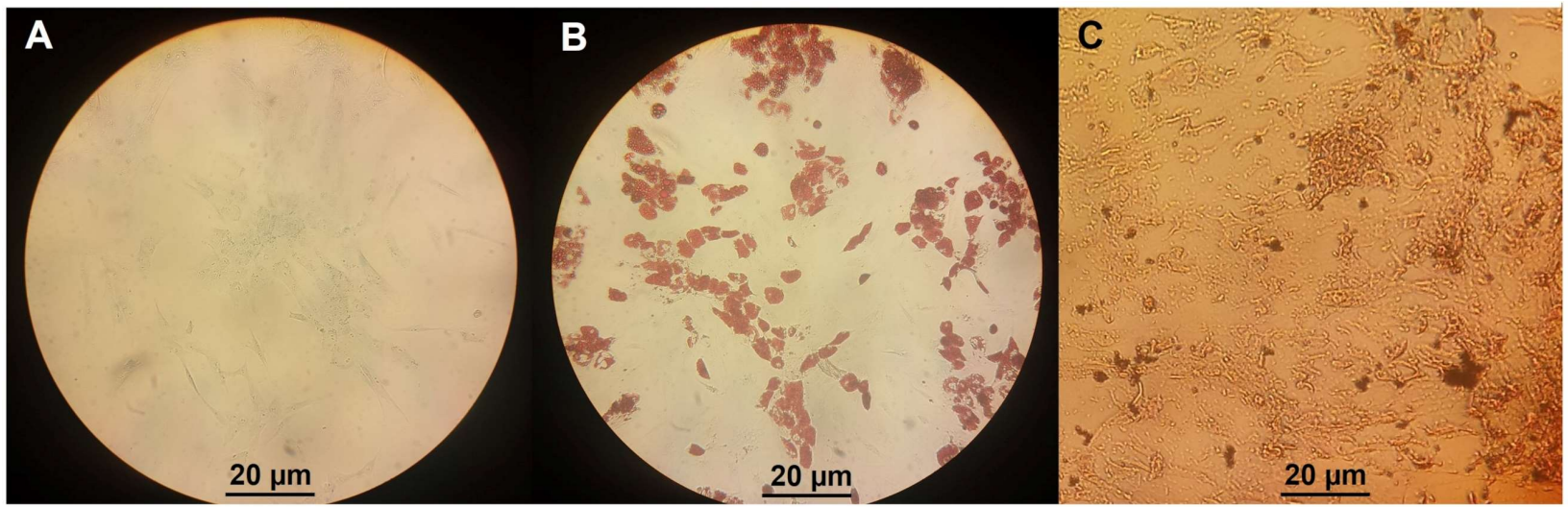


Table 1

Patient	Age at collection (years)	Weight (kg)
Case 1	35	68
Case 2	33	65
Case 3	35	70
Case 4	34	72
Case 5	36	72
Case 6	36	67
Case 7	38	62
Case 8	50	63
Case 9	37	65

Height (meter)	BMI*
1.64	25.28
1.65	23.88
1.68	24.8
1.64	26.77
1.69	25.21
1.64	24.91
1.53	26.49
1.68	22.32
1.58	26.04

Table 2

Patient	Volume collected (mL)	SVF Cellular yield (cell/mL) (x 10^5)	Volume in culture (mL)	Average percentage of confirmed ADSC (%)
Case 1	96	9.2	2	na
Case 2	100	25.2	1	38
Case 3	100	26.2	1	na
Case 4	105	21.1	1	55.9
Case 5	110	23.7	1	61.4
Case 6	100	13.3	1.1	78.9
Case 7	98	6.8	2	na
Case 8	100	9.7	1.1	44.2
Case 9	100	6.1	2	43.8
SD	3.89	7.81	0.46	13.75

Number of cells in initial culture (x 10^5)	Estimated number of ADSC in culture (x 10^5)	Days to P1	Cellular yield before going to P1 (x 10^5)
18.4	na	10	18
25.2	9.6	12	10.8
26.2	na	12	6.6
21.1	11.8	3	13.1
23.7	14.5	4	16.1
14.6	11.5	10	13.5
13.6	na	8	10.5
10.7	4.7	11	6.9
12.2	5.3	6	15.9
5.55	3.53	3.2	3.79

Table 3

SAMPLE	% Of ADSC DETERMINED BY MONOCLONAL ANTIBODIES					% OF HEM BY M
	CD45-(*)	CD73+/CD90+	CD73+/CD105+	CD105+/CD90+	Mean	CD34+
Case 2	52.34%	31.97%	25.36%	56.52%	37.95%	63.16%
Case 4	48.02%	61.62%	40.93%	65.25%	55.93%	82.94%
Case 5	27.74%	54.02%	49.72%	80.42%	61.38%	73.33%
Case 6	55.52%	79.52%	67.70%	89.52%	78.91%	86.86%
Case 8	57.28%	46.84%	30.88%	57.65%	45.12%	78.47%
Case 9	56.14%	47.52%	36.30%	47.69%	43.83%	88.10%

ATOPOIETIC CELLS DETERMINED MONOCLONAL ANTIBODIES			CELL VIABILITY ASSAY AND TIME OF SVF CRYOPRESERVATION
HLA-DR+	**CD11b+**	**CD19+**	**LIVE/DEAD +**
12.87%	2.41%	0.21%	39.54% (2 years)
26.62%	0.00%	0.16%	38.30% (2 years)
51.31%	0.05%	0.00%	23.06% (2 years)
8.83%	0.18%	1.06%	56.76% (2 years)
26.97%	0.03%	0.00%	55.56% (1 year)
26.94%	0.05%	0.24%	72.34% (8 months)

To
Dr. Nilanjana Saha, Ph.D.
Review Editor
JoVE

December 7th, 2021

Ref: JoVE63036R1 Revision

Dear Dr. Nilanjana,

Thank you for considering our manuscript "Technique for Obtaining Mesenchymal Stem Cell Derived from Adipose Tissue and Stromal Vascular Fraction Characterization in Long-Term Cryopreservation", submitted for publication.

We have made revisions based on the corrections/comments/suggestions of you and Reviewers #1, #2, #5, and #6. The comments of each reviewer are numbered below, with our response (clarifications and changes) following.

We appreciate and thank you and the reviewers for all your helpful comments. We feel that the manuscript is now greatly improved.

Please, find enclosed the revised version of the manuscript, considering the comments and suggestions received, with the modifications in the text highlighted in red.

Editorial comments:

1) The editor has formatted the manuscript to match the Journal's style. Please retain it and use the attached version for revision.

Author's reply: Thank you for the effort. We have used the formatted manuscript for revision.

2) Please address the specific comments marked in the manuscript. Also, please don't delete the Editor's comments in the attached manuscript; instead, provide a very brief reply to each comment regarding your actions.

Author's reply: **We did that.**

3) Please do not abbreviate the journal names in the References.

Author's reply: The journal names were given without abbreviations.

Reviewers' comments:

Reviewer #2:

Thank you for revising the manuscript based on the comments.

Author's reply: We thank you a lot for all your brilliant suggestions.

Reviewer #5:

Manuscript Summary:

The manuscript "Technique for Obtaining Mesenchymal Stem Cell Derived from Adipose Tissue and Stromal Vascular Fraction Characterization in Long-Term Cryopreservation" describes a method to obtaining and characterizing the adipose derived stem cells (ADSC) from human lipoaspirate samples.

The Authors demonstrated that the indicated method is a more effective in term of cell viability and of total time than others previously reported in literature. In my opinion, the topic is not entirely innovative but, anyway, is of interest because the optimization of ADSC recovery is a crucial step in regenerative medicine.

The manuscript title entirely represents data enclosed. In the Statistical analysis should be specify the standard deviation (SD) or the standard error mean (SEM). In example, how many flow cytometer analysis have been made for every SVF samples? If the showed data are relative to more analysis should be reported the SD or SEM.

Author's reply: For each case, one immunophenotyping was performed using the relevant set of clusters of differentiation (CDs) markers to confirm the presence of mesenchymal stem cells. Because of this, we did not present standard deviation nor standard error mean for these values. On the other hand, standard deviations (SD) were included in Table II.

Major Concerns:

not reported

Minor Concerns:

- Line 101: Authors reported "After eight weeks of expansion, medium changes, and passages, ADSCs represent the great majority of the cell population in the flasks". Approximately how many passages are eight weeks?

Author's reply: Eight weeks represent about 8 to 10 passages, considering a medium time of cell growth. But it is known by those handling cell cultures that there are factors that can slow down it, reaching 80% to 90% of cell confluence, or even accelerate, such as the case when the patient uses medication that interferes with the cell cycle.

- Throughout the entire manuscript the definition "mesenchymal adipose tissue-derived stem cells" could be abbreviated in ADSCs

Author's reply: This point was corrected.

- Line 185: "….. 5 uL of Cell viability monoclonal…" Correct the mistake

Author's reply: "Cell viability monoclonal" was changed for "Cell viability assay - 5 uL of fluorescent reactive dye."

- Line 196: Since this is a detailed protocol I suggest to specify what antibiotic/antimycotic has been used in this work.

Author's reply: It was specified in the text: "Add 12 mL of DMEM low glucose + 20% of FBS + 10% antibiotic/antimycotic (with 10,000 units penicillin, 10 mg streptomycin and 25 µg amphotericin B per mL, 0.1 µm)."

- Line 247: Why the Authors report the "adipose tissue" … is lipoaspirate sample.

Author's reply: The term was corrected in the text.

- Figure 2: Quality of figure 2 should be improved

Author's reply: Figure 2 quality was improved.

- Reference section could be improved replacing older articles with some recent ones. In example the cited article n.52 ("According to literature, isolation of adipose-derived stem cells can take about 8h) should be replaced. Moreover, to improve the quality of this article, about the influence of different handling methods on SVF cell yield and viability I suggest to add the review "Methods of Isolation, Characterization and Expansion of Human Adipose-Derived Stem Cells (ASCs): An Overview. Int J Mol Sci. 2018 Jun 28;19(7):1897. doi: 10.3390/ijms19071897.)

Author's reply: We are very grateful for the suggestion, and we have included the suggested reference and remodeled the sentence.

Reviewer #6:

Manuscript Summary:

I have reviewed with great interest the revised paper from Pola et al. I think main previous issues have been addressed, from previous reviewing. However, some minor revisions remain to perform, and i've also a major concern regarding ADSC characterization.

Author's reply: We appreciate the alert to points to be improved.

Major Concerns:

- In vitro differentiation in adipocytes, chondrocytes and muscle cells should be provided, in addition to flow cytometry - the latest being by the way very well conducted. The authors have justified this by financial and time issues, which is not receivable. Another justification was made using a paper about international recommendations... but this paper was released in 2006 ... unless then provide the most recent international and accepted by community up to date ADSC validation points, this cannot be received neither. Somehow, even if more justifications are provided, such a paper of protocol should include all criteria used from community, to be accepted as a valid method and be used as reference. This will strongly assess that none of the cell processing and innovation harmed the ability of cells to differentiate: even if they don't aim at this, most of researchers will.

Author's reply: Truly, we conducted the differentiation assay in the pilot case (case 1) and demonstrated positive results for *in vitro* differentiation in adipocytes, chondrocytes, and osteocytes (accepted as well by The International Society for Cellular Therapy position statement) cells. We have added this important information to the manuscript.

- Infiltration before liposuction: plastic surgeons always use adrenaline in a clinical setting, and often they are using xylocaine to diminish postoperative pain: the impact of xylocaine on ADSC viability is highly discussed - please discuss if the following protocol is available if xylocaine is used as well - this is of tremendous importance for practical applications. The ideal would be to include such group is the analysis.

Author's reply: We are grateful for highlighting this relevant point associated with the clinical practice. In the protocol performed in this study, xylocaine was not used to diminish postoperative pain because patients were under general anesthesia.

When performing abdominoplasty, it is quite unusual to perform liposuction before lipectomy: if most cases of clinical sources of lipoaspirates, these are different indications. Please discuss. This should be realistic when discussing clinical source of human cells.

Author's reply: In the protocols for the therapeutical use of mesenchymal stem cells, it is indicated that obtaining these cells must be through liposuction and not from lipectomy. In this work, the main objective was to test the efficiency of a protocol for ADSC isolation from liposuction and not exactly from lipectomy. In this way, liposuction was performed before lipectomy.

Minor Concerns:
- Summary section: "ADSC", acronym should be explicited here.

Author's reply: It was corrected.

- Protocol:
* Line 117 : please confirm the etiology from abdominoplasty, post-pregnancy, pendulum, surgery after massive weight loss. In the latest case, please provide maximum BMI before weight loss - indeed this is a very special population, and should be highlighted if there are in the study.

Author's reply: The etiology of patients' abdominoplasty was from excess skin after pregnancy.

* Lines 125-137: avoid bullets 1.1.1, 1.2.1 and 1.3.1 if there are no other bullets: reader is then expecting a 1.1.2, 1.2.2 etc...

Author's reply: The steps and bullets were better connected in the text.

* Line 143: "DPBS", i'm not sure acronym has been defined before in the main text.

Author's reply: The acronym "DPBS" has been previously defined in line 45.

* Step 2.2 : which type of collagenase? even if manufacturers are given at the end of paper, the extensive information about products should be better linked.

Author's reply: The collagenase used was type I. This information was added to the text.

* Line 203: bullet 7.1 isolated, same remark as below.

Author's reply: This point was corrected.

- Discussion:
* I don't totally agree about the fact that cells donors are females: indeed the true explanation is that patients requiring abdominoplasties are very frequently women. Liposuction procedures are more balanced. To be discussed further.

Author's reply: Yes. We agreed with this observation, and we rewrote the sentence.

* Lines 295-296: BMI point is not well conducted here - plastic surgeons don't perform abdominoplasties when BMI is superior to 30, so in most cases you won't have any overweight patients in the population described in this study. This is less true for liposuction, when BMI is less taken in account. Moreover, if the patient has encountered massive weight loss, the maximum BMI should be indicated, as it is an important information.

Author's reply: The information about BMI was better described to clarify this important information.

* Line 302: indeed this point is very important, as the absence of adrenaline infiltration almost never occur in clinical practice. This statement should appear, as it can represent a bias and weaken the applicability of the method for researchers working with surgeons.

Author's reply: The information about adrenaline infiltration was added to the manuscript.

On behalf of my co-authors, I would like to thank you and the reviewers for taking the time to review our manuscript entitled "Technique for Obtaining Mesenchymal Stem Cell Derived from Adipose Tissue and Stromal Vascular Fraction Characterization in Long Term Cryopreservation" for possible publication in JoVE.

We sincerely appreciate the comments we received. They certainly contributed to improving our paper. We have critically reviewed and addressed point-by-point all the concerns and suggestions, as it is reflected in the revised version of our manuscript and our responses. We believe this paper will contribute to the current clinical practice. Therefore, we are submitting the revised version. We will fully respect your final decision and we thank you in advance for kindly reconsidering our paper for possible publication.

Hoping that now our paper meets all the requirements for publication, and thanking you for all your attention,
I remain
Sincerely yours,

Maria Isabel

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To
Dr. Amit Krishnan, Ph.D.
Review Editor
JoVE

September 13rd, 2021

Ref: JoVE63036 Revision

Dear Dr. Krishnan,

Thank you for considering our manuscript "Technique for Obtaining Mesenchymal Stem Cell Derived from Adipose Tissue and Stromal Vascular Fraction Characterization in Long-Term Cryopreservation", submitted for publication.

We have made revisions based on the corrections/comments/suggestions of you and Reviewers #1, #2, #3, and #4. The comments of each reviewer are numbered below, with our response (clarifications and changes) following. **Please, note that the revised text is in red.**

We appreciate your helpful comments and those from the reviewers.
We feel that the manuscript is now greatly improved.

Please, find enclosed the revised version of the manuscript, considering the comments and suggestions received.

Editorial comments:

Editorial Changes
Changes to be made by the Author(s):

1. Please take this opportunity to thoroughly proofread the manuscript to ensure that there are no spelling or grammar issues.
Author's reply: The manuscript was thoroughly proofread and grammar and language revised.

2. Please revise the following lines to avoid previously published work: 75-77, 159-162, 222-225,305-309. Please refer to the iThenticate report attached.
Author's reply: These sentences were rewritten.

3. Please revise the text to avoid the use of any personal pronouns (e.g., "we", "you", "our" etc.).
Author's reply: All personal pronouns were removed.

4. JoVE cannot publish manuscripts containing commercial language. This includes

trademark symbols (™), registered symbols (®), and company names before an instrument or reagent. Please remove all commercial language from your manuscript and use generic terms instead. All commercial products should be sufficiently referenced in the Table of Materials. For example: Falcon, LIVE/DEAD®, etc.
Author's reply: In the text and Table, commercial names were rewritten.

5. Please adjust the numbering of the Protocol to follow the JoVE Instructions for Authors (The style guide is attached for your reference). For example, 1 should be followed by 1.1 and then 1.1.1 and 1.1.2 if necessary. The Protocol should be made up almost entirely of discrete steps without large paragraphs of text between sections. Please simplify the Protocol so that individual steps contain only 2-3 actions per step and a maximum of 4 sentences per step.
Author's reply: All Protocol session was remodeled according to the JoVE Style Guide.

6. Please ensure that all text in the protocol section is written in the imperative tense as if telling someone how to do the technique (e.g., "Do this," "Ensure that," etc.). The actions should be described in the imperative tense in complete sentences wherever possible. Avoid usage of phrases such as "could be," "should be," and "would be" throughout the Protocol. Any text that cannot be written in the imperative tense may be added as a "Note." However, notes should be concise and used sparingly. Please include all safety procedures and use of hoods, etc.
Author's reply: all Protocol session was remodeled according to the JoVE Style Guide.

7. Please add more details to your protocol steps. Please ensure you answer the "how" question, i.e., how is the step performed? Alternatively, add references to published material specifying how to perform the protocol action.
Author's reply: All Protocol session was remodeled to be as JoVE Style Guide ask.

8. 1: How was the skin preparation and asepsis done? Please mention.
Author's reply: This step was clarified.

9. 1.1: How was the adrenaline solution injected. What was used to inject. Please provide the specifications.
Author's reply: This step was clarified.

10. 1.2: Was the 60 mL syringe attached for aspiration or injecting adrenaline? How was the fat aspirated manually?
Author's reply: This step was rewritten.

11. 2: What was the concentration of DPBS and calcium? Please specify.
Author's reply: This information was added.

12. 3: What was the concentration of calcium chloride used? What was the volume of DMEM added? Please specify.

Author's reply: This information was added.

13. 4: Please provide microscope settings and parameters. Was 20% FBS used here as well. Please specify.
Author's reply: The settings and parameters were added and information regarding FBS was specified, as requested.

14. 5.2: How was immunophenotyping performed. Please detail the step or consider including the appropriate reference which will enable readers to understand how this is done.
Author's reply: This step was rewritten.

15. 6: What were the marker concentrations, dilutions and volume used? Please include more details on the Flow cytometer settings.
Author's reply: This step was rewritten.

16. 8: Please mention how the statistical analysis was performed. What was compared?
Author's reply: This statement was rewritten.

17. For SI units, please use standard abbreviations when the unit is preceded by a numeral throughout the protocol. Abbreviate liters to L to avoid confusion. Examples: 10 mL, 8 µL, 7 cm2.
Author's reply: These abbreviations were rewritten.

18. For time units, please use abbreviated forms for durations of less than one day when the unit is preceded by a numeral throughout the protocol. Do not abbreviate day, week, month, and year. Examples: 5 h, 10 min, 100 s, 8 days, 10 weeks.
Author's reply: These abbreviations were rewritten.

19. Please include a single line space between all the steps and ensure that the highlight is up to 3 pages of the Protocol (including headings and spacing) that identifies the essential steps of the protocol for the video, i.e., the steps that should be visualized to tell the most cohesive story of the Protocol.
Author's reply: This session was remodeled to be as JoVE Style Guide requests.

20. As we are a methods journal, please revise the Discussion to explicitly cover the following in detail in 3-6 paragraphs with citations:
a) Critical steps within the protocol
b) Any modifications and troubleshooting of the technique
c) Any limitations of the technique.
Author's reply: This information were added.

21. Figure 2: Please include scale bars in all the images of the panel.
Author's reply: This information was added.

22. For in-text formatting, corresponding reference numbers should appear as numbered superscripts after the appropriate statement(s).
Author's reply: The references were remodeled to be as JoVE Style Guide ask.

23. Please ensure all the essential items are included in the Table of Materials. Please remove trademark (™) and registered (®) symbols from the Table of Equipment and Materials.
Author's reply: This session was revised.

In addition to the aforementioned modifications, we provide the p values for the Spearman's coefficient data.

Reviewers' comments:

Reviewer #1:
Major points:
* I do appreciate the efforts which the authors put into preparing the manuscript, but in many places the sentences are "ornamented" with long and complicated statements. They really should be simplified; there are also multiple gramma- my suggestion would be either ask a native speaker for helps, or an independent proofreading company (many to be found online and their services are inexpensive).
Author's reply: The manuscript was revised by an English-speaking professional.

* The paper is long; I get an impression that this was a part of a PhD thesis which was squeezed into a methods format.
Author's reply: The paper was remodeled to be more concise.

* The manuscript does not contain substantial novelty compared to what is already known in the literature for the extraction of SVF from adipose tissue
Smaller Points *28/5000 Minor concern: Unclear figures and tables
Author's reply: All figures and tables are in the resolution asked by JoVE.

Reviewer #2:

Manuscript Summary:
The authors have described the methodology for collection and isolation of mesenchymal stem cells derived from adipose tissue and evaluated the effect of this improved technique after long-term SVF cryopreservation.
I would like to congratulate the authors for the good-quality article, the literature used to write the paper, and for the clear and appropriate structure. The manuscript is well written, presented and discussed, and understandable to a specialist readership. In general, the

organization and the structure of the article are satisfactory and in agreement with the journal instructions for authors. The subject is adequate with the overall journal scope. The work shows a conscientious study in which a very exhaustive discussion of the literature available has been carried out. The introduction provides sufficient background, and the other sections include results clearly presented and analyzed exhaustively.

Minor Concerns:
The authors have chosen nine female patients. Can sex of the patient affect the parameters like viability, yield etc.?
Author's reply: Yes, sex can affect cell parameters as it is written in the paper (line 122-124). The patients were all chosen by female sex beyond other parameters cited to reduce sex bias.

Please include a brief discussion on the impact of various factors on the viability of SVF cells and yield.
Author's reply: In the sentence about this issue (line 290), more information was included.

Do the authors have any suggestion for further studies or scope for further evaluation that may further improve this method? Please discuss at the end.
Author's reply: Apart from the information given in line 270 that suggested a trial performing this method with a larger sample, a new paragraph was added concerning other evaluations to improve this method.

Reviewer #3:

Manuscript Summary:
The authors have described a protocol for obtaining viable ADSCs after cryopreservation. They have demonstrated a shorter duration for processing the adipose tissue in order to isolate the ADSCs.

Major Concerns:
The stem cells characterization do not meet the minimal criteria as per international norms. Only flow cytometry and viability are insufficient to ascertain the quality of cells.
Author's reply: In line 294 the minimal criteria were cited as a reference. Since these studied cells would not be infused in real patients and the major objective of the work was to assess whether the method of collection and isolation of the cells were feasible, and because the project is limited with research funds, all the techniques were carried out for only one of the cases (assay *in vitro* differentiation to osteoblasts, adipocytes and chondroblasts for Case 2). So, the authors decided not to present it in the paper.

Line: 166-167: In cell culture practice it is not recommended to store cells at -80°C for 1 year. Usually it is kept overnight at -80°C and transferred the next day to liquid nitrogen vapor phase. This is a serious flaw in the protocol and may account for the low viability. For

transplantation, viability of >80% is the minimum requirement (before or after cryopreservation).
Author's reply: In fact, in line 346 there is a sentence explaining cells viability decreasing after cryopreservation with literature reference. The method cited for the storage of cells used in this study was already published in the literature.

Lines 219-225: It is not clear if the ADSCs are hematopoietic or non-hematopoeitic. For Mesenchymal stem cells irrespective of source the non-hematopoietic markers should be >95% and hematopoietic markers should be <2% as per the minimal criteria described by Dominici et al (2006). There appears to be a large heterogeneity among the cells studied which is not desirable and this is very evident in Fig. 2B and Table 3.
Author's reply: It was demonstrated by the percentage of monoclonal antibodies for CD45-, CD34+ and combinations of CD105, CD73, and CD90 in table 3 that ADSC are non-hematopoietic. The sentence in lines 302-304 was rewritten to clarify this information.

Minor Concerns:
Line 136: what was the time taken to reach the lab and before processing the adipose tissue? What was the temperature during transportation of tissue?
Author's reply: In lines 136-137 it was written: "room temperature". It was added the specific temperature and a sentence about the time taken during the transportation to complete the information that it is "immediately" taken to the laboratory.

Lines 143 and 147: what was the concentration of Calcium added to the DPBS?
Author's reply: This information was added in the sentence (calcium chloride (1 g/L)).

Line: 155: was the DMEM high or low glucose?
Author's reply: This information was added in the sentence (low glucose).

Other queries:

1. Please clarify at which passage numbers the cells were evaluated before and after cryopreservation.
Author's reply: This information was included in the sentence.

Reviewer #4:

Manuscript Summary:
The authors describe a protocol to isolate adipose-derived stromal cells (ASCs) from lipo-aspirates and indicated that the protocol results in a faster isolation time of ASCs. They also investigated viability of ASCs after cryopreservation. The protocol described is standard protocol for isolating ASCs which is well described in literature. With the only exception: usually the lipo-aspirate will be decanted into centrifuge tube. The authors opted to perform the initial washing steps in the collection bag which will result in saving time during

the isolation procedure. However, the claim that the isolation described is faster is subjective as the volume of lipo-aspirate processed will potentially influence the digestion time. A volume of 100 mL lipo-aspirate as starting material is a relatively small volume and naturally will take quicker to process. The collection bags used in this manuscript may also not be standard as different collection cannisters are used by surgeons globally. Several other factors standard to isolation of ASCs is also not described or mentioned. Digested lipo-aspirate is usually filtered to get rid of undigested tissue fragments. No mention of this was made by the authors. No mention of seeding density was made in the manuscript. Another important aspect that should have been standardized between the different donors.
Author's reply: brilliant appointments. The purpose of using 100 mL is to demonstrate, precisely, that with little adipose tissue collected, it is possible to obtain good cell volume with good viability. The use of different inputs such as the collection bag or the non-use of filter for undigested tissue fragments is to test new ways of working. The information about the seeding density was in line 188 and it was rewritten to clarify the information.

The introduction can better structured and organized to limit unnecessary repetition.
Author's reply: The introduction was remodeled, as suggested.

The method is poorly described and is unclear and should be revised.
Author's reply: The methods were rewritten.

Major Concerns:
The manuscript is poorly written and needs significant editorial attention, especially regarding construction and phrasing of sentences.
Author's reply: The manuscript was revised and rewritten, considering the editor's comments and corrections.

Minor Concerns:
Please consider replacing 'cellularity' with 'cellular yield' throughout the manuscript.
Author's reply: Done.

Full term of DPBS should be introduced at first use.
Author's reply: Done.

Line 37: 'first cultured cells' It is unclear what this refer to. Does it refer to the adherent fraction of the SVF. Please clarify and rephrase.
Author's reply: The sentence was rewritten to become clearer.

Lines 51 - 53. Structure and phrasing of sentence should be reconsidered.
Author's reply: The text was revised.

Line 162.'equilibrate' Please consider replacing with 'resuspend'.
Author's reply: Done.

Line 162. 'maintained'. Please consider replacing with 'stored'.
Author's reply: Done.

Line 177. It is unclear what was used as negative control - unstained cells or cells stained with isotypic controls. Please clarify. Please also describe how negative/positive boundaries were set.
Author's reply: This information was added, as requested.

Point 6 of methodology is unclear and needs to be rewritten.
Author's reply: Done.

On behalf of my co-authors, I would like to thank you and the reviewers for taking the time to review our manuscript entitled "Technique for Obtaining Mesenchymal Stem Cell Derived from Adipose Tissue and Stromal Vascular Fraction Characterization in Long Term Cryopreservation" for possible publication in JoVE.

We sincerely appreciate the comments we received. They certainly contributed to improving our paper. We have critically reviewed and addressed point-by-point all the concerns and suggestions, as it is reflected in the revised version of our manuscript and our responses. We believe this paper will contribute to the current clinical practice. Therefore, we are submitting the revised version. We will fully respect your final decision and we thank you in advance for kindly reconsidering our paper for possible publication.

Hoping that now our paper meets all the requirements for publication and thanking you for all your attention,
I remain
Sincerely yours,

Maria Isabel

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