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In vitro multilineage potential and immunomodulatory properties of adipose derived stromal/stem cells obtained from *nanofat* lipoaspirates

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Abstract

Background: **Among stem cell sources for regenerative therapy, human adipose derived stromal/stem cells (hASCs) have attracted a lot of attention over the last decade. hASCs are easily accessible, available in high numbers and their use does not pose ethical concerns. These cells can be obtained with minimal manipulation methods and have been used extensively in the autologous setting. However, the methods for tissue harvesting and grafting remain a crucial issue.**

This study aims at evaluating the biological features of hASCs obtained with *nanofat*, a novel liposuction technique, and at comparing them with those of hASCs obtained with the classical *Coleman* technique of adipose tissue harvesting.

Materials and Methods: **The *nanofat* methodology is a procedure alternative to the classical *Coleman* technique of fat harvesting and is characterized by the use of a thinner cannula. We analyzed hASCs derived from lipoaspirate samples collected using these two techniques. We assessed the immunophenotype, the rate of cell proliferation, adipogenic, osteogenic and chondrogenic differentiation potential and immunomodulatory properties of *nanofat*- and *Coleman*-processed hASCs.**

Results: **Both liposuction procedures enabled isolation and expansion of hASCs with high efficiency. The immunophenotypic characterization showed an antigen profile similar to the pattern expressed by mesenchymal stem cells. hASCs obtained with the two methods displayed multilineage differentiation potential into the adipogenic, osteogenic and chondrogenic commitments. Finally, both *nanofat*- and *Coleman*-derived hASCs displayed immunomodulatory properties, as evidenced by their tolerogenic phenotype and their ability to inhibit lymphocyte proliferation *in vitro*.**

Conclusions: **These results indicate that the stem cell component in the stromal-vascular fraction is preserved in lipoaspirates obtained with the *nanofat* technique.**

Introduction

Adipose tissue contains a heterogeneous stromal cell population that includes uncommitted mesenchymal stromal/stem cells (MSCs). These cells, termed human adipose-derived stromal/stem cells (hASCs) due to their tissue origin, are endowed with multilineage differentiation potential and immunomodulatory properties. These characteristics candidate them for use in a variety of clinical applications. Since the discovery of hASCs in

2001, adipose tissue has been recognized not only as an energy reservoir, but also as a rich source of multipotent MSCs[1-5]. These cells exhibit fibroblastic morphology and possess MSC characteristics. According to the International Society for Cellular Therapy, MSCs are defined as plastic-adherent multipotent cells that can differentiate *in vitro* into mesodermal lineages such as osteoblasts, adipocytes and chondrocytes[6,7]. Similarly to MSCs from other sources, hASCs express the cell surface mesenchymal markers CD44, CD73, CD90, CD105, whereas they present minimal or no expression of the hematopoietic markers CD11b, CD14, CD19, CD34, CD45, CD79a and of the histocompatibility antigens HLA-DR-DP-DQ[8-11].

hASCs have attracted the attention of investigators for their immunomodulatory properties, as demonstrated by several *in vitro* and *in vivo* studies[12]. This feature is relevant for their possible use in cell therapy applications. hASCs can interact with the immune system to delay or prevent rejection and maintain a state of tolerance in allogeneic transplantation[13]. hASCs inhibit phytohemagglutinin (PHA)-stimulated lymphocyte proliferation and display a low immunogenic profile (e.g. low expression of HLA-DR)[14]. Recent studies[15-18] have demonstrated potent immunomodulatory effects from mesenchymal cell therapy in solid organ transplantation, although more preclinical and clinical testing will be needed before translating this cell therapy into an option for clinical transplantation.

The most common method used for collecting adipose tissue is liposuction. Several liposuction procedures have been developed during the last two decades. In the first medical applications, autologous fat was used as a filling after trauma, disease or aging, and relatively large cannulas (2 mm diameter) were used to perform macrofat grafting. As studies proceeded, the reduction of the volume of tissue injected was found to improve both engraftment and survival of the tissue in the transplant site. The use of cannulas of smaller size for grafting marked the transition from macrofat (more than 2 mm diameter) and microfat graft (0.9-0.7 mm diameter) to the most recent *nanofat* (0.5 mm diameter) grafting techniques[19-21].

At this point, issues such as the ideal cannula size and fat harvesting technique, as well fat processing methods to ensure maximum uptake and viability of the graft, are all aspects that require clarification[22]**.** The harvesting technique may have an effect on the yield, viability and functions of processed cells, and certain tissue sources may be accessible only with specific methods[23]. The harvesting technique may ultimately affect the performance of hASC-based tissue engineering for regenerative medicine purposes[24,25]. The use of a thinner cannula for harvesting and grafting could disaggregate the tissue and release hASCs more efficiently, which could result in improved outcomes in plastic and reconstructive surgeries[26]. When compared to conventional lipofilling, this method could stimulate tissue regeneration and neovascularization, which could result from the secretion of angiogenic, antiapoptotic and anti-inflammatory factors by hASCs[27-31]. The *Coleman* technique of lipoaspirate harvesting is based on cannulas with a diameter of 3 mm, with large side holes of 2x7 mm diameter (classic *Coleman* technique)[32]. *Nanofat*, a very recent liposuction technique for adipose tissue harvesting, is based on the use of a cannula of 2 mm diameter with sharp side holes with a diameter lower than 1 mm. This procedure allows to obtain fat tissue preparation that is more suitable for injection through 25G or 27G needles in order to obtain a skin biostimulation in delicates areas such as eyelids and lips.

hASCs with MSCs characteristics can be isolated and expanded in culture from the stromal-vascular fraction (SVF) of lipoaspirates obtained with the *Coleman* technique[33]. We decided to investigate the availability and function of these cells in *nanofat* lipoaspirates.

The aim of this study was to isolate hASCs from *nanofat* lipoaspirates and to compare these cells with hASCs derived from *Coleman* lipoaspirates. We performed a characterization of *nanofat*-processed cells and analyzed mesenchymal cell features, including stemness state, multilineage differentiation potential and immunomodulatory properties, after *in vitro* culture.

Materials and Methods

Tissue collection

Human adipose tissue was harvested after abdominoplasty from five donors through liposuction procedures. Informed consent was obtained from patients according to the policy of the local Ethical Committee (University Hospital St. Orsola-Malpighi, n. 29/2015/U/Tess).

For the surgery, body areas of the donor were identified and marked, the donor's skin areas were disinfected with Betadine, and a sterile surgical field was prepared. The donor's areas were infiltrated with a modified Klein solution (lidocaine 800 mg/L and adrenaline 1:1,000,000) to achieve anesthesia and tissue tumescence, according to the tumescent liposuction technique[32]. Lipoaspirates were obtained from each donor by using both *Coleman* and *nanofat* harvesting techniques during the same surgery.

Coleman's fat tissue harvesting technique was performed using a cannula produced by Mentor Medical System (MENTOR, Santa Barbara, CA, USA). This cannula measures 150 mm in length and has an outer diameter of 3 mm. It has a single suction hole at its peak and this hole has a diameter of 2.5 mm. After physiological solution rinsing and filtering, the adipose tissue obtained was centrifuged at 1200 g for 2 minutes.

The cannula used for *nanofat* harvesting, also produced by Mentor Medical System (MENTOR, Santa Barbara, CA, USA), shows different characteristics. This cannula, normally used for the infiltration of the anesthetic solution of the donor site, measures 150 mm in length and has an outer diameter of 1.5 mm with six holes of 0.5 mm arranged in a spiral. After saline rinsing and filtering through a sterile nylon cloth with 0.5 mm pore size, the adipose tissue obtained was treated as above. Then, mechanical emulsification of the fat was performed to obtain the nanograft preparation, shifting the fat 30 times[26] between two 10 cc syringes connected to each other by a female-to-female Luer-Lock connector (Mentor Medical System, Santa Barbara, CA, USA)

The samples obtained from the two methods were immediately transported in sterile conditions to the laboratory and processed.

Cell isolation of hASCs

Isolation of human Adipose-derived Stromal/Stem cells (hASCs) from tissues collected with the *Coleman* and *nanofat* techniques was started immediately after receiving lipoaspirates in the cell processing facility. Isolation from the two samples was performed in parallel and in sterile conditions. An equal volume of sterile-filtered 0.075% collagenase type II (Sigma-Aldrich Co., St. Louis, MO, USA) prepared in Dulbecco's Modified Eagle's Medium (DMEM, Lonza, Walkersville, MD, USA) was added to the lipoaspirates. The mixtures were incubated for 30 minutes at 37°C with constant shaking. The samples were centrifuged at 400 g for 10 minutes, after which the overlying fluid and adipose phases were discarded. The stromal cell pellet was resuspended and filtered through a 100 μm cell strainer. Cells were counted with Trypan blue (Sigma-Aldrich Co., St. Louis, MO, USA) and cultured at a density of 20,000 cells/cm^2 in DMEM supplemented with 10% heat-inactivated fetal bovine serum (FBS), 2 mM L-glutamine and 1% Penicillin-Streptomycin solution (Lonza, Walkersville, MD, USA).

Cell isolation without enzymatic digestion

To isolate cells from lipoaspirate tissue specimens without enzymatic digestion, 300 μL of both samples were seeded directly on culture with 2 mL of complete medium, DMEM H. 10% FBS, and incubated at 37°C with 5% of CO_2. Then, the medium was changed every 3 days until the 80% of confluence was reached.

Proliferation assay

hASCs derived from *Coleman* and *nanofat* harvesting methods, from passages 1 to 6, were analyzed for their cumulative population doubling. Cells were seeded at an initial concentration of 5,000 cells/cm^2 in T25 culture flasks. At every passage cells were harvested with 0.25% Trypsin-EDTA (Lonza, Walkersville, MD, USA) solution for 3 minutes at 37°C, counted with a hemocytometer by Trypan blue exclusion and seeded again at the initial concentration.

Flow cytometry characterization

Freshly isolated and cultured cells obtained from *Coleman* and *nanofat* lipoaspirates were analyzed by flow cytometry (FACS Navio FC, Beckman-Coulter, Fullerton, CA, USA) to investigate their immunophenotypic profile. Data obtained were analyzed by Kaluza FC Analysis software. The antibodies used were for hematopoietic markers anti-CD14-fluorescein isothiocyanate (FITC), anti-CD34-FITC and anti-CD45-allophycocyanin (APC); for endothelial-perivascular markers anti-CD31-phycoerythrin (PE) and anti-CD146-PE; for stromal markers anti-CD44-FITC, anti-CD73-PE, anti-CD90-phycoerythrin-cyanine 5 (PC5) and anti-CD105-PE (all these primary monoclonal antibodies were purchased from Beckman-Coulter,

Fullerton, CA, USA). We also analyzed the expression of stem cell markers Oct-4-FITC (Santa Cruz Biotechnology, Santa Cruz, CA, USA) and SSEA4-APC (R&D System, Minneapolis, MN, USA). To reveal Oct-4, the cells were permeabilized with the IntraPep Kit (Beckman-Coulter, Fullerton, CA, USA).

In vitro differentiation

Cells obtained with the two techniques were culture-expanded in DMEM 10% FBS and passaged with Trypsin-EDTA. At passage 3, cells were tested for their ability to differentiate into the three classical mesenchymal lineages: osteogenic, adipogenic and chondrogenic lineages. Control cells were cultured in a standard medium, DMEM 10% FBS.

Adipogenic differentiation

For adipogenic differentiation, hASCs were cultured on 24-well plates (40,000 cells/well) with adipogenic medium (hMSC Adipogenic Differentiation Medium, Lonza, Walkersville, MD, USA). The medium was changed twice a week for two weeks. At the end of the induction, differentiation was assessed using Oil Red O staining. Cells fixed in 10% formalin at room temperature for 15 minutes were washed in distilled water and incubated with Oil Red O solution. Subsequently, the cell monolayer was washed three times with demineralized H_2O. Finally, Oil Red O was extracted by incubation with isopropanol for 10 minutes. The dye was removed and 50 μL aliquots were transferred to a 96-well plate prior to reading absorbance at 495 nm with a spectrophotometer (Victor 2, Perkin Elmer Wallac, Milan, Italy).

Osteogenic differentiation

For osteogenic differentiation, hASCs were cultured on 24-well plates (15,000 cells/well) with osteogenic medium (StemPro Osteogenesis Differentiation kit, Gibco, Invitrogen, Carlsbad, CA, USA). The medium was changed twice a week for two weeks. At the end, differentiation was assessed using Alizarin Red stain (AR-S, Sigma-Aldrich Co., St. Louis, MO, USA). Cells fixed in 10% formalin at room temperature for 15 minutes were washed in distilled water and incubated with AR-S (40 mM, pH 4.1). The monolayer was then washed three times with dH_2O. AR-S was extracted by incubation with a cetylpyridinium chloride solution 10% for 10 minutes. The dye was removed and 50 μL aliquots were transferred to a 96-well plate prior to reading absorbance at 595 nm with a spectrophotometer (Victor 2, Perkin Elmer Wallac, Milan, Italy).

Chondrogenic differentiation

To induce chondrogenic differentiation, aliquots of 250,000 cells were pelleted in polypropylene conical tubes in chondrogenic medium (StemPro Chondrogenesis Differentiation kit, Gibco, Invitrogen, Carlsbad, CA, USA).

This medium was replaced twice a week for 3 weeks. Pellets were formalin-fixed, embedded in paraffin, examined morphologically and assessed using Alcian Blue stain. Glycosaminoglycan quantification was performed using BlyScan Sulfated Glycosaminoglycan Assay (Biocolor, Carrickfergus, UK).

RT-PCR Analysis

Total RNA of *Coleman* and *nanofat* hASCs was isolated using TRIzol (Invitrogen, Carlsbad, CA, USA). RNA concentrations were measured by absorbance at 260 nm with a NanoDrop instrument, and 2 μg of each sample were used as a template for a Cloned AMV First-Strand cDNA Synthesis Kit (Invitrogen, Carlsbad, CA, USA). The cDNA was subjected to 40 cycles of PCR using the pri-

Table 1. Reverse transcriptase polymerase chain reaction: primers and conditions.

Gene		Primer sequence 5'→3'	Annealing temp./time	Extension temp./time	Product size (bp)
OCT 4	F	GGTGAAGCTGGAGAAGGAGAAGCTG	57°C 1 min	72°C 1 min	246
	R	CAAGGGCCGCAGCTTACACATGTTC			
SOX 2	F	ACCAGCTCGCAGACCTACAT	57°C 1 min	72°C 1 min	150
	R	TGGAGTGGGAGGAAGAGGTA			
NANOG	F	AAGGCCTCAGCACCTACCTA	57°C 1 min	72°C 1 min	236
	R	ACATTAAGGCCTTCCCCAGC			
B ACTIN	F	GGACTTCGAGCAAGAGATGG	57°C 1 min	27°C 1 min	233
	R	AGCACTGTGTTGGCGTACAG			

mers and parameters shown in Table 1. The mRNA expression of the following genes was investigated: OCT-4, SOX2, NANOG and β-actin (control). The PCR products were visualized on a 1.5% agarose gel stained with ethidium bromide.

Immunomodulatory activity

To evaluate the immunomodulatory potential of hASCs obtained with the two harvesting methods, the cells were cocultured with activated human Peripheral Blood Mononuclear Cells (hPBMCs). hASCs were plated in 6-well plates (10,000 cells/cm^2), and were allowed to adhere in culture for 1 day. Subsequently, hPBMCs were isolated from healthy donors by density gradient centrifugation (Ficoll-Paque, Sigma-Aldrich Co., St. Louis, MO, USA) and plated on an hASC monolayer with a ratio of 10:1 (1,000,000 cells per well) in 2 mL of RPMI, 10% FBS (Lonza, Walkersville, MD, USA). hPBMCs were activated by addition of phytohemagglutinin (PHA, 1 μg/ml, Sigma-Aldrich Co., St. Louis, MO, USA) and incubated for 72h at 37°C, 5% CO_2. The negative control consisted of hPBMCs without PHA stimulation and the positive control consisted of hPBMCs with PHA in the absence of hASCs. Finally, hPBMCs were fixed with 70% ethanol at 4°C and subsequently stained with Propidium Iodide (Beckman-Coulter, Fullerton, CA, USA) at room temperature for 10 minutes. Cell cycle phase distribution of hPBMCs was analyzed by flow cytometry. The immunomodulatory activity of hASCs was also analyzed by measuring the BrdU incorporation in hPBMCs. The supernatant containing hPBMCs was resuspended. 100 μl of the hPBMCs resuspension recovered were seeded in triplicate in a 96-well plate. Finally, BrdU incorporation was evaluated using a colorimetric immunoassay, according to the manufacturer's instructions (Cell Proliferation ELISA, BrdU – colorimetric kit, Roche, Basel, Switzerland).

Moreover, the expression of the immunomodulatory molecule HLA-G was analyzed. hASCs cultured with and without activated hPBMCs were detached by trypsinization and fixed for flow cytometric analysis. hASCs were then incubated for 20 min at room temperature with the anti-HLA-G-FITC antibody (MEM-G/9 ab7904, Abcam, Cambridge, UK) and the expression of membrane-bound HLA-G was measured by flow cytometry.

Statistical Analysis

Quantified data are presented as means ± standard deviation. Data were analyzed by one, two-way Anova test and the *t*-test using GraphPad Prism software (GraphPad Software, Inc., La Jolla, CA, USA). The significance threshold was $p<0.05$.

Results

Tissue collection

In this study, we describe the stem cell features of a mesenchymal cell population derived through a novel liposuction technique based on *nanofat* harvesting. We compared the cell populations obtained with this method to the hASCs populations obtained with the classical *Coleman* procedure. The cannulas used to harvest the *Coleman* and *nanofat* samples, and the emulsification procedure of *nanofat* samples are showed in Figure 1. The characteristics of *Coleman*'s cannula maximized the vitality of adipose lobules, useful in reconstructive cases where it is crucial to fill large volumes. On the contrary, the features of the cannula used for the nanofat technique, combined with the emulsification procedure, disaggregated completely the adipose tissue structure and allowed to obtain a liquid fat preparation easier to inject into the receiving site by using 25-G or 27-G needles.

Cell isolation and proliferation

Stromal vascular cells were successfully isolated from lipoaspirates obtained with both techniques. The yield was similar: we isolated approximately 500,000 cells per mL of processed tissue. After isolation, adherent fibroblast-like cells were expanded and maintained in culture for at least six passages and did not show noticeable changes in their morphology during this culture expansion (Figure 2). The cumulative population doubling of hASCs isolated from samples collected with *Coleman* and *nanofat* technique displayed no significant difference (Figure 3).

We observed that cultures of fibroblast-like cells could be established using both techniques even in the absence of enzymatic digestion by simply plating the mechanically processed adipose tissue (Figure 4). After three days in culture, a small number of cells appeared in attachment to the plate and the cells reached confluence after 14 days of culture. We observed a higher yield of cultured cells in the *nanofat*-derived samples, but given the low number of samples tested, the difference was not statistically significant (data not show).

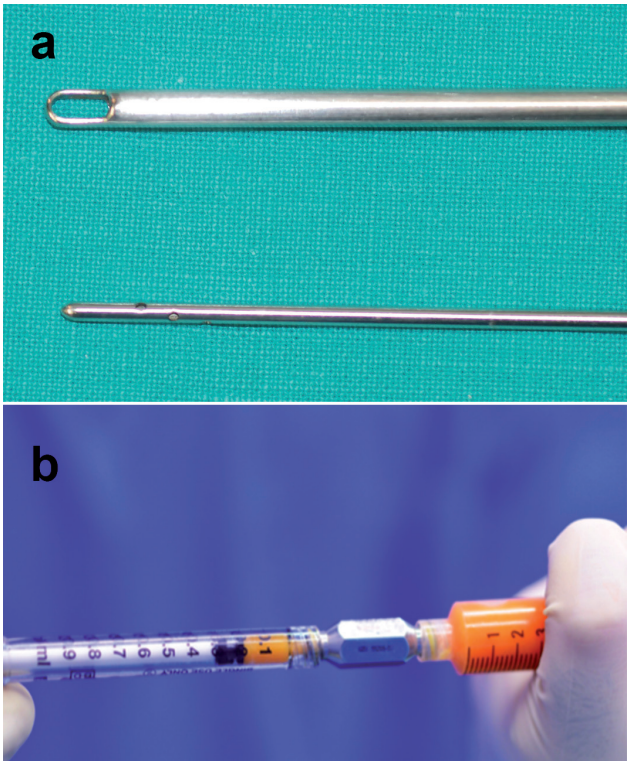

Figure 1. Harvesting technique: in panel *(a)* the first cannula is a standard Coleman's cannula of 150 mm length with an outer diameter of 3 mm and a single hole of 2.5 mm diameter at his peak. The second cannula of 150 mm length with an outer diameter of 1.5 mm and six side holes arranged in spiral of 0.5 diameter was used for the nanofat technique. Panel *(b)* shows the phase of mechanical emulsion of adipose tissue obtained with the nanofat technique: 30 passes between the 2 syringes were performed. This phase is extremely important to allow the change of adipose tissue into liquid fat and also to release the stromal/stem component contained in SVF.

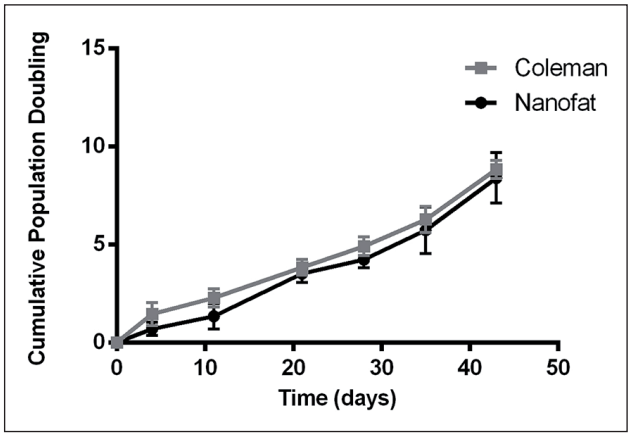

Figure 3. Cumulative population doubling of cells isolated with the two techniques. No significant differences were observed in the rate of proliferation. Data are presented as means ± SD and were analyzed by the t-test using Graph Pad Prism software.

Immunophenotypic characterization

Cell surface antigens were assessed by flow cytometry analysis for a variety of markers associated with hematopoietic (CD14, CD34 and CD45), mesenchymal stromal (CD44, CD73, CD90 and CD105), mature endothelial (CD31) and pericytic (CD146) cells at isolation and after three culture passages. As shown in Table 2, cells expanded from the Stromal Vascular Fraction of *Coleman* or *nanofat* lipoaspirates exhibited similar immunophenotypes, with low expression of hematopoietic, endothelial and pericytic markers and high expression of stromal markers. The immunophenotype of these populations was consistent with hASCs and

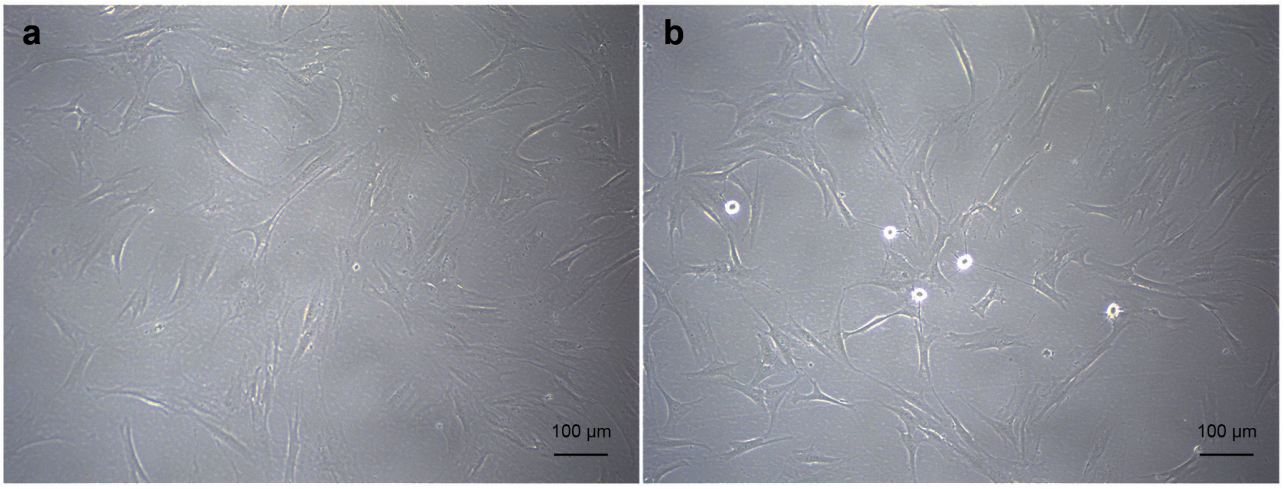

Figure 2. Representative light microscopic images. Cultured cells at passage three obtained from *Coleman (a)* and *nanofat (b)* lipoaspirates displayed fibroblast-like morphology and robust proliferation in DMEM 10% FBS medium.

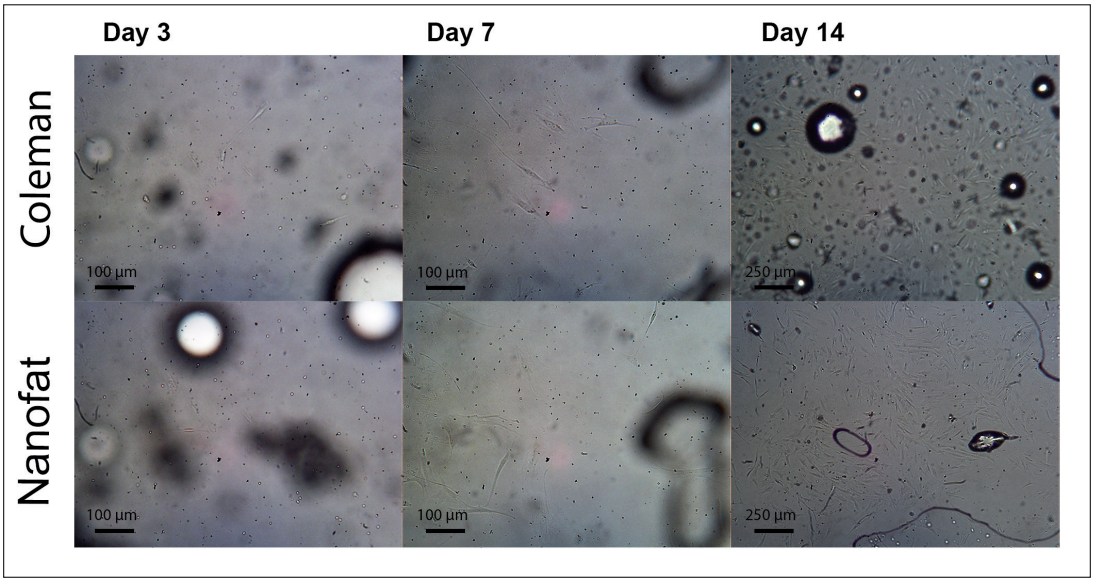

Figure 4. Cells isolation in absence of enzymatic digestion. Representative light microscopic images of the cultured cells at different times post-isolation. In both *Coleman* and *nanofat* samples, fibroblast-like cells appeared in attachment and were able to proliferate, growing to confluence in 14 days.

indicated mesenchymal origin. The percentage of $CD34^+$ cells varied among different SVF samples, and decreased during passaging, but a small fraction of the cultured cells could maintain a low level of CD34 positivity, in accordance with published findings[7].

Expression of Stemness markers

To test the expression of stemness markers in the cells populations obtained with the two techniques, the expression of the transcription factor Oct-4 and the surface antigen SSEA4 was analyzed by flow cytometry. As shown in Figure 5-a, hASCs express these typical markers of embryonic stem cells. Furthermore, RT-PCR analysis showed that cells obtained with both procedures presented expression of the stemness transcription factors OCT-4, SOX2 and NANOG, as shown in Figure 5-b.

Differentiation potential

To complete the characterization of hASCs and analyze the multi-differentiation potential of the cells derived with the two harvesting methods, we stimulated differentiation into the three classical mesodermal commitments (adipogenic, osteogenic and chondrogenic) with *in vitro* protocols. At the completion of each differentiation protocol, we tested the acquisition of more mature phenotypes with the following methods.

Adipogenic differentiation was analyzed and quantified via Oil Red O staining, a dye that stains neutral triglycerides and lipids. hASCs matured into cells containing multiple lipid-rich vacuoles in the cytoplasm, vacuoles that increased in size and number during the two weeks of induction and were intensely stained red with Oil Red O (Figure 6-a). The quantification showed increased red staining in the induced cells compared with controls, $p<0.001$ (Figure 6-d).

Table 2. Immunophenotypic characterization of hASCs. Antigenic profile of freshly isolated cells in the Stromal Vascular Fraction (SVF) and of hASCs expanded for at least three passages indicated respectively as *Coleman* and *nanofat*.

	SVF Coleman	**SVF Nanofat**	**Coleman**	**Nanofat**
CD45	-/+	-/+	-	-
CD14	-	-	-	-
CD34	+	++	-/+	-/+
CD31	++	++	+	-/+
CD146	++	++	-/+	+
CD44	+/++	+/++	++	++
CD73	-/+	-/+	++	++
CD90	+++	++/+++	+++	+++
CD105	+/++	+/++	+++	+++

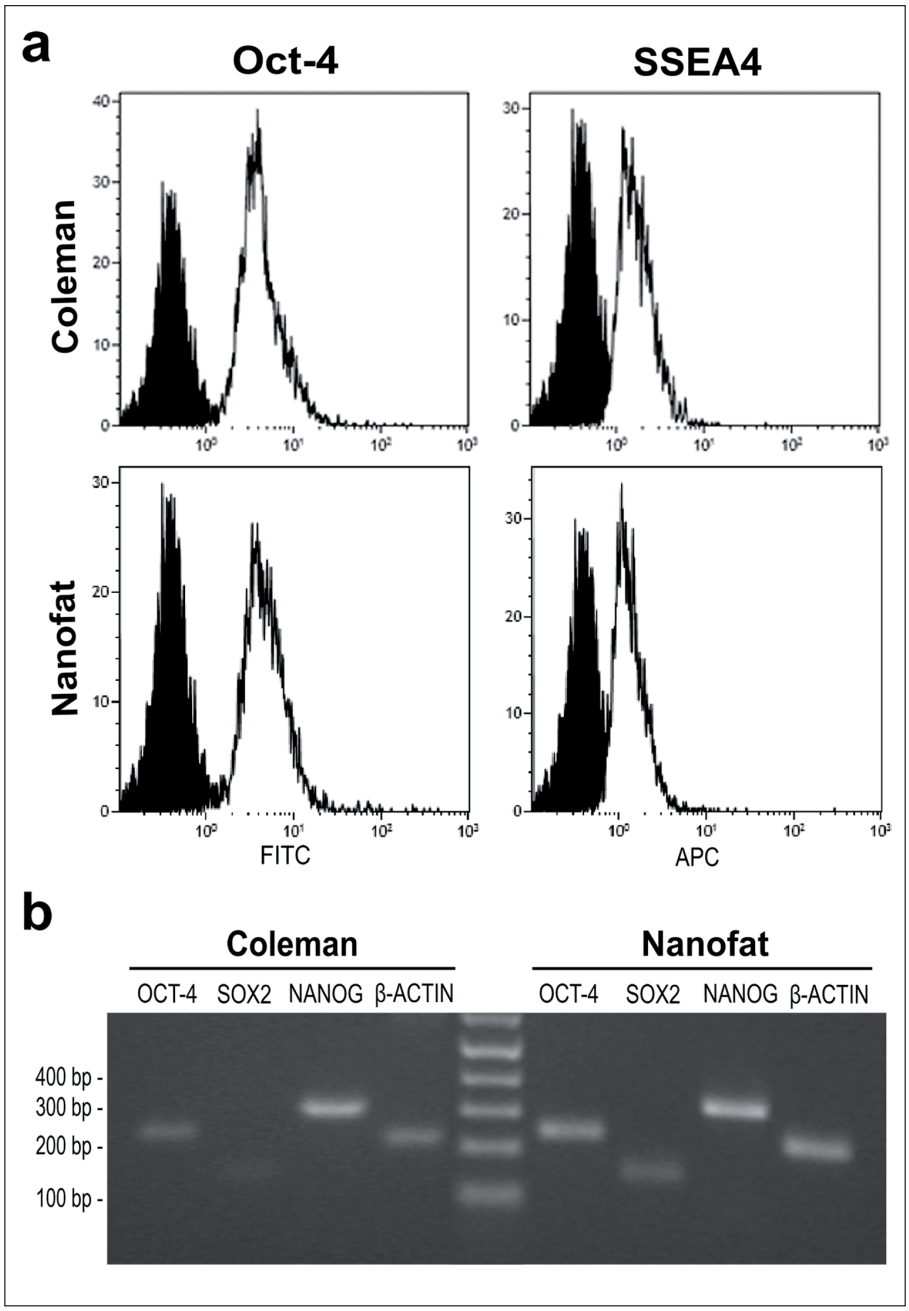

Figure 5. Stem cell markers. *(a),* The expression of Oct-4 and SSEA4 was analyzed by flow cytometry. Isotype controls are presented as filled black histograms, the specific cell markers as white histograms. *(b),* Expression of Oct-4, SOX2 and NANOG mRNAs. β-actin was used as a control. *Nanofat*- and *Coleman*-derived cells expressed stem cell markers at comparable levels.

Osteogenic differentiation was tested after two weeks of induction with Alizarin Red staining, a staining for calcium accumulation. Intense red staining was observed in induced cells indicating extracellular mineralization, a key step toward the formation of calcified matrix. Uninduced cells did not show Alizarin Red positive deposits (Figure 6-b). Quantification of the staining confirmed the qualitative observation, $p<0.001$ (Figure 6-d).

The chondrogenic differentiation was documented by using Alcian Blue staining and glycosaminoglycan quantification. hASCs displayed a robust potential for chondrogenic differentiation. Differentiated cells were embedded in a rich extracellular matrix that proved highly positive for the presence of a proteoglycan component in the extracellular matrix, as evidenced by Alcian Blue staining in the induced cells, but not in the control cells (Figure 6-c). Quantification showed the presence of glycosaminoglycans in the extracellular matrix of induced cultures $p<0.001$ (Figure 6-d).

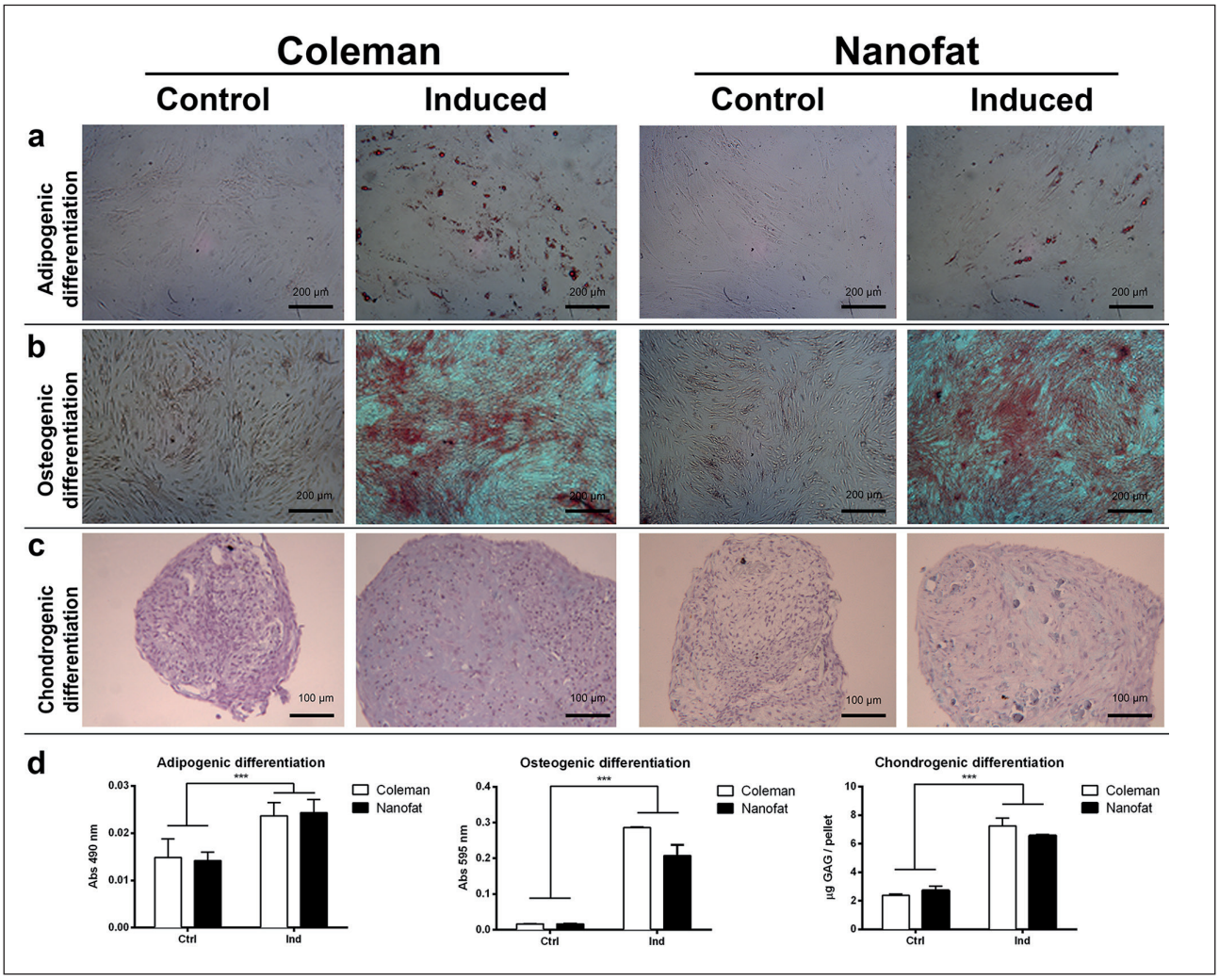

Figure 6. hASC multilineage potential: Oil Red O staining was performed for adipogenic differentiation *(a)*, Alizarin Red staining for osteogenic differentiation *(b)* and Alcian Blue staining for chondrogenic differentiation *(c)*. Histograms present the results of quantification assays (histological staining and glicosaminoglycans content): open bars represent data for *Coleman*-derived hASCs, black bars for *nanofat*-derived hASCs *(d)*. Data are presented as means ± SD and were analyzed by the t test using Graph Pad Prism software, p<0.001.

hASCs obtained with the two harvesting techniques presented consistent levels of maturation towards the three lineages tested, confirming that the intrinsic multipotency of mesenchymal cells in adipose tissue was maintained in *nanofat* lipoaspirates.

Immunomodulation

A pivotal feature of hASCs, of great interest for clinical applications, is their immunomodulatory capacity. We compared the ability of hASCs obtained with the two methods to inhibit proliferation of PBMCs activated with PHA and to arrest them in the G0/G1 phase of the cell cycle. We also studied the surface expression of HLA-G, a well-known tolerogenic molecule capable of mediating this immunomodulatory effect. In the co-culture with hPBMCs, hASCs were able to reduce approximately 50% of the hPBMC proliferation, *p<0.001* (Figure 7-a). Moreover, PBMCs cultured in contact with hASCs, displayed an increase in the G0/G1 phase of the cell cycle, *p<0.05* (Figure 7-b). Finally, we observed an increase in HLA-G expression by hASCs when cultured in the presence of hPBMCs activated with PHA (Figure 7-c). hASCs obtained with the two different methods exerted comparable immunomodulatory effects. Therefore, the use of a thinner cannula for adipose tissue collection by liposuction did not affect the immunomodulatory capability of mesenchymal cells contained in the stromal vascular fraction of fat tissue.

Discussion

In the present study, we have analyzed the possibility to isolate human adipose derived stromal/stem cells (hASCs, a form of mesenchymal stromal cells) from lipoaspirate samples collected by *nanofat*, a novel procedure of fat harvesting and processing. This technique has sparked great interest in the last few years in plastic and reconstructive surgery. Until now only one research group has started to investigate the cellular component and the biological effects of *nanofat* lipoaspirates in medical aesthetic treatments[26]. Thus, the aim of our study was to investigate the biological features of *nanofat*-derived hASCs, a cellular component of the stromal vascular fraction of *nanofat* lipoaspirates, and compare them with hASCs derived with the standard method of harvesting described by Coleman.

In this study, we demonstrate the possibility to isolate hASCs from *nanofat* samples with a similar yield similar to the classical *Coleman* method. We established cultures of mesenchymal-like cells

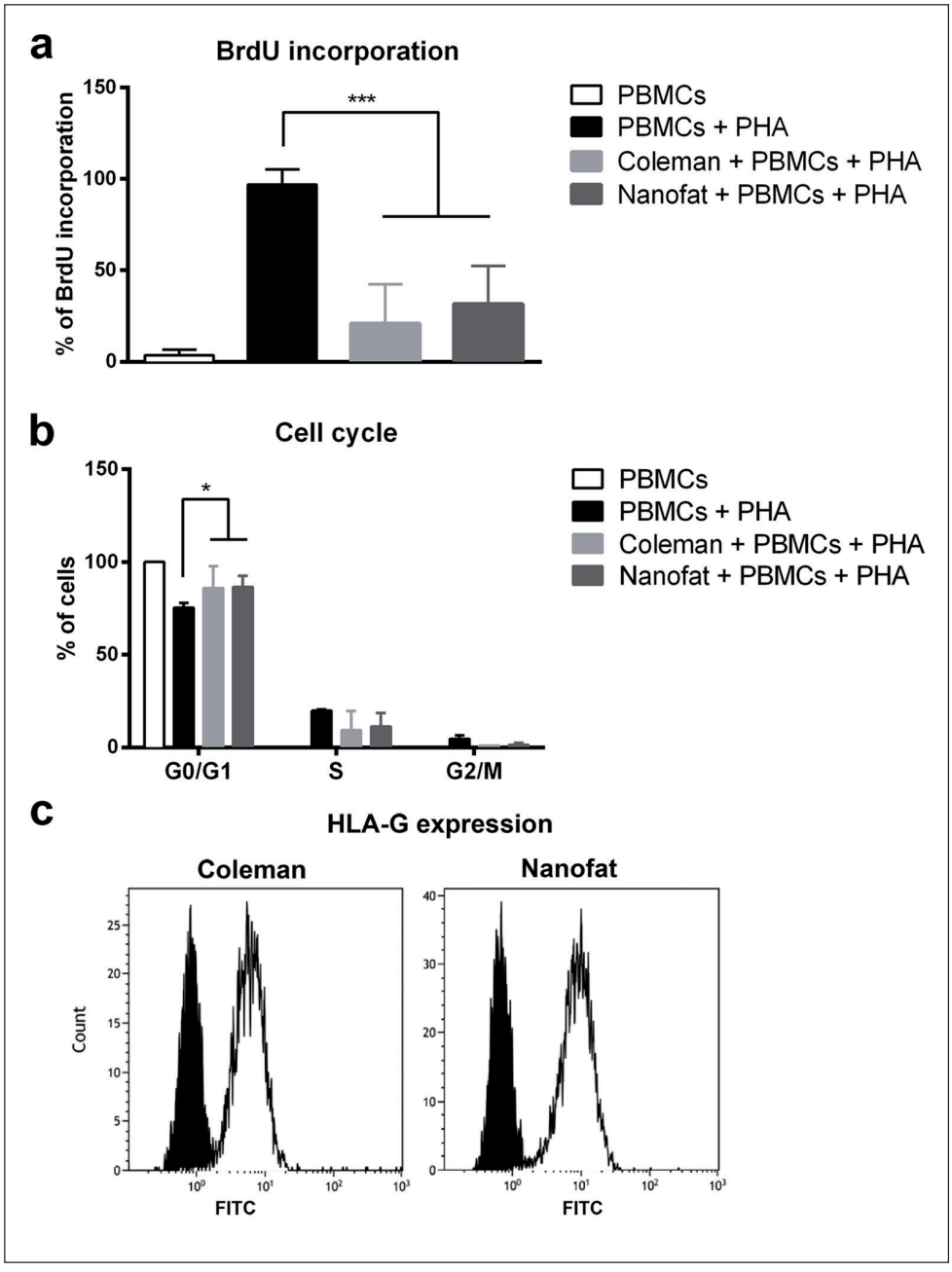

Figure 7. Immunomodulation was assayed by BrdU incorporation *(a)* and cell cycle analysis *(b)* on hPBMCs activated with PHA, after co-culture with hASCs obtained with *Coleman* and *nanofat* methods. Comparison of basal HLA-G expression on hASCs (black bar) with HLA-G expression on hASCs after co-culture with hPBMCs activated with PHA (open bar) *(c)*. Data are presented as means ± SD and were analyzed respectively by one, two Anova tests and t-test using Graph Pad Prism software. We found no statistically significant differences in the suppression of hPBMC proliferation between *Coleman* and *nanofat*-derived hASCs.

with fibroblast morphology from both *nanofat* and *Coleman* lipoaspirates. These cells grow in adhesion to plastic and proliferate extensively when cultured in DMEM 10% FBS. The immunophenotypic profile was consistent with the one of hASCs. We observed the expression of the mesenchymal markers CD44, CD73, CD90, CD105, along with CD34, a typical marker of this cell population. We observed a low expression of the hematopoietic markers CD14 and CD45. The expression of the endothelial marker CD31 and pericytic marker CD146 was detected at stable levels until passage 3, indicating the presence of vascular and perivascular populations characteristics of the adipose tissue SVF.

To analyze the stemness of ASCs, we studied the expression of OCT-4 SOX2 and NANOG and the ability of these cells to differentiate in three mesenchymal lineages (adipogenic, osteogenic, and chondrogenic). Lastly, we investigated the immunomodulatory properties of hASCs derived from *nanofat* and *Coleman* lipoaspirates. We observed that hASCs derived using both methods were able to inhibit hPBMCs proliferation and express HLA-G, one of the possible mediators of this effect.

Conclusions

We observed that hASCs could be efficiently isolated from lipoaspirates obtained with the *nanofat* method of adipose tissue harvesting. Our results support the argument that the *nanofat* method does not impact negatively the hASCs cellular component residing in the adipose tissue. hASCs from *nanofat* lipoaspirates are similar to those from *Coleman* lipoaspirates: the yield of hASCs is comparable for the two methods, the cells display similar stemness characteristics, *in vitro* proliferation, differentiation and immunomodulatory properties. The potential of *nanofat*-derived hASCs extends beyond their use as simple structural filling: these cells could sustain adipogenesis and tissue regeneration over long periods of time and could modulate immunity. These findings are relevant to the development of *nanofat*-based plastic, reconstructive and aesthetic surgery treatments, because this method enables obtainment of a readily injectable preparation and it enables grafting in sites that cannot be targeted with other methods. Many commercially available instruments and cannulas can harvest adipose tissue, but this tissue subsequently needs to be minced in order to obtain an injectable fluid preparation. In this study, we demonstrated that the *nanofat* method of adipose tissue processing does not affect the viability and the characteristics of hASCs. For these reasons, the *nanofat*-processed adipose tissue and the *nanofat*-derived hASCs are suitable for use in tissue- and cell-therapy strategies. *Nanofat* grafts, thus, present important features for application in surgeries targeted at the brow and upper sulcus, at the orbit hollow and at the perioral area, where the regeneration of the subcutaneous space of the skin represents a main objective[19,34].

Conflict of Interests:

The Authors declare that they have no conflict of interests.

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