

An alternative method for the isolation of mesenchymal stromal cells derived from lipoaspirate samples

Leandra S. Baptista^{1,2}, Ronaldo J. F. C do Amaral^{1,3}, Rosana B. V. Carias⁴,
Marcelo Aniceto⁵, Cesar Claudio-da-Silva⁵ and Radovan Borojevic^{1,2,3}

¹APABCAM, Clementino Fraga Filho University Hospital, Federal University of Rio de Janeiro, RJ, Brazil, ²DIPRO, INMETRO, Rio de Janeiro, RJ, Brazil, ³Institute of Biomedical Sciences, Federal University of Rio de Janeiro, RJ, Brazil, ⁴Excellion Biomedical Services, Petrópolis, RJ, Brazil, and ⁵Medical Clinics Department, Clementino Fraga Filho University Hospital, Federal University of Rio de Janeiro, RJ, Brazil

Background aims

Since initial methods were developed for isolating cells from adipose tissue, little has been done to improve mesenchymal stromal cell (MSC) yield. The aim of the present study was to isolate a population of MSC from lipoaspirate samples without tissue digestion and to assess the possibility of cryopreserving the freshly isolated cells.

Methods

A population of MSC was isolated from 13 patients' lipoaspirate samples by mechanical dissociation. Mechanically processed lipoaspirate adipose tissue (MPLA) cells were characterized after *in vitro* cell expansion by morphologic analysis, expression of MSC surface markers and differentiation assays.

Results

Mechanical dissociation yielded a large quantity of adherent MSC both after standard and vibro-assisted liposuction. Preservation of lipoaspirate samples at 4°C for 1 or 2 days until the mechanical

procedure did not change the MPLA cell content. It was possible to store freshly isolated MPLA cells by cryopreservation without loss of the MSC population. Adherent MPLA cells were negative for CD45 and CD31 and positive for CD34, CD105, CD44 and CD90. They also showed adipogenic, osteogenic and chondrogenic potentials similar to MSC populations from other sources as already described in the literature.

Conclusions

MSC can be isolated from human lipoaspirate samples by the mechanical procedure described in this study with a significant reduction in time and cost. Together with cryopreservation of freshly isolated MPLA cells, this has made it easier to harvest and store MSC for therapeutic applications such as soft-tissue augmentation and tissue engineering.

Keywords

lipoaspirate, mesenchymal stromal cells, tissue engineering.

Introduction

The methods for isolating cells from adipose tissue were initially proposed by Rodbell and colleagues in the mid-1960s [1]. They minced rat fat pads, washed them extensively to remove contaminating blood cells, incubated the tissue fragments with collagenase and centrifuged the digest, separating the floating population of mature adipocytes from the pelleted stromal cells. The population of stromal cells includes adipocytes, pre-adipocytes, endothelial cells, macrophages and fibroblasts [2]. Similar to the

bone marrow (BM) stroma, a subpopulation of multipotent mesenchymal stromal cells (MSC) also dwells in the adipose stroma. They are excellent candidates for tissue engineering of bone, cartilage, skin and other tissues [3].

In initial studies, fragments of human tissue were manually minced. The development of liposuction surgery simplified the process of cell harvesting, as it generates finely minced tissue [4], allowing incubation of the fat sample directly with collagenase in order to isolate MSC. Liposuction procedures also improve the soft-tissue

Correspondence to: Leandra S. Baptista, APABCAM, Clementino Fraga Filho University Hospital, Federal University of Rio de Janeiro, Cidade Universitária, Ilha do Fundão, 21941-902 Rio de Janeiro, RJ, Brazil. E-mail: leandra.baptista@gmail.com.

augmentation techniques using immediate autologous fat transfer. This is an attractive therapy, the success of which depends upon the techniques of harvesting, cleansing and reinjection of cells [5]. Recently, Yoshimura *et al.* [6] achieved optimal human MSC yield, resulting in subsequent formation of adipose tissue following implantation in immune-deficient mice.

In tissue engineering, synthetic or tissue-derived scaffolds can provide mechanical support to cells, promoting their differentiation, proliferation and tissue growth. MSC have been described as a better source for tissue engineering than differentiated cells, such as chondrocytes or osteoblasts. This approach requires large numbers of MSC. For example, an *in vivo* chondro-induction requires at least 10^7 cells/cm³ of scaffold [7]. Several donor sites can be used for harvesting MSC, BM and adipose tissue being the most frequent. The latter is easily harvested by liposuction and, in most patients, a large quantity of MSC can be obtained without harm to the donor [2]. Liposuction is frequently done for aesthetic reasons, allowing using MSC for reparative processes extemporaneously. They can be also cryopreserved and stored for future use.

The enzymatic digestion that is the current protocol for isolating MSC limits the volume of lipoaspirate that can be processed for two main reasons: difficulties of the method *per se* when applied to large volumes, and the high cost of collagenase. The aim of the present study was to isolate a population of MSC from lipoaspirates with simultaneous red blood cell lysis without tissue digestion, in order to reduce both time and procedure costs. The mononuclear cell population obtained by mechanical procedure contains blood, endothelial and stromal cells. Cryopreservation was tested in this fresh mononuclear cell population to avoid *in vitro* expansion before a therapeutic approach. The adherent cell fraction was characterized after *in vitro* cell expansion by morphologic analysis and expression of MSC surface markers. The proliferation profile and adipogenic, osteogenic and chondrogenic potentials were monitored. Cells isolated in this study by the mechanical procedure showed MSC properties similar to lipoaspirate cells isolated by the enzymatic procedures of others [3] as well as our group [8].

Methods

Human lipoaspirate sampling

Lipoaspirates were harvested by vibro-assisted and standard liposuction from 13 healthy female donors that

underwent abdominal liposuction, after approval given by the research ethics committee of the University Hospital Clementino Fraga Filho, Federal University of Rio de Janeiro, RJ, Brazil. All donors signed an informed consent form. Adipose tissue samples were preserved at 4°C after surgery until the mechanical processing.

Isolation and culture of mechanically and enzymatically processed lipoaspirate

Adipose tissue samples were distributed into tubes containing ACK buffer solution (1/1, v/v). The mixture was shaken and maintained at 37°C for 15 min for red blood cell lysis. Cells were separated from adipose tissue fragments, oil and debris by centrifugation at 900 g for 15 min at room temperature. The pellets obtained were resuspended in fetal bovine serum (FBS; Cultilab, Campinas, SP, Brazil) supplemented with 10% dimethylsulfoxide (DMSO; Sigma Chemical Co., St Louis, MO, USA) for cryopreservation (10^7 cells/mL). The vials were kept overnight at -70°C, transferred and kept at -196°C until thawing. Alternatively, they were resuspended and plated into culture dishes (10^5 cells/cm²) in low-glucose Dulbecco's modified Eagle's medium (DMEM; LGC, Cotia, SP, Brazil) containing 20% FBS, 100 U/mL penicillin and 100 µg/mL streptomycin. Samples obtained by vibro-assisted and standard liposuction followed the same protocol in order to compare the isolated cell populations.

For cell thawing, the vials were placed at 37°C until the ice had been thawed. Approximately 10 mL fresh DMEM was added to the cell suspension from each vial and centrifuged at 400 g for 7 min. The cell pellets were resuspended in DMEM containing 20% FBS, 100 U/mL penicillin and 100 µg/mL streptomycin and plated into culture dishes (10^5 cells/cm²). Cultures of fresh and thawed cells were maintained at 37°C in a humid atmosphere containing 5% CO₂, and the medium was changed every 3–5 days until cells reach pre-confluence. Adherent cells were detached with 0.78 mM EDTA and 0.125% trypsin (Gibco BRL, Rockville, MD, USA) and cell suspension was centrifuged at 400 g for 7 min, counted and replated into culture dishes (10^4 cells/cm²). This was considered to be 'one passage'. For monitoring cell proliferation, cells in the third and 10th passages were plated (10^4 cells/well; $n = 3$) in 24-well plates and cultured in DMEM containing 20% FBS for up to 2 weeks. At days 1, 3, 6, 9, 12 and 15, viable cells were quantified using trypan blue staining, which allows dead cell exclusion.

The same lipoaspirate samples underwent mechanical and enzymatic processes in order to compare the cell population yield. Adipose tissue samples were incubated with collagenase IA (Sigma Chemical Co.). Cells were harvested by centrifugation and plated in tissue culture flasks with low-glucose DMEM containing 20% FBS, 100 U/mL penicillin and 100 µg/mL streptomycin. Cultures were maintained at 37°C in a humid atmosphere with 5% CO₂, and the medium was changed every 3–5 days until cells reach pre-confluence.

Flow cytometry analysis

Cells were monitored for surface marker expression at the moment they were isolated, and at the first and third passages ($n = 3$ for each analysis) using flow cytometry. Cells were washed with phosphate-buffered saline containing 3% bovine serum albumin (PBS-BSA 3%) and incubated for 30 min at 4°C with monoclonal antibodies conjugated with fluorescent dyes: CD14–phycoerythrin (PE), CD16–PE–Cy7, CD31–PE, CD34–APC, CD34–PerCP–Cy5.5, CD44–PE, CD45–fluorescein isothiocyanate (FITC), CD73–PE, CD90–APC (BD Biosciences, Franklin Lakes, NJ, USA), CD105–FITC and CD105–PE (R&D Systems, Minneapolis, MN, USA). Subsequently, cells were washed with PBS-BSA 3%. When necessary, the cell suspensions were incubated at room temperature in the absence of light for 10 min with FACS lysing solution (BD Biosciences). Flow cytometry analyzes were performed using a FACScanto (BD Biosciences) equipped with the software FACS Diva 4.0.

Cell differentiation assays

Osteogenic and adipogenic potentials were investigated using the appropriate inducing media ($n = 3$, for each assay), as described previously [3]. Briefly, for adipogenic assays, cells were cultured in DMEM containing 20% FBS, 10 µM insulin (Biohulin, Montes Claros, MG, Brazil), 0.5 µM isobutylmethylxanthine (IBMX), 1 µM dexamethasone and 200 µM indomethacin (Sigma) for 14 days. For osteogenic assays, cells were cultured in DMEM containing 10% FBS, 5×10^{-6} M ascorbic acid, 0.01 µM dexamethasone and 10^{-2} M β-glycerolphosphate (Sigma) for 14 days.

Following lineage differentiation induction, cells were fixed in 10% formaldehyde for 60 min. Adipogenic differentiation was assessed using Oil Red O staining (Sigma) as an indicator of intracellular lipid accumulation. Cells were pre-incubated in propylene glycol (Merck, Whitehouse

Station, NJ, USA), incubated in 0.5 mg/mL Oil Red O solution for 30 min at room temperature and excess staining removed with 85% propylene glycol. Osteogenic differentiation was assessed by a modified Von Kossa's method [3] as an indicator of extracellular calcium deposition. Cells were rinsed with distilled water and then covered with a 2% silver nitrate solution (Sigma), in the absence of light, for 60 min. The cells were washed several times with distilled water and the reaction was developed under ultraviolet (UV) light for 30 min.

Chondrogenic potential was monitored in pellet cultures as described previously [9]. Briefly, 1.5×10^5 cells were centrifuged at 400 *g* in 15-mL polypropylene conical tubes and the resulting pellets cultured for 21 days. To induce chondrogenic differentiation, cultures were grown in human MSC chondrogenic differentiation BulletKit supplemented with 10 ng/mL recombinant human transforming growth factor (TGF)-β3 (Cambrex, Charles City, IA, USA). Control cultures were grown in the same medium without dexamethasone and TGF-β3. After 21 days, pellet cultures were fixed in 10% formaldehyde for 6 h and embedded routinely in paraffin. Paraffin sections were stained with periodic acid-Schiff (PAS) with nuclei counterstained by hematoxylin, or with alcian blue at pH 1.0 with nuclei counterstained by neutral red.

Statistical analysis

Unpaired and non-parametric ANOVA tests were performed when necessary to evaluate differences between groups. Results are expressed as mean ± standard deviation (SD) and *P*-values less than 0.05 were considered statistically significant.

Results

Ex vivo isolation of MSC with a minor red blood cell contamination

Mechanical dissociation with simultaneous lysis buffer treatment resulted in *ex vivo* cell suspensions, called mechanically processed lipoaspirate adipose tissue (MPLA), that contained two different major mononuclear cell subpopulations, CD45⁺ and CD45⁻. Cells positive for CD45, a pan-hematopoietic marker, were also positive for CD16, CD14, CD31, surface markers of granulocytes, monocytes–macrophages and endothelial cells (Figure 1B–F). They were also positive for CD44, a cell-adhesion molecule that participates in a wide variety of cellular functions, including lymphocyte activation, recirculation and homing,

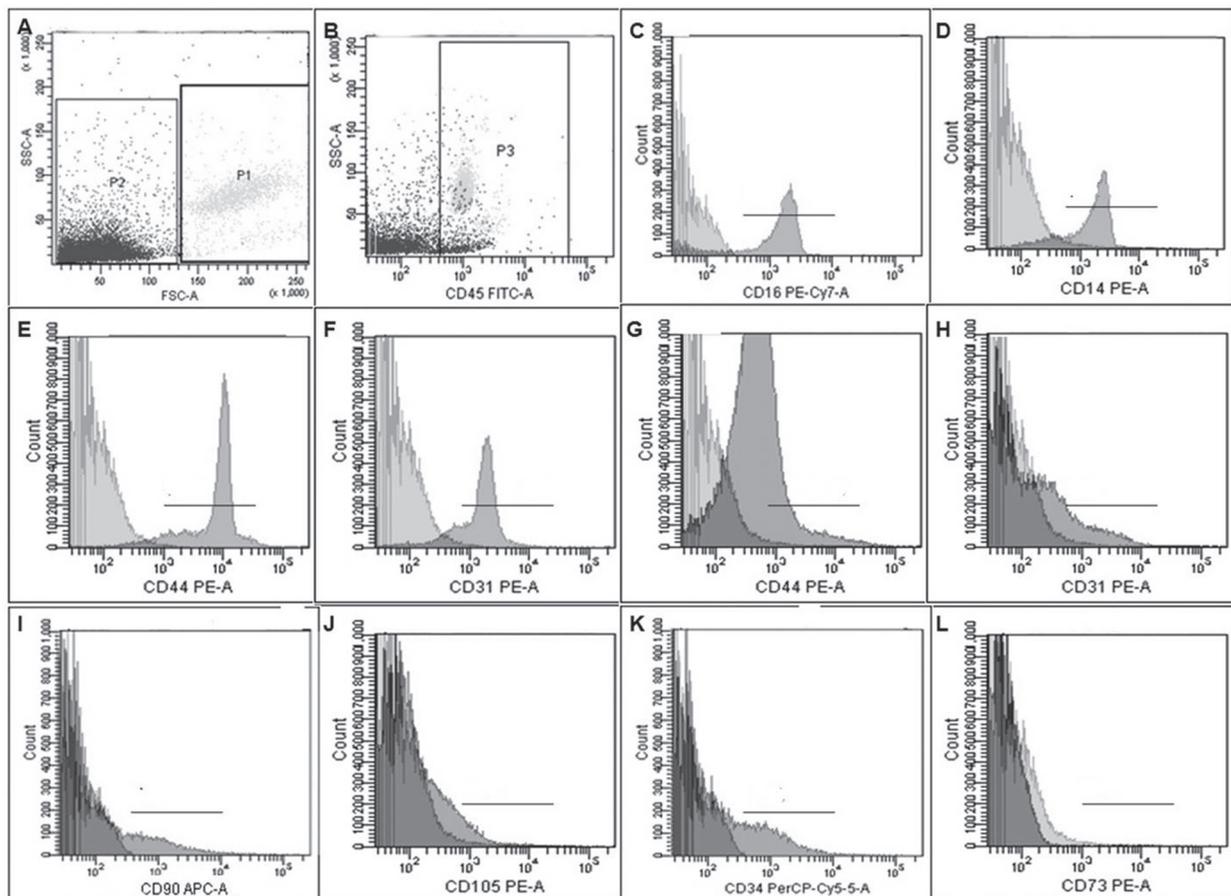


Figure 1. Phenotypic characterization of freshly isolated total MPLA cells. (A) FSC/SSC distribution of all MPLA cells that can be subdivided into two subpopulations, P1 and P2. (B) P1 is basically constituted by blood cells ($CD45^+$) gated in P3. The histogram of cells within P3 is shown in (C–F), and outside P3 is shown in (G–L). Note that $CD45^-$ cells express MSC surface markers except CD73 (L). Isotype controls are overlaid in all histograms.

hematopoiesis and tumor metastasis [10]. As $CD45^+$ cells present in lipoaspirate samples are peripheral blood contaminants, they were removed after plating by washing, leaving a population of adherent cells with fibroblastoid morphology. In this study, this population was called adherent MPLA cells. The presence of macrophages in the adherent population was very low. Being resistant to trypsin, they were lost at replating, as shown by the absence of cells bearing macrophage markers at flow cytometry analysis. These adherent MPLA cells were negative for CD45 and CD73 and positive for CD44, CD31, CD90 and CD105 (Figure 1G–J), surface markers described in MSC populations of different origins. They were also positive for CD34, which is reported only in adipose tissue-derived mesenchymal cells [11].

The mechanical process was accomplished in a reduced time compared with the enzymatic digestion, and it was

possible to isolate viable cells from lipoaspirate samples harvested by distinct liposuction procedures. The total number of MPLA cells harvested from the vibro-assisted lipoaspirates samples was superior to that obtained from the standard one. Adherent MPLA cells from these two samples showed no difference in adhesion and proliferation (data not shown).

Lipoaspirate samples can be stored without damage to MPLA cells

In order to monitor possible damage or loss of MPLA cells during their storage and transportation from surgery facilities to a cell-processing unit, lipoaspirate samples were stored at 4°C for up to 2 days in a lipoaspirate collection recipient containing approximately 10% fresh DMEM. Total MPLA cells were subsequently cultured in low-glucose DMEM supplemented with 20% FBS and

Table I. Number of total MPLA cells and adherent MPLA cells obtained from fresh and preserved lipoaspirate samples by mechanical dissociation. Data represent the mean \pm SD of five samples.

	Number of MPLA cells per milliliter of lipoaspirate sample ($\times 10^4$)	Number of adherent MPLA cells per milliliter of lipoaspirate sample ($\times 10^4$)
Fresh lipoaspirate sample	24.0 \pm 7.4	1.2 \pm 0.37
Preserved lipoaspirate sample for 1 day	22.6 \pm 10.1	0.51 \pm 0.2
Preserved lipoaspirate sample for 2 days	18.9 \pm 9.1	0.39 \pm 0.18

antibiotics at 37°C in 5% CO₂ atmosphere. Although the number of total MPLA cells did not change, the number of adherent MPLA cells isolated from preserved samples (for 1–2 days) was reduced (not statistically significant) compared with fresh samples (samples preserved at 4°C for up to 4 h) (Table I and Figure 2). This could be attributed to a reduction of the total MPLA cell adhesion and/or viability, but no change in the isolated adherent cells morphology was observed. Lipoaspirate samples obtained by the enzymatic process, called here adherent MPLA cells, yielded a higher number of cells (Table II and Figure 2).

We also monitored the possibility of cryopreserving the total MPLA cells for a long time, without a previous *in vitro* expansion. Immediately after the mechanical procedure, fresh MPLA cells were cryopreserved and, after thawing, morphologic (Figure 3A) and phenotype characteristics (Figure 3B) of the adherent fraction were the same as the non-cryopreserved MPLA cells.

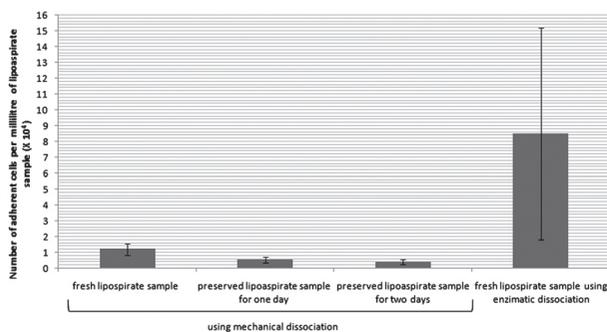


Figure 2. Graph representing the mean values of adherent MPLA cells obtained from fresh and preserved lipoaspirate samples using mechanical dissociation, and of PLA cells from fresh lipoaspirate samples using enzymatic dissociation. No statistical significance was detected among the fresh and preserved lipoaspirate samples or between preserved lipoaspirate samples ($P > 0.05$). Statistical significance was only detected among adherent MPLA and PLA cell numbers (enzymatic dissociation versus fresh lipoaspirate sample using mechanical dissociation, $P < 0.05$; enzymatic dissociation versus the preserved lipoaspirate samples for 1 and 2 days using mechanical dissociation, $P < 0.01$).

Adherent MPLA cells showed *in vitro* MSC properties

When adherent MPLA cells reached pre-confluence, they were harvested and replated in low-glucose DMEM containing 20% FBS and antibiotics until the 10th passage. *In vitro* expansion altered neither the MPLA cell proliferation (Figure 4A) nor their spindle-shaped morphology (Figure 4B,C). Adherent expanded MPLA cells were negative for CD45 (Figure 5A), as expected, as well as the mature endothelial cell surface marker CD31 (Figure 5B). They were positive for MSC-associated markers CD105 (Figure 5D), CD44 (Figure 5E) and CD90 (Figure 5F), which were up-regulated during *in vitro* expansion. At the third passage, CD34 expression was strongly reduced (Figure 5C). For differentiation assays, adherent MPLA cells were cultured under adipogenic- or osteogenic-inducing medium for 14 days. As expected, MPLA cells were able to accumulate lipid droplets (Figure 6B) and deposit extracellular calcium (Figure 6D), which were both observed under the microscope by specific stainings. MPLA cell pellets formed solid three-dimensional tissue structures that could be harvested and processed for histology. In the presence of TGF- β 3, pellets grew in size, almost doubling the control cultures (Figure 6E,F). This growth could be attributed to cartilage matrix accumulation, as shown by alcian blue staining, which reacts mainly with sulfated proteoglycans (Figure 6H).

Discussion

We have described a novel method of isolating MSC, derived from lipoaspirate samples, based on mechanical tissue dissociation. The usual enzymatic digestion procedure for adipose tissue consists of at least four main steps: washing, digestion, centrifugation and red blood cell lysis. This procedure generates tissue fragments that should be removed before cell plating through a 100–150 μ m nylon mesh. Conversely, mechanical dissociation consists basically of two steps, dissociation of adipose tissue concomitantly with red blood cell lysis, followed

Table II. Number of total PLA cells and adherent PLA cells obtained from fresh lipoaspirate samples by enzymatic dissociation. Data represent the mean \pm SD of five samples.

	Number of PLA cells per milliliter of lipoaspirate sample ($\times 10^4$)	Number of adherent PLA cells per milliliter of lipoaspirate sample ($\times 10^4$)
Fresh lipoaspirate sample	58.4 \pm 17.8	8.5 \pm 6.7

by centrifugation. There are no visible tissue fragments, and it is not necessary to filter cell suspension. The ease of mechanical digestion reduces considerably both time and cost, and does not interfere with cell viability.

Compared with the enzymatic process, the mechanical process generates a significantly lower number of adherent MPLA cells. However, compared with other studies, the MSC yield by enzymatic process [12] remains close to the adherent MPLA cells. Moreover, processes based on enzymatic digestion seem to be less reproducible, as there is no consensus in the literature regarding the MSC yield. We observed a large standard deviation among cell numbers isolated with the enzymatic digestion process. Adding reproducibility to the low costs, the mechanical process is proposed as a preferable method for large volumes of samples. The reproducibility of the mechanical process described here should be tested for a larger number of lipoaspirate samples and by other research groups.

This alternative procedure was also tested with lipoaspirate samples preserved at 4°C for up to 2 days. Recently, it has been shown that adipose tissue-derived stem cell yield from tissue digests is significantly reduced by preservation at room temperature for 24 h and even by preservation at 4°C for 2–3 days [13]. In our study, preservation for 1–2 days did not make a difference in surface marker profile or the number of total MPLA cells. Moreover, no significant difference was observed among adherent MPLA cells isolated from fresh and preserved lipoaspirate samples, and after 2 weeks it was possible to obtain monolayers of MSC from all samples.

Liposuction surgery often generates large volumes of samples to be processed, so it is important not to waste them. In order to facilitate preservation of total MPLA cells for a long time, they were cryopreserved immediately after being isolated. It was possible to thaw them without loss of cell viability. Interestingly, another study has investigated a method for cryopreserving human adipose-derived stem cells isolated by an enzymatic process. Fresh human cells

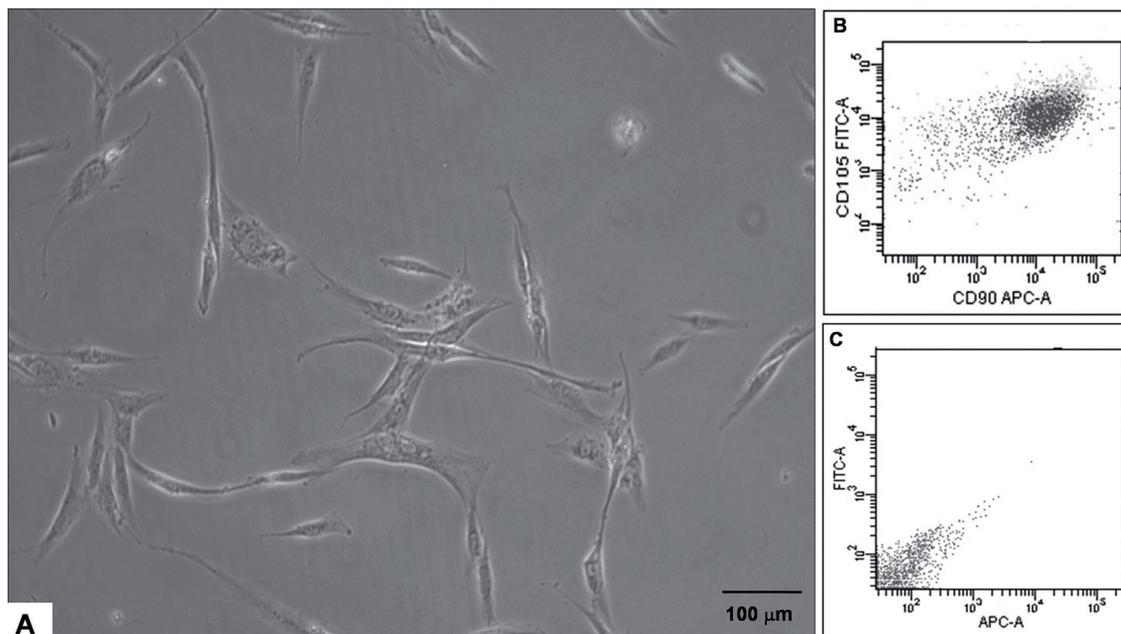


Figure 3. Culture of thawed MPLA cells. (A) Adherent cells showed a typical morphology and (B) expression of mesenchymal surface markers. (C) Isotype control. Bar size, 100 μ m.

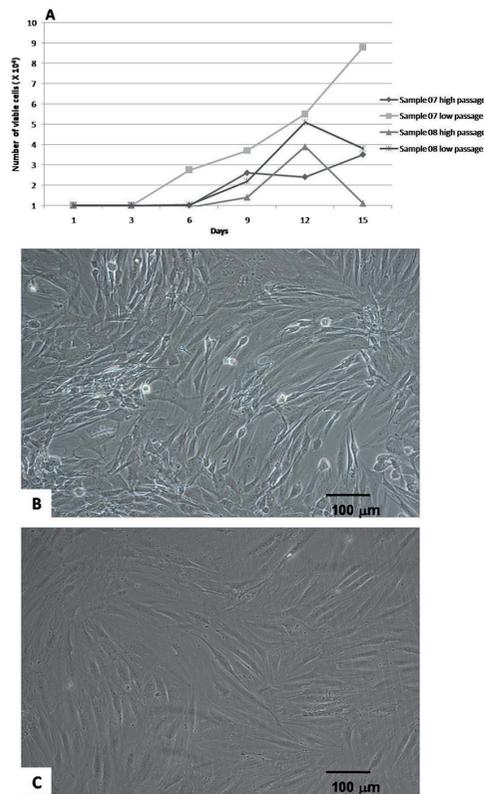


Figure 4. The proliferation of adherent MPLA cells was evaluated by trypan blue exclusion assay. (A) No considerable differences in cell numbers generated by adherent MPLA cells in low and high passages. Monolayer of adherent MPLA cells cultured in the presence of 20% FBS in low (B) and high (C) passages. Bar size, 100 μm .

were cryopreserved using Me_2SO as the cryoprotective agent at a density of 10^6 cells/mL [14], 10 times lower than the cell quantity cryopreserved in our study. Cryopreservation is interesting because it reduces labor costs and avoids possible loss of cell viability and senescence after long-term cultures [15,16]. It is possible to thaw total fresh MPLA cells as needed for cell therapy approaches.

The yield of adipose tissue cells derived from liposuction procedures depends directly upon the surgical procedure. It has been reported for ultrasound-assisted liposuction that the number of MSC recovered from tissue digests is reduced, as is their proliferative capacity [17]. In the present study, MSC could be isolated from vibro-assisted liposuction, maintaining their biologic properties similar to the standard liposuction procedure. Vibro-assisted liposuction has already been reported to reduce the duration of surgery because of its large rate of aspiration [18]. Using a mechanical approach with vibro-assisted lipoaspirate samples, it was possible to obtain a large MSC population, not described for enzymatic processes.

The loss of cell viability caused by the use of enzymes such as collagenase during cell harvesting and processing has already been described in several protocols of primary cell cultures. Rodeheaver *et al.* [19] observed a loss of tubular morphology and an increase in the rate of Na-K-ATPase activity in rabbit renal proximal tubules subjected to incubation with enzymes that were attributed to the disruption

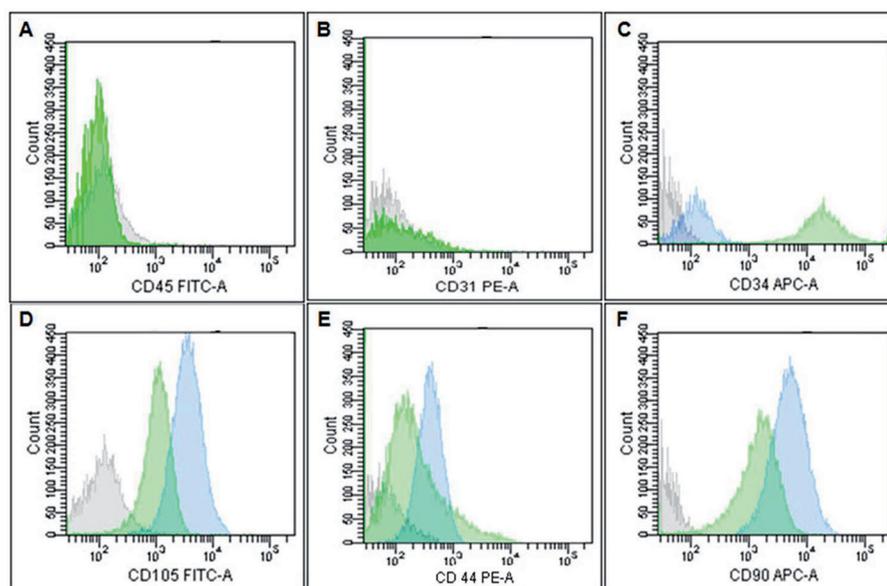


Figure 5. Surface marker profile of adherent MPLA cells harvested after first (green solid histograms) and third passage (blue solid histograms). The population of adherent MPLA cells was negative for CD45 (A) and CD31 (B). Expression of CD34 (C) was down-regulated, unlike CD105 (D), CD44 (E) and CD90 (F) that were up-regulated at the third passage. Gray solid histograms represent negative controls (isotype controls).

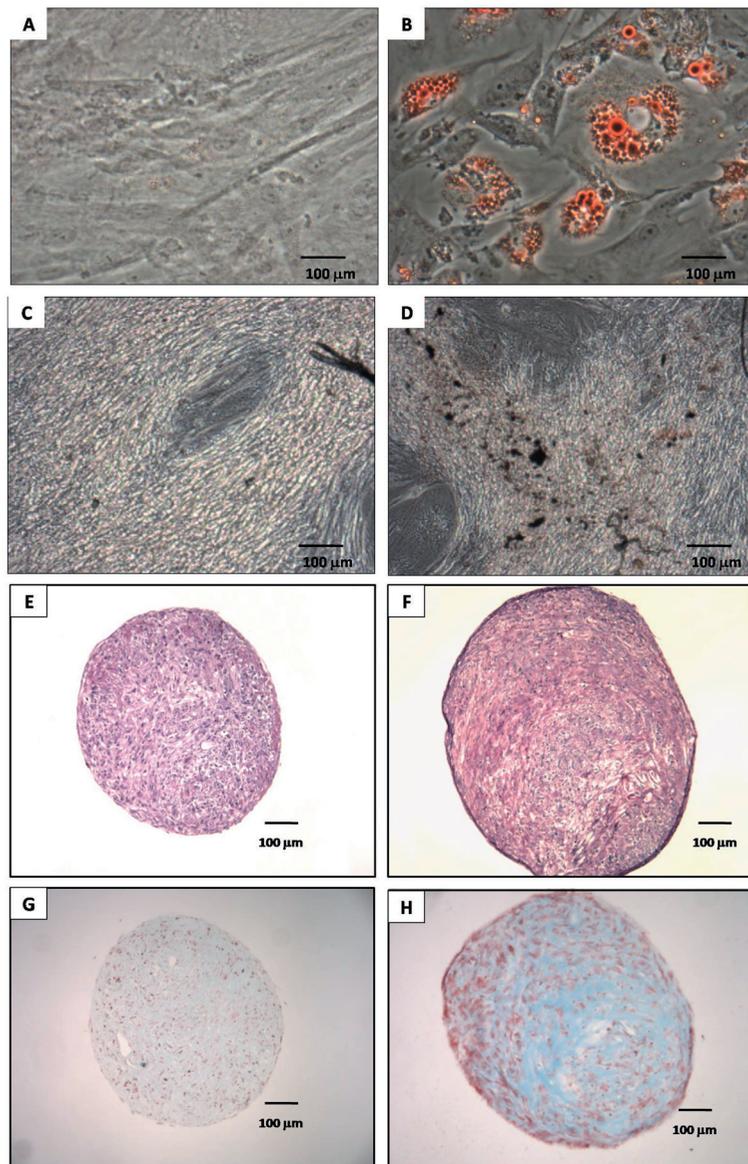


Figure 6. Adherent MPLA cells showed in vitro multipotentiality. Monolayers of cells were maintained under adipogenic or osteogenic inducing media for 14 days (A–D). After this period, they were fixed and stained with Oil Red O to show the lipid droplets, and with the Von Kossa method to reveal calcium deposits. Control media (A, C); inducing media (B, D). Pellet cultures formed by adherent MPLA cells under control (A, C) and chondrogenic (B, D) media for 21 days. (A, B) PAS staining with nuclei stained by hematoxylin. (C, D) Alcian blue staining with nuclei stained by neutral red. Matrix accumulation is typical of cartilage. Bar size, 100 μm.

of the tubular basement membrane. Recently, it has been shown that isolation of chondrocytes by enzymatic digestion disturbs their viability and differentiated phenotype [20]. We hypothesize that the described mechanical process of cell harvesting does not chemically destabilize or disrupt cell membranes, and for this reason it was possible to obtain a full cell yield even from vibro-assisted and not fresh (preserved) lipoaspirate samples. We believe that the process based on mechanical dissociation also explains the successful cryopreservation of freshly isolated cells.

Flow cytometry analysis revealed that the mechanical procedure was able to isolate CD45⁻ cells with a profile of surface markers typical of MSC [21], except for the absence of CD73 and presence of CD34. The lack of CD73 expression may reflect a specific characteristic of MPLA cells. In addition, MSC when in culture adhere to plastic dishes, which can modulate their expression of surface markers [8]. With respect to CD34, several studies have described it as a surface marker for MSC from adipose tissue [8,11], in agreement with our study. We also found a

slightly increased expression of MSC-associated markers and a down-regulation of CD34 by adherent MPLA cells after several passages *in vitro*, in accordance with previously reported data [8,22]. Moreover, we observed the same surface marker profile on adherent MPLA cells isolated from preserved lipoaspirate samples.

Differentiation assays to adipogenic, osteogenic and chondrogenic lineages support the idea that adherent MPLA cells from fresh and preserved lipoaspirate samples are a population of MSC. At third or fourth passages these cells were able to accumulate lipid droplets, calcium deposits and cartilage matrix under appropriate inducing media in a manner similar to MSC from adipose tissue isolated by enzymatic digestion procedures [3,8].

Autologous progenitor cells, such as those derived from BM or adipose tissue, can be used clinically for regenerative cell therapy or for tissue engineering only when isolated in a reproducible manner and in sufficient quantities. Despite the major differences between the enzymatic and mechanic processes, similar populations of MSC have been isolated. MPLA cells can be processed easily from lipoaspirate samples and provide a significant quantity of adherent cells with minor time and costs for the procedure. Furthermore, MPLA properties support their use for diverse therapeutic applications. The possibility of cryopreservation of freshly isolated MPLA cells abrogates culture-associated changes found in cells after prolonged expansion, and provides the possibility of generating extemporaneously a large stock of cells using a relatively simple method.

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Declaration of interest: The authors report no conflicts of interest. The authors alone are responsible for the content and writing of the paper.

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