Journal of Cellular Physiology

In Vitro Evaluation of Different Methods of Handling Human Liposuction Aspirate and Their Effect on Adipocytes and Adipose Derived Stem Cells

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Nowadays, fat tissue transplantation is widely used in regenerative and reconstructive surgery. However, a shared method of lipoaspirate handling for ensuring a good quality fat transplant has not yet been established. The study was to identify a method to recover from the lipoaspirate samples the highest number of human viable adipose tissue-derived stem cells (hADSCs) included in stromal vascular fraction (SVF) cells and of adipocytes suitable for transplantation, avoiding an extreme handling. We compared the lipoaspirate spontaneous stratification (10-20-30 min) with the centrifugation technique at different speeds (90-400-1500 × g). After each procedure, lipoaspirate was separated into top oily lipid layer, liquid fraction, "middle layer", and bottom layer. We assessed the number of both adipocytes in the middle layer and SVF cells in all layers. The histology of middle layer and the surface phenotype of SVF cells by stemness markers (CD105+, CD90+, CD45−) was analyzed as well. The results showed a normal architecture in all conditions except for samples centrifuged at 1500 × g. In both methods, the flow cytometry analysis showed that greater number of ADSCs was in middle layer; in the fluid portion and in bottom layer was not revealed significant expression levels of stemness markers. Our findings indicate that spontaneous stratification at 20 min and centrifugation at 400 × g are efficient approaches to obtain highly viable ADSCs cells and adipocytes, ensuring a good thickness of lipoaspirate for autologous fat transfer. Since an important aspect of surgery practice consists of gain time, the 400 × g centrifugation could be the recommended method when the necessary instrumentation is available.

J. Cell. Physiol. 230: 1974–1981, 2015. © 2015 The Authors. *Journal of Cellular Physiology* published by Wiley Periodicals, Inc.

Autologous fat grafting is a widely used technique in regenerative and reconstructive surgery to ameliorate tissues damaged or failed by burns, radiation, surgery, and generally, atrophy of soft tissues (Zhu et al., 2008). Several findings showed that this procedure is minimally invasive, effective, and completely safe for body contouring. In particular, lipoaspirate to be implanted represents a source of different growth factors, cytokines, and heterogeneous cell subpopulations including adipose tissue-derived stem cells (ADSCs) helpful in fat grafting enhancement (Folgiero et al., 2010; Casteilla et al., 2011; Lindroos et al., 2011). Lipoaspirate is characterized by heterogeneous stromal vascular fraction (SVF) subpopulations (Zuk et al., 2002; Sliwa et al., 2009) including stromal cells, hematopoietic progenitors, circulating blood cells, leukocytes, macrophages, pericytes, endothelial cells, fibroblasts, adipocyte progenitors, and ADSCs (Gimble et al., 2007; Zimmerlin et al., 2010), the latter with the ability to differentiate into cells of several lineages such as adipocytes, osteoblasts, chondrocytes, myocytes, endothelial cells, hematopoietic cells, hepatocytes, and neuronal cells (Zuk et al., 2001; Huang et al., 2004; Nicoletti et al., 2015). Of note, the amount of ADSCs in autologous lipotransfers seems to affect the long-term survival of fat grafting (Rodriguez et al., 2004; Mojallal et al., 2011). The use of fat grafts, enriched with autologous isolated ADSCs, has been reported to increase the longevity and the volume of these implants (Yoshimura et al., 2008; Zhu et al., 2010).

The various procedures for fat collection and injection have a main pitfall: the high rate of resorption and low survival rate of the transferred adipose tissue, possibly due to the poor quality of adipocytes at the time of transplantation, as reported in the Peer's study (Peer, 1955). Although it is generally agreed that fat graft survival is affected by fat collection methods and by procedures of handling and reinjection and despite several studies conducted to improve harvesting, processing, and implantation techniques, a standard practice shared by clinicians is yet to be defined (Kurita et al., 2008; Conde-Green et al., 2010; Ferraro et al., 2011; Pulsfort et al., 2011; Pu, 2012; Tabit et al., 2012).

It is known that the centrifugation procedure is widely used just before the autologous fat transfer (Ferraro et al., 2011)

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Contract grant sponsor: Abruzzo earthquake relief fund.

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Manuscript Received: 26 November 2014
Manuscript Accepted: 17 February 2015

Accepted manuscript online in Wiley Online Library (wileyonlinelibrary.com): 3 March 2015.
DOI: 10.1002/jcp.24965

even if the conclusions drawn by several studies are different mainly due to the variability of the cell viability detection methods. Some works have previously described different lipoaspirate handling techniques such as decantation or spontaneous stratification under the action of gravity, washing, and centrifugation at different speeds, in order to obtain viable and intact adipocytes as well as a significant number of viable SVF cells (Kurita et al., 2008; Padoin et al., 2008; Conde-Green et al., 2010; Ferraro et al., 2011; Son et al., 2013). However, to date, there are no clear and defined indications on autologous lipoaspirate handling procedures. In the present work, we endeavored to compare two common fat processing techniques: lipoaspirate spontaneous stratification at different times and centrifugation at several speed forces, by evaluating the effects of each procedure on a) adipocytes and SVF cells number, b) number of isolated ADSCs, c) ADSC plastic adhesion ability, and d) ADSC differentiation potential. Throughout, the main goal was to standardize the working conditions of surgeons and enhance their clinical practice also considering the time required for each procedure.

Materials and Methods

Reagents

DMEM (Dulbecco's Modified Eagle Medium, high glucose), Phosphate-buffered saline (PBS), Fetal Bovine Serum (FBS), Glutamine, Penicillin, and Streptomycin were obtained from EuroClone (West York, UK). Collagenase (type IA), Oil Red-O, Trypan blue solution, Haematoxylin/Eosin, Bovine Serum Albumin (BSA, fraction V), Triton X-100, VR(−)-N6-(2-Phenylisopropyl) adenosine (PIA), Insulin, and Hepes were purchased from Sigma Chemical Co. (St. Louis, MO). Lympholyte cell separation media (Ficoll) was acquired by Cedarlane (Ontario, Canada). Sterile catheter tip syringes were acquired from BD Plastipak (Franklin Lakes, NJ). Ten percent neutral buffered ready to use formalin, dehyol absolute, dehyole 95, and paraffin were purchased from Bio-Optica (Milan, Italy). Anti-Vimentin antibody was acquired from Abcam (ab92547); the secondary used was an HRP conjugated goat anti-rabbit polyclonal (Millipore AP106P). Advanced smart processor (ASP300), automatic stainer (5010 Autostainer XL), and rotative microtome (RM2135) were purchased from Leica Microsystems (Nussloch, Germany). Direct light microscopy (Nikon Eclipse 50i) and inverted optical microscope (Nikon Eclipse TS100) were from Zeiss (Jena, Germany). Monoclonal anti-human fluorochrome-conjugated antibodies CD105-phycoerythrin (PE) and CD45-fluorescein isothiocyanate (FITC) were purchased from Immunotools (Altenoyther Strasse, Germany), anti-human CD90-allophycocyanin (APC) was acquired from eBioscience (San Diego, CA).

Patient selection

Five women (average age: 46.6; age range 35–58) undergoing to liposuction in the Plastic and Reconstructive Surgery (Director: Prof. Maurizio Giuliani; Casa di Cura Di Lorenzo, Avezzano, L'Aquila – Italy) were enrolled in the present study. To standardize the sample, making the experimental results reproducible, we collected and used for experimental session only the lipoaspirate obtained from the thighs' medial aspect. Before treatment, all patients received an accurate screening to exclude contraindications, gave their consent to the surgery and signed the agreement for the experimental procedure on the excised tissues. Subjects presenting other comorbidities, such as hypertension, diabetes, or smoking were excluded from the study. In order to make the results reproducible and reduce the sample bias, the same specimen was used to perform all experimental procedures. To this end, all enrolled patients were subjected to liposuction procedure for collection of about of 500 cc lipoaspirate.

Sample collection

With the patient in upright position, the inner aspect of the thighs was marked. The liposuction was performed under peripheral blocks obtained by using hyperbaric bupivacaine (10 mg) in epidural space. After the surgical area was set up, tissues were infiltrated with Klein solution respecting the ratio of 1:1 (wet liposuction). In all cases, Klein solution, containing lidocaine (0.05%), epinephrine (1:1,000,000), and sodium bicarbonate (12.5 meq/L), was administered 20 min before liposuction (Klein, 1988).

Through a 4 mm skin incision, the solution was administered with slow fan shape movements in fat thickness using a 15 cm blunt cannula (diameter: 1 mm) designed for infiltration with 3 holes per side along its length. The surgical procedure was conducted through the same surgical access using a 2 mm blunt cannula connected to an electric negative pressure generator. In accordance with the available literature, liposuction was performed with slow fan shape movements starting from the deeper layer moving up to the subcutaneous space (Zoccali et al., 2012). Immediately after surgery, the lipoaspirate was transferred in sterile case and sent to the laboratory.

Spontaneous stratification and centrifugation methods

Lipoaspirate from each subject was divided in 20 ml aliquots which were then processed by spontaneous stratification and centrifugation methods. All experiments were performed in duplicate: total n. 120 tubes (n. 24 tubes/each lipoaspirate). *Spontaneous stratification*: sample aliquots of lipoaspirate were left to decant for 10, 20, and 30 min at room temperature to obtain the sample stratification under the gravity effect.

Centrifugation technique: sample aliquots of lipoaspirate were centrifuged at 90 × g, 400 × g, and 1500 × g for 3 min at room temperature.

After spontaneous stratification and centrifugation techniques, as expected, four layers were observed: free released oils (oily fraction) on the top, a "middle layer" consisting of purified fat with adipocytes and connective tissue, an aqueous layer containing Klein solution and a bottom layer composed by erythrocytes and SVF cells. After discarding the oily fraction, the Klein solution fraction and the bottom layer were subjected to Ficoll gradient centrifugation for 40 min at 900 × g. In order to obtain SVF cells and adipocytes, the middle layers, collected in sterile conditions, were incubated with 1.5 mg/ml crude collagenase type I in PBS solution at 37 °C for 45 min in a water bath by gentle stirring. The collagenase activity was then neutralized by adding DMEM supplemented with 10% FCS and the digested tissue was centrifuged at 90 × g for 3 min to recover adipocytes and at 400 × g for 10 min to recover SVF cells. SVF cell viability was determined by the trypan blue exclusion (Chung et al., 2013), while the adipocytes count was performed by Oil Red-O staining (Palumbo et al., 2010).

The SVF cells were washed twice and plated with DMEM high glucose supplemented with 10% FBS, 2mM glutamine, 100 U/ml penicillin and 100 mg/ml streptomycin, and grown in sterile conditions at 37 °C with 5% CO_2; the obtained adherent cells were observed with an inverted optical microscope.

Histology of lipoaspirate samples

Multiple small specimens of middle layer obtained from both methods were fixed in 10% neutral buffered formalin, at room temperature, over a 48 h period. The samples were washed under running water for 2 h, dehydrated in the ethanol ascendant series with an automatic processor (Leica ASP 300) and, afterwards,

manually embedded in paraffin. Four micro meter tick sections were obtained with a rotative microtome and then stained with haematoxylin–eosin using a Leica 5010 Autostainer XL. Vimentin was also analyzed by immunostaining with anti-vimentin antibody.

Flow cytometry analysis

All SVF cells, obtained from each layer of lipoaspirate following to spontaneous stratification and centrifugation methods, were freshly examined phenotypically by the following markers: CD105, CD90, and CD45, using FACSCalibur flow cytometry (Beckton Dickinson, Immunocytometry System, San Jose, CA).

CD105 is a highly expressed marker in both human vascular endothelial cells and mesenchymal stem cells (Anderson et al., 2013). CD90 (also known as Thy-1), is a major marker for stem and progenitor cells, detected on the endothelium of capillaries; CD45, finally, that is expressed on all leucocytes is undetectable on ADSCs (Zimmerlin et al., 2010).

At least 5×10^5 cells for each experimental condition were simultaneously stained with fluorochrome-conjugated human monoclonal antibodies for 1 h at room temperature in the dark. Samples were centrifuged at $400 \times g$ for 10 min and after washing the cells were analyzed. To identify the ADSCs, we analyzed the co-expression of CD105/CD90 markers on cell surface of the CD45 negative population (Bourin et al., 2013; Kokai et al., 2014). Data were analyzed with the CellQuest software (BD Biosciences).

Multilineage differentiation assay

Adherent cells isolated from digested middle layer samples were grown in culture and differentiated using commercially available differentiation kit (hMesenchymal Func Ident Kit, product code SC006). Cells were seeded at densities of 2.1×10^4 cells/cm^2, 4.2×10^3 cells/cm^2, and 25×10^4 cells/cm^2 for adipogenic, osteogenic, and chondrogenic differentiation (the latter in a 15 ml conical tube), respectively. The differentiation was performed for three weeks (in accordance with the manufacturer's instructions) and differentiation media were changed every 3 days. After 16 and 21 days of culture, the medium was aspirated and the cells were washed twice with PBS and fixed with 4% paraformaldehyde in PBS for 20 min at room temperature, for immunostaining. In particular, a panel of antibodies, including goat anti-mouse FABP-4, goat anti-human Aggrecan, and mouse anti-human Osteocalcin, were used to identify the adipogenic, chondrogenic, and osteogenic differentiation, respectively. Following staining, cells were examined at 10× and 40× magnifications with a fluorescent microscope (Nikon Eclipse 50i). Nuclei were counterstained with DAPI (blue) fluorescent dye.

Statistical analysis

Statistical analysis of data was performed by using one-way analysis of variance ANOVA followed by the Student's *t* test (Prism 3.0 GraphPad Software, San Diego, Ca). Results were expressed as mean ± SEM. *P* values less than 0.01 were considered statistically significant.

Results

Comparison between spontaneous stratification and centrifugation methods on yield of adipocytes from lipoaspirate

The effect of both spontaneous stratification and centrifugal forces on lipoaspirate samples was analyzed on yield of adipocytes. The middle layers obtained from each spontaneous stratification time (see the Methods section) were enzymatically digested. Of interest, we observed that the four layers were not well defined in stratified samples for 10 min and the thicknesses of middle layer were not suitable, therefore we decided to eliminate this experimental condition. Our results demonstrated the presence of viable adipocyte in the middle layer in both experimental time points ($4.61 \times 10^4 \pm 0.08$ cells and $3.99 \times 10^4 \pm 0.07$ cells after 20' and 30', respectively). No statistically significant difference was revealed in adipocyte number when 30 min was compared to 20 min (P=0.07) (Fig. 1A).

When lipoaspirate samples, after enzymatic digestion, were centrifuged, a relevant and significant adipocyte number decrease was observed at $1500 \times g$ for 3 min

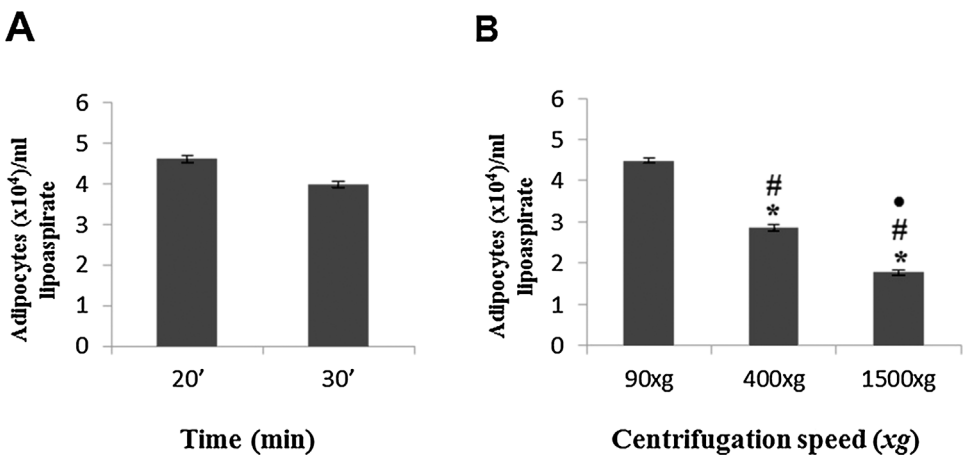


Fig. 1. Effect of spontaneous stratification and centrifugation techniques on adipocytes. (A) Adipocyte numbers ($\times 10^4$/ml lipoaspirate) after spontaneous stratification at 20 and 30 min and (B) after several centrifugation speeds $90 \times g$, $400 \times g$, $1500 \times g$ for 3 min. Values represent mean ± SEM of five independent experiments in duplicate (*P value < 0.01 when compared $1500 \times g$, $400 \times g$ and $90 \times g$; •P value < 0.01 when compared $400 \times g$, $500 \times g$; #P value < 0.01 when compared 20 min and 30 min to $400 \times g$ and $1500 \times g$, respectively).

($1.77 \times 10^4 \pm 0.05$ cells/ml lipoaspirate) when compared to $90 \times g$ and $400 \times g$ ($^*P < 0.01$) (Fig. 1B). This result was in accordance with the appearance of an oily layer on the top of the sample centrifuged at $1500 \times g$, resulting from unmistakeable adipocyte damage. Although, at $400 \times g$ a significant decrease of adipocyte number was observed ($2.86 \times 10^4 \pm 0.07$ cells/ml lipoaspirate) when compared to $90 \times g$ ($4.48 \times 10^4 \pm 0.05$ cells/ml lipoaspirate) ($^*P < 0.01$) (Fig. 1B), the recovered middle layer had a better thickness and seemed better cleansed than centrifugation at $90 \times g$.

When the two different techniques were compared, no significant difference between adipocyte number recovered after 20 min spontaneous stratification or $90 \times g$ centrifugation speed was observed ($P = 0.66$). When 20 min, as well as 30 min, was compared to $400 \times g$ and $1500 \times g$ a significant reduction of adipocyte number was observed, as showed in Figure 1B ($\#P < 0.01$).

Figure 2 shows representative images of the histology of adipose tissue sections after spontaneous stratification (Fig. 2 panel a) and centrifugation (Fig. 2 panel b). In particular, a normal lobular architecture with regular and undamaged adipocyte membranes and intact nuclei could be observed after spontaneous stratification at 20 min and 30 min by hematoxylin–eosin staining (Fig. 2a images A and C) and immunostaining of vimentin (Fig. 2a images B and D). Vimentin is a ubiquitous intermediate filament that surround the lipid droplets, so that adipose tissue sections appeared as a regularly spaced cage-like structure (Lieber and Evans, 1996). The histology of centrifuged lipoaspirate samples was preserved at $90 \times g$ and $400 \times g$, showing largely uninjured cell nuclei and continuous adipocyte membranes, as revealed through hematoxylin–eosin staining (Fig. 2b images E and G) and vimentin immunostaining (Fig. 2b images F and H). These results were similar to those obtained after spontaneous stratification handling technique at 20 min and 30 min. Instead, at $1500 \times g$ centrifugal force, a heavily injured lipoaspirate tissue was observed with collapsed adipocyte morphology, disrupted, and folded cell membranes and haemorrhagic extravasation (Fig. 2b image I, hematoxylin–eosin staining, and image L, vimentin immunostaining).

Comparison between spontaneous stratification and centrifugation methods on yield, viability, and phenotype of SVF cells from lipoaspirate

The number of nucleated SVF cells after performing spontaneous stratification (20 and 30 min at room temperature) and centrifugation ($90 \times g$, $400 \times g$, $1500 \times g$ at room temperature for 3 min) techniques was evaluated. Figure 3 shows the amount of SVF cells obtained from the middle layer, the aqueous layer (fluid portion or "Klein solution") and the bottom layer following both spontaneous stratification and centrifugation. Specifically, the number of viable SVF cells obtained from spontaneous stratification was enough comparable among all layers at 20' and 30' time point (Fig. 3A). In order to characterize the phenotype of cell populations in each layer, the cells were analyzed using flow cytometer for the absence or presence of specific antigens for ADSCs identification, such as CD45, CD105, and CD90. After spontaneous stratification, the cytofluorimetric analysis revealed that the amount of CD45−/CD105+/CD90+ cells/ml lipoaspirate was relevant only in the middle layer samples at all analyzed times (Fig. 3B). No significant difference ($P = 0.72$) was observed between the number of CD45−/CD105+/CD90+ cells/ml lipoaspirate, obtained from digestion of middle layer after 20 min ($2.85 \times 10^4 \pm 0.11$ cells/ml lipoaspirate) or 30 min ($2.77 \times 10^4 \pm 0.14$ cells/ml lipoaspirate). Thus, the spontaneous stratification at 20 min, providing a number of ADSCs similar to 30 min, should be preferred for the time savings.

When centrifugation procedures were performed on lipoaspirate aliquots, the number of SVF cells/ml lipoaspirate in the middle layers was similar among the samples differently centrifuged (Fig. 3C). As expected, at all centrifugal speeds, the number of cells obtained from the fluid portion was negligible (not shown). The cells collected from the bottom and middle layers were phenotypically analyzed and the results clearly demonstrated that, at all centrifugal forces, CD45−/CD105+/CD90+ cells/ml lipoaspirate were mainly present in the middle layer being this population, on the other hand, poorly detectable in the bottom fractions ($P < 0.01$) (Fig. 3D). The number of CD45−/CD105+/CD90+ cells/ml lipoaspirate, recovered in middle layers after centrifugation

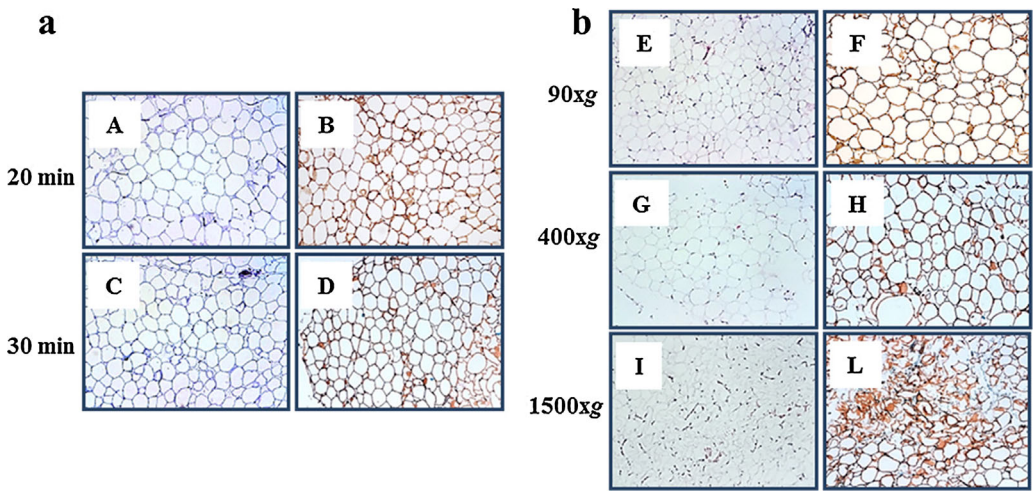


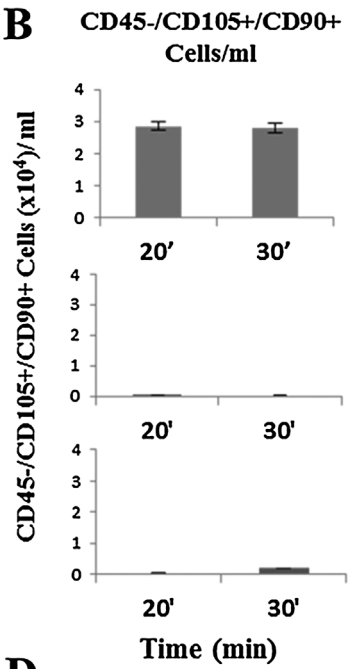
Fig. 2. Histology of middle layer sections obtained after handling methods. Representative images of middle layer histology after (A) spontaneous stratification at 20 and 30 min and (B) centrifugation techniques for 3 min at $90 \times g$, $400 \times g$, and $1500 \times g$. The middle layer sections (A-C-E-G-I) were stained with the Haematoxylin–Eosin and (B-D-F-H-L) sections were immunostained for vimentin. All magnifications were at 10×.

A

SPONTANEOUS STRATIFICATION

	Stratification time	SVF cells/ml lipoaspirate tissue
Middle layer	20 min	$41x10^4 \pm 0.73$
	30 min	$45x10^4 \pm 1.29$
Bottom layer	20 min	$54x10^4 \pm 0.82$
	30 min	$53x10^4 \pm 0.54$
Fluid portion	20 min	$45x10^4 \pm 0.65$
	30 min	$52x10^4 \pm 0.97$

B



C

CENTRIFUGATION

	Speed centrifugation	SVF cells/ml lipoaspirate tissue
Middle layer	90*xg*	$46x10^4 \pm 0.84$
	400*xg*	$52x10^4 \pm 0.76$
	1500*xg*	$62x10^4 \pm 1.34$
Bottom layer	90*xg*	$83x10^4 \pm 1.20$
	400*xg*	$81x10^4 \pm 0.95$
	1500*xg*	$88x10^4 \pm 1.10$

D

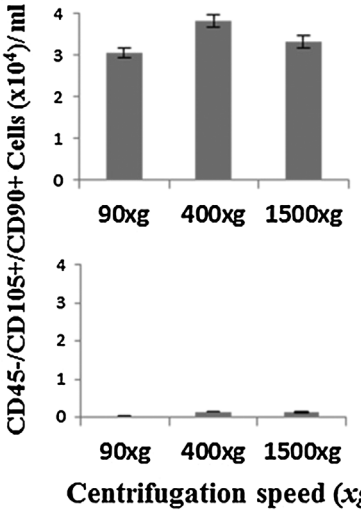


Fig. 3. Cell yield and immunophenotype of SVF cells after handling methods. (A) Number of SVF cells/ml human lipoaspirate and (B) CD45−/CD105+/CD90+ cells/ml human lipoaspirate both obtained after spontaneous stratification at 20, 30 min. (C) Number of SVF cells/ml human lipoaspirate and (D) CD45−/CD105+/CD90+ cells/ml human lipoaspirate both obtained after centrifugation speeds at 90 × g, 400 × g, 1500 × g. Exposed data represent the mean of five independent experiments in duplicate ± SEM. There were no statistical differences when comparing 20 min, as well as 30 min, to 90 × g, 400 × g, 1500 × g, respectively ($P > 0.01$).

($3.05 \times 10^4 \pm 0.11$ cells/ml lipoaspirate at 90 × g, $3.83 \times 10^4 \pm 0.14$ cells/ml lipoaspirate at 400 × g and $3.32 \times 10^4 \pm 0.16$ cells/ml lipoaspirate at 1500 × g), did not significantly change when the different speeds were compared ($P = 0.10$ comparing 90 × g to 400 × g; $P = 0.37$ comparing 400 × g to 1500 × g; $P = 0.52$ comparing 90 × g to 1500 × g) (Fig. 3D).

Moreover, we observed that there were no significant differences in ADSCs number, recovered by middle layers digestion, when compared spontaneous stratification to centrifugation ($P > 0.01$ when 20 min, as well as 30 min, was compared to 90 × g, 400 × g, and 1500 × g, respectively).

The Figure 4 shows the cytofluorimetric profiles from a representative experiment: the main level of CD45−/CD105+/CD90+ cells (blue population) was clearly detected in digested middle layers from each experimental condition.

Identification of ADSCs after spontaneous stratification and centrifugation: morphology and differentiation ability

Then, the above results suggested that the spontaneous stratification at 20 min and the centrifugation at 400 × g ensured satisfactory results in both adipocytes and ADSCs number. We investigated whether the 20 min spontaneous

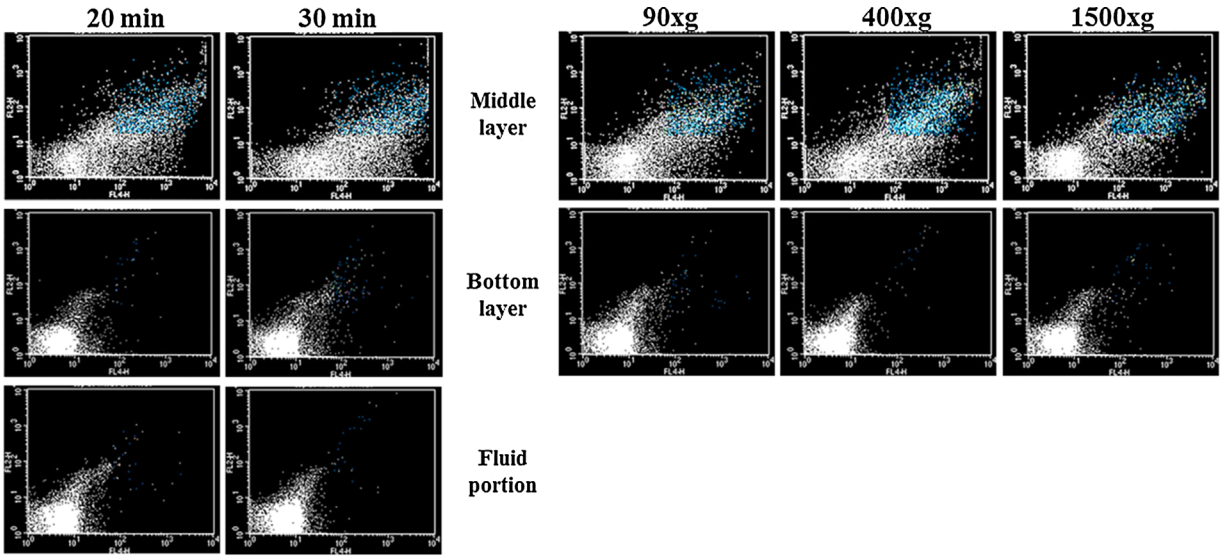


Fig. 4. Representative flow cytometric profiles for the quantification of CD45−/CD105+/CD90+ cells. The SVF profiles were obtained in all layers, after spontaneous stratification at 20 min and 30 min and after centrifugation speeds at 90 × g, 400 × g, 1500 × g. The blue population represents the CD45−/CD105+/CD90+ cells.

stratification and 400 × g centrifugation methods, could influence characteristics of ADSCs in terms of plastic adhesion ability such as the fibroblastic-like, spindle-shaped morphology. The cell populations recovered by middle layer digestion were plated and then daily observed for 2 weeks under a phase contrast microscopy at 10× magnification. Figure 5 shows the representative images of an adherent cell culture at 1st and 10th day. In particular, a low cellular density and undefined morphology at 1 culture day could be observed in both spontaneous stratification for 20 min and centrifugation at 400 × g speed (Fig. 5 A and B), while a typical elongated fibroblastic-like morphology was acquired by adherent stem cells after 10 culture days (Fig. 5C and D).

To confirm the stemness potential of adherent cells derived from spontaneous stratification at 20 min and from centrifugation at 400 × g, we analyzed their ability to differentiate toward the adipogenic, osteogenic, and chondrogenic lineages after 16 days, in the presence of specific

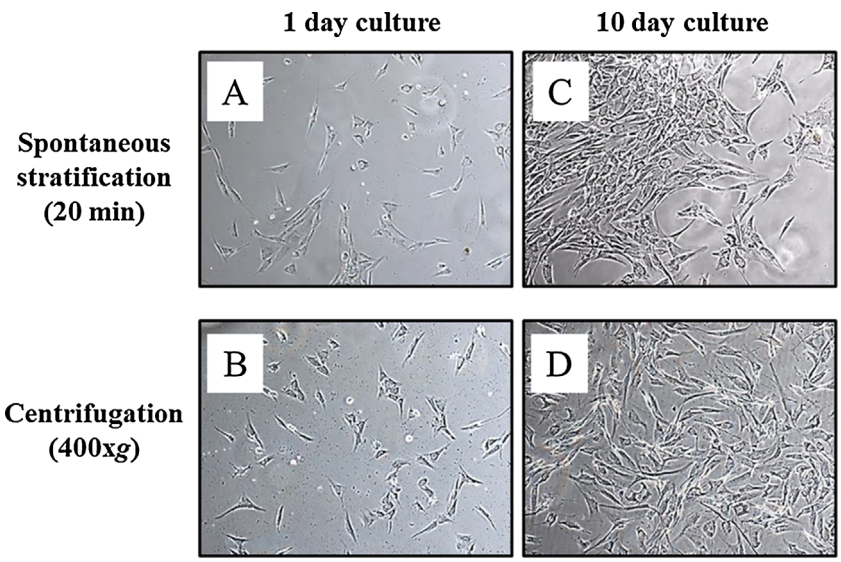


Fig. 5. Morphology of adherent cells from digestion of middle layer. Adherent cells with an undefined morphology at 1st day culture (A) after 20 min spontaneous stratification technique and (B) after 400 × g centrifugation, respectively; adherent cells with fibroblastic-like morphology at 10th day culture obtained (C) from 20 min spontaneous stratification and (D) from 400 × g centrifugation, respectively. All images were acquired with a phase contrast microscope at 10× magnification.

differentiation culture media. The cells successfully differentiated into adipogenic, osteogenic, and chondrogenic lineages, suggesting that the ability to differentiate was not influenced by any of the handling methods. The Figure 6 shows the images from a representative experiment.

Discussion

The different lipoaspirate handling protocols, commonly described in regenerative and reconstructive medicine, could affect the quality of fat grafting, reducing the amount and viability of adipocytes, increasing the reabsorption rate, and selecting cell types. The fat grafting should be characterized by high adipocyte viability and integrity, a good density of adipose portion and low levels of debris, fluids, and oil fractions. Nevertheless, a standardized, reproducible, and ideal method shared by surgeons has yet to be identified, leading to disagreement in the literature on the best method of lipoaspirate handling.

In the present study, we investigated the effects of lipoaspirate spontaneous stratification techniques performed at three different times (10 min, 20 min, and 30 min) in comparison with the centrifugation technique at several speeds (90 × g, 400 × g, and 1500 × g) on viable adipocytes and SVF cell number, to identify the most effective method to maintain and preserve the quantity and quality of the sample. The stratification technique for 10 min was excluded because the four layers were not well defined, and so, the results were not acceptable. The obtained data suggested that the spontaneous stratification method allowed to recover a comparable, no statistically different, amount of viable adipocytes after both 20 min and 30 min. The comparison between the different centrifugation forces, 1500 × g speed, respect to 90 × g, and 400 × g, caused the appearance of an upper oily layer and a significant decrease of cell number due to injured adipocyte membranes. Unlike reports from other authors (Kurita et al., 2008; Pulsfort et al., 2011; Son et al., 2013), our histological results suggested a more relevant damage in the fat cell membranes and the alteration of histological architecture in the samples centrifuged at 1500 × g speed, thus confirming results of viable cell counts. On the other hand, 90 × g centrifugation, although preserving the adipocyte integrity, showed low thickness when compared to 400 × g. In this context, it seemed useful to point out that an a good thickness of middle layer, acting as scaffold that is able to support ADSCs growth, is considered an important requirement for autologous fat grafting (Luo et al., 2013).

Comparing the two handling methods, we observed that the spontaneous stratification at 20 and 30 min causes a slight and significant decrease in the adipocytes number respect to 400 × g and 1500 × g centrifugation. So, we suggest that the results at 20 min are comparable to 90 × g, but the lipoaspirates centrifuged at 400 × g are much more packed and clean from debris. Then the 400 × g centrifugation technique is a good compromise to get an appropriate viable adipocytes amount and a good compactness of lipoaspirates for transplant.

It's well known that ADSCs represent an important and remarkable cell population so that adipose tissue is particularly attractive in regenerative medicine (Nicoletti et al., 2015). In particular, these cells improve the survival of fat grafts generally impaired by tissue atrophy (Tabit et al., 2012).

Besides retaining plastic-adherence ability, the mesenchymal stem cell population is known to be positive for several antigens, such as CD105, CD90, CD73, CD44, and negative for

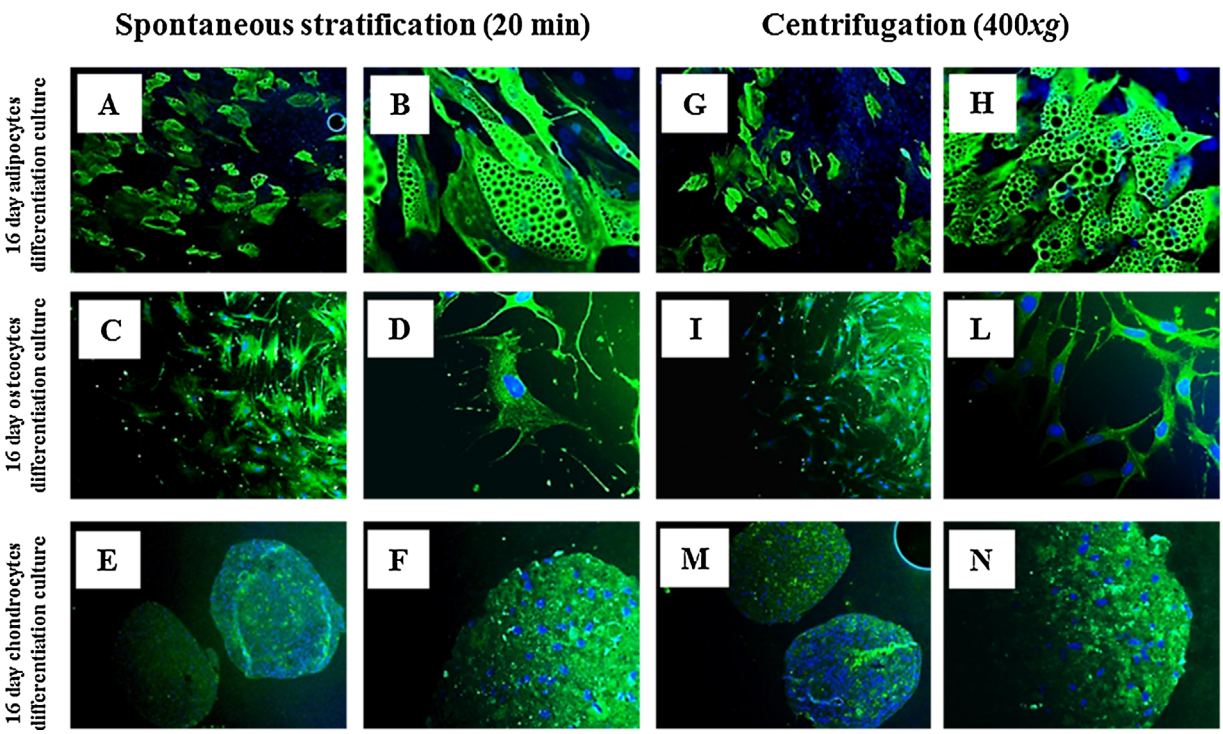


Fig. 6. Immunofluorescence staining of differentiated hADSCs. Spontaneous stratification at 20 min induced toward (A, B) adipocytes, (C, D) osteocytes, and (E, F) chondrocytes, respectively at 10× and 40× magnification. Immunofluorescence staining of differentiated hADSCs after centrifugation at 400 × g induced toward (G, H) adipocytes, (I, L) osteocytes, and (M, N) chondrocytes, respectively at 10× and 40× magnification. Immunostaining antibodies were described in Methods section. Nuclei were counterstained with DAPI (blue) fluorescent dye.

CD31, CD45 (Kern et al., 2006; Mitchell et al., 2006). In our hands, the phenotypic analysis of freshly isolated SVF cells, composed by a heterogeneous population of cells such as erythrocytes, endothelial cells, haematopoietic cells, lymphocytes, etc, reported that the number of CD45−/CD105+/CD90+ cells/ml lipoaspirate (ADSCs) was significantly higher in the middle layer. On the other hand, the presence of this sub-population recovered from the bottom layer and the fluid portion, in both methods, was unremarkable, suggesting, unlike other reports (Conde-Green et al., 2010), the poor efficiency to implanting the middle layer mixed with the bottom portion. The results indicated that the technique of spontaneous stratification, to 20 such as 30 min, is an effective method to obtain a relevant quantity of ADSCs and to preserve, simultaneously, the integrity and number of adipocytes.

Of note, the lipoaspirate samples centrifuged at 1500 × g, through showed an amount of CD45−/CD105+/CD90+ cells/ml lipoaspirate similar to the other centrifugation speeds, displayed the heavily damaged adipocytes. Whereas, the centrifugation at 90 × g and 400 × g for 3 min preserved the integrity of the adipocyte membrane and allowed to maintain a good percentage of cells with stemness characteristics (ADSCs population). We observed that samples centrifuged at 90 × g, if compared with 400 × g, were less thick with the presence of red blood cells. Kurita et al., stated that centrifugative forces, greater or equal than 400 × g, caused a better separation of the different layers ensuring a purified and cleaner fat graft (Kurita et al., 2008). In this context, the transplant of cellular debris into healthy tissue cause an inflammatory response that negatively affecting the transplant effectiveness, as previously reported (Pulsfort et al., 2011).

Taken together, our results suggest that the replanting of the middle layer obtained from lipoaspirate samples after either spontaneous stratification at 20 min or centrifugation at 400 × g, is enough to provide a good amount of ADSCs and to preserve the adipocyte integrity, showing that both approaches are effective. Obviously, if suitable equipment is available, the use of the technique of centrifugation can certainly allow the surgical team a significant time savings for fat grafting.

Acknowledgments

The Authors thank Gasperina De Nuntiis (Department of Life, Health and Environmental Sciences) for technical assistance. The study has been performed in the framework of the "Research Centre for Molecular Diagnostics and Advanced Therapies" supported by the "Abruzzo earthquake relief fund" (Toronto, Ontario).

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