SEFFI (Superficial Enhanced Fluid Fat Injection) for Aesthetic and Clinical Regenerative Treatments

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Abstract: *Aim*: In the field of regenerative applications, new fat preparations are an interesting perspective for easily available sources of highly potent staminal cells. In order to support the development of new robust techniques for regenerative purposes, stemness and proliferative properties of cellular content should be addressed.

Methods: SEFFI is standardized technique and published by two of the Authors (AG, PFB). These technique aim to harvest and re-inject autologous microfragmented adipose tissue with minimum manipulation. In this study the harvesting and preparation procedure was performed by disposable kits SEFFILLER™ and SEFFICARE™ (Produced by SEFFILLINE srl Bologna Italy). These cannulas performed a selection of clusters dimension and therefore microfragmented adipose tissue were immediately plated in colture dishes to allow liberated cells to attach to plastic. Cellularity from both cannulas was calculate and adipose stem cells (ASCs) from SEFFILLER™ and SEFFICARE™ were characterized by proliferation assay and differentiation capacity towards mesenchymal lineages. Moreover, as a quality control system, a new technology named Celector® was used to identify cell heterogeneity and viability of expanded ASCs which can be used for further cell therapy approaches.

Results: Microfragmented tissue, harvested by both cannulas, showed good number of adherent cells. Cells were vital and with an optimal proliferation ability. Celector® analysis confirmed the highly cell viability and sharing physical properties between ASCs from both cannulas. Isolated cells showed stemness characteristics due to their ability to differentiate towards adipogenic and chondrogenic lineages.

Conclusion: These results confirmed the presence of regenerative elements in autologous graft of SEFFI tissue. With perspectives of applications in aesthetic and clinical field.

Keywords: Liposuction cannula, fat graft, ASCs, regenerative medicine, cell production quality control system.

INTRODUCTION

Advance therapy using stem cells is a promising treatment for degenerative diseases or disorders that cannot yet be successfully managed through conventional care. The most used cells in current cell-based approaches are the Mesenchymal Stem Cells (MSCs) which are multipotent stem cells present in almost every organ and tissue. Adipose tissue is a promising source of MSCs since Zuk *et al.* describe it in 2001 as a stem cell population similar to the one from bone marrow [1-4]. Adipose derived mesenchymal stem cells (ASCs) are isolated through enzymatically digestion from the stromal vascular fraction (SVF) which it contains large numbers of cells composing interrelated cell populations: adipocyte progenitors, pericytes, endothelial progenitor cells, and transit amplifying cells [5]. ASCs have shown the possess differentiation potential towards different lineages like osteogenic, chondrogenic, myogenic, hepatogenic and to endothelial cells – both *in vitro* and *in vivo* [6,7]. Moreover, like all MSCs, they exhibit antifibrotic and immunomodulatory characteristics [8,9] and they stimulate angiogenesis and revascularization of fat grafts [10,11]. Thanks to these characteristics, adipose tissue implantation has been used to improved skin trophism, accelerated closure of complex wounds or ulcers, and enhancement of skin appearance after damage from radiotherapy [11-14]. Therefore, the combination of adipocytes and SVF/ASCs-enriched fat grafts are considered a valuable approach in the aesthetic rejuvenation treatment to give volumization and skin regeneration effects [11,15-23]. Moreover, to obtain an efficient engraftment and regenerative effect, superficially (subdermal plane injection) injection of smaller adipose tissue's clusters is suggested [24-26]. In SEFFI (Superficial Enhanced Fluid Fat Injection) [18-

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19-20-43]. and MicroSEFFI [21-31] techniques we proved that it's possible to obtain a good potential regenerative tissue without any substantial manipulation; using micro cannulas with very small side port holes (1-0.8-0.5-0.3 mm) we selected the clusters dimension during the harvesting procedure hence we don't need any substantial manipulation in order to thin the tissue.

In the light of these evidences, in our technique we used two disposable kits (SEFFILLER™ and SEFFICARE™), which provide two different small cannulas (diameter of the side port holes: 0.8mm and 1 mm) for tissue harvesting. These kits derive from the SEFFI technique and offer the advantages to be disposable, all-in-one and guided. In particular the Italian patented Guide allow to harvest the tissue in a very superficial layer and offer the opportunity to physicians without a skills in liposuction to perform the procedure in a safe, standardized and effective way . We consider this step of the procedure as a "cell cluster dimension selection", the small cannulas select micro fragmented tissue ready to be injected without any substantial manipulation.

METHODS

Sample Collection and Experimental Setup

Adipose tissue was harvested from 3 consecutive healthy patients after informed consent was obtained in accordance with the Declaration of Helsinki guidelines. Subcutaneous adipose tissue was harvested from different fat depot areas trough 0.8 mm diameter side port holes cannula (SEFFILLER™ disposable kit produced by SEFFILINE™ srl, Bologna, Italy) and 1 mm diameter side port holes cannula (SEFFICARE™ disposable kit produced by SEFFILINE™ srl, Bologna, Italy) (Figure **1A**) after informed consent and in accordance with the Declaration of Helsinki guidelines. The SEFFILLER™ and SEFFICARE™ are disposable kit that provide the selecting tissue cannulas and a special guide to lead the adipose tissue selection (Figure **1B**). This guide allows to any physician even without a specific skill in fat harvesting or liposuction, to perform the procedure in the safe, effective and standardized superficial subdermal plain. Fat aspiration is performed while the patient is maintained under local anesthesia. The following protocol has been standardized. Cold Ringer's lactate solution (100 mL) was mixed with lidocaine 2% (200 mg), sodium bicarbonate (5 mEq) and epinephrine (1ml/1 mg), then injected into the selected donor site with a ratio of 1:1

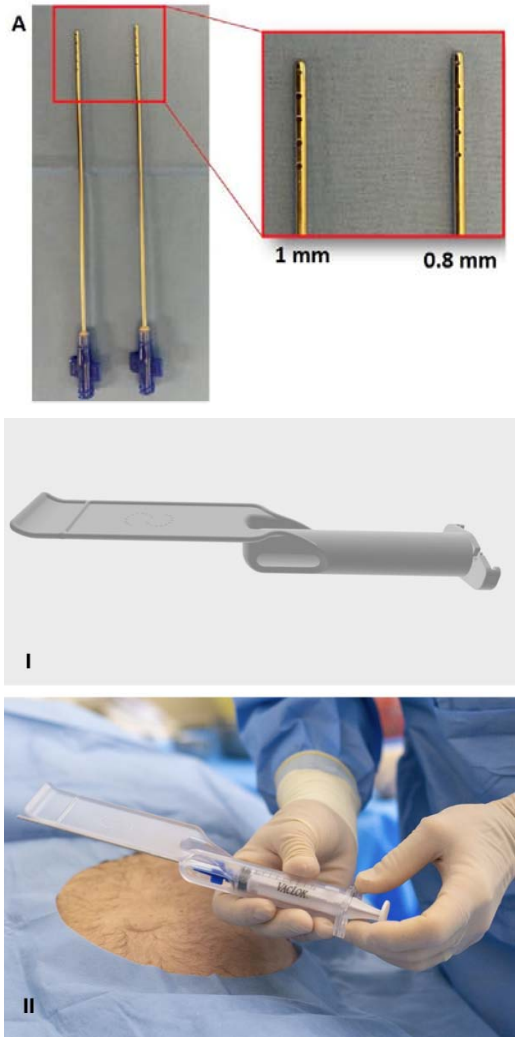

Figure 1: Lipoaspirate cannulas. (**A**) These cannulas, 1 mm and 0.8 mm sideport holes respectively, select adipose tissue in small clusters without need of substantial manipulation before injection. (**B**) (**i**) The special guide allows to select the adipose tissue in the superficial and standardized plain even if the physician has not specific liposuction skills. (**ii**) The guide assembled with syringe and cannula.

of the average amount of harvesting tissue. Manual aspiration/selection of the adipose tissue was performed with a 10 mL syringe with plunger lock (provided into the kits) mounted alternatively with the two different multi-perforated side port cannulas and inserted into the special guide (Figure **2 i**, **ii**, **iii**, **iv**). The fat depots were chosen as preferred harvesting sites in abdomen, hip and trochanteric region. After aspiration, the fat was mixed with cold Ringer's solution to rinse it from the anesthetic and to facilitate tissue precipitation (Figure **2 v**, **vi**, **vii**). The syringe was then capped and maintained in a dark environment, under a sterile cloth, to reduce the possibility of light oxidation of adipocytes. The tissue harvested 0.8 mm and 1mm side port cannulas were kept in separate, labeled syringes. The

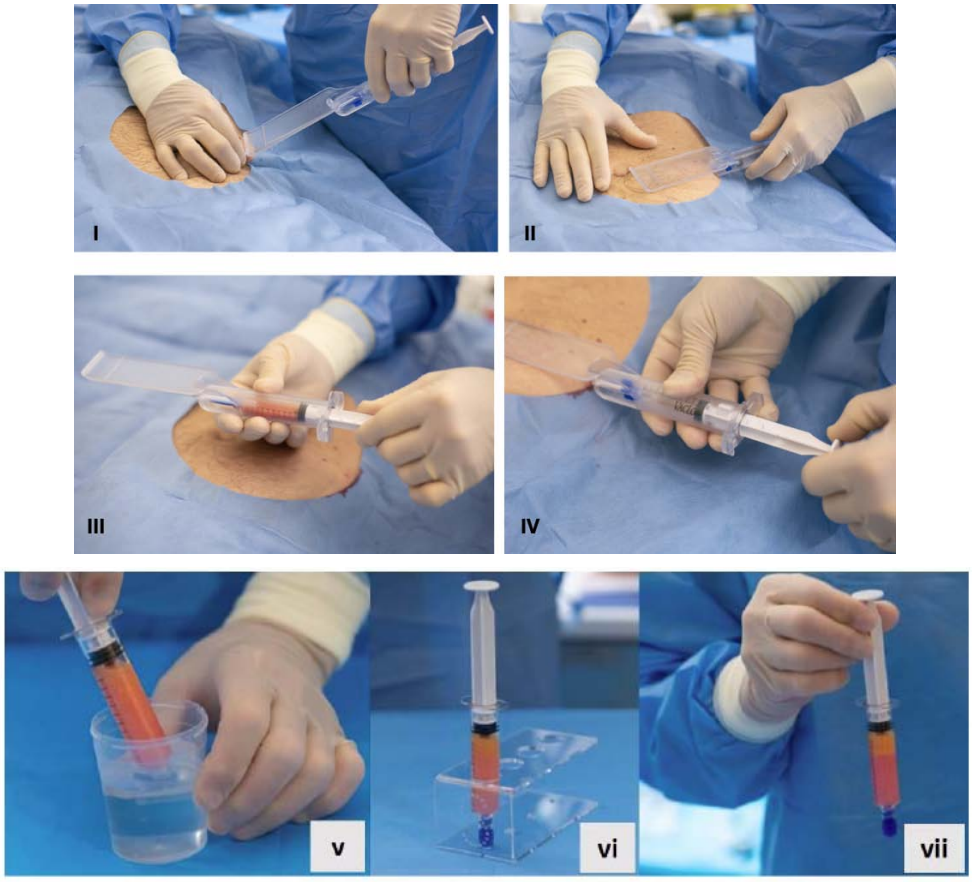

Figure 2: Manual aspiration/selection of the adipose tissue. (**i**) Cannula introduction perpendicular to the skin through a hole in the skin performed with a 18G needle; (**ii**) Rotation of the guide 90 degree and introduction of the cannula in the superficial subcutaneous plain; (**iii**) lock the plunger in aspiration position and move back and forth to select the adipose tissue clusters; (**iv**) micro fragmented adipose tissue in the syringe. (**v**) After aspiration, the fat was mixed with cold Ringer's solution to rinse it from the anesthetic and to facilitate tissue precipitation (**vi, vii**).

tissue was centrifuged for 1 minute at 3500 rpm, with an estimated sedimentation force of 448 g. The liquid portion, collected at the bottom of the syringe, was then eliminated as well as the free oil on top of the tissue. Samples were transferred at 4°C to the lab and processed.

Cell Isolation

Adipose tissue was measured with a serological pipette and each milliliter of tissue was immediately plated in 20 cm^2 of cell culture surface in expansion medium composed of Dulbecco's Modified Eagle's Medium High Glucose (DMEM-H), 10% fetal bovine serum (FBS) and 1% Penicillin and Streptomycin (PS) (all material from Lonza, Walkersville, MD, USA). After 3 days, medium was replaced and micro-fragmented adipose tissue was left another 3 days in culture to allow cells to be released form tissue and adhere to plastic surface. After one week, cell culture was washed with PBS to remove adipose tissue, debris and red blood cells. Generally, attached cells were trypsined after 14 days of culture, counted and plated in new plate at cell density of 5,000 cells/cm^2. Cellularity was calculated as number of counted cells after 14 days and divided for the milliliter of plated tissue.

Cell Analysis

Attached cells harvested using both systems, 0.8 mm side port holes cannula (SEFFILLER™ kit) and 1mm side port holes cannula (SEFFICARE™ kit), were monitored under light microscope and pictures were taken to show cell morphology at different passage in culture.

Cell Proliferation Assay

Cell proliferation was measured using colorimetric assay AlamarBlue following manufacturer's instructions (AlamarBlue® Cell Viability Assay - Thermo Fisher Scientific, Waltham, MA, USA). Cell culture triplicate were plated at a density of 3700 cells/cm2 in growth

medium for 24 hours and the day after medium was replaced with 10% of AlamarBlue solution in expansion medium. Fluorescence was measured 2 hours after medium replaced and plate was read every day at the same time until cells reached confluence. Fluorescence was monitored at a 530-560nm excitation wavelength and 590nm emission wavelength directly from the plate (Victor system, Victor Multilabel Plate Reader - Perkin Elmer, Boston, MA, USA).

Differentiation Potential Towards Mesenchymal Lineages

To prove stemness characteristics of the tissue selected with the two different cannulas 0.8 and 1 mm, the derived cells were differentiated towards the mesenchymal adipogenic and chondrogenic lineages.

Adipogenic Differentiation

ASCs were cultured in growth medium until reached 90% of confluence then medium was replaced with the adipogenic medium (StemPro Adipogenic Differentiation kit, Gibco). Differentiation assay lasted 2 weeks and medium was replaced every 3-4 days until the end of the treatment. To visualize fat droplets, cells were fixed in 10% formalin for 10 minutes and stained with Oil Red O solution.

Chondrogenic Differentiation

100˙000 cells were resuspended in 10 µl of PBS and plated for 1 hours and placed in incubator at 37˚C, 5% CO2. After 1 hour, growth medium was added for one day and then replaced with the chondrogenic medium (StemPro Chondrogenesis Differentiation kit, Gibco). Differentiation assay lasted 21 days and medium was changed every 3-4 days. Micro-masses and differentiated adherent cells were visualized using Alcian blue staining using light microscope.

Celector® Characterization

Celector® is a new instrument for cell characterization, QC and cell sorting that has the key advantage to sort both cells from rough tissues and ex vivo cultured cells without any additional manipulation like antibody biding27-30(Stem Sel ltd., Italy). Celector® instrumental setup consists of a fluidic system and a biocompatible capillary separation device that implement a patented technology for cell analysis. The device made of biocompatible plastic material allows the analysis of dispersed tissue or cells carried in a biocompatible fluid. A camera with a microscopic objective is placed at the end of the separation channel (USB 2.0 board-level camera - mvBlueFOX-MLC, Matrix Vision, Oppenweiler, Germany) and it monitors the elution process, generating a recorded plot of the eluted cell number as a function of time (the fractogram). Cells will elute at given retention time intervals through a fraction collector in sterile tubes for further studies or use.

Fractionation Procedure

System conditioning and instrumental settings were previously described [30]. Before cell analysis, decontamination of the fractionation system was performed by flushing cleaning solution; then the system was washed copiously with sterile, demineralized water. The fractionation system was flushed with a sterile coating solution in order to block cells' unspecific interaction sites on the plastic walls. Finally, the fractionation system was filled with sterile mobile phase (all solutions were provided by Stem Sel Ltd, Italy). Enzymatically detached cells were prepared in a solution of $3x10^6$ cells/ml and 100 µL was injected into the system and eluted with a flow of 1 mL/min. Both cells from SEFFILLER™ and SEFFICARE™ were analysed and profiles were compared.

Statistical Analysis

All data were plotted using Excel and Graph Pad software which was used to performed statistical analysis using t-test.

RESULTS

Cell Characterization

We were able to derive cells from both adipose tissues harvested using the SEFFILLER™ and SEFFICARE™ system. After one-week, adherent, viable cells were observed, and showed a fibroblastoid shape, with small cytoplasm and long extrusions (Figure **3A-i**). After *in vitro* expansion, cells maintained the fibroblastoid morphology with an enlargement of the cytoplasm, especially at passage 4 (Figure **3A-iii**). No differences were noted among samples from tissue selected with the two different cannulas 0.8 and 1 mm, indicating the similar quality of isolated cells. After two weeks in culture, 450000 cells could be harvested per milliliter of tissue using both systems, demonstrating the same cellularity from different cannula.

The homogeneity in the morphological aspect was correlated to the similar proliferative potential between cells derived from both systems. Cells showed a good

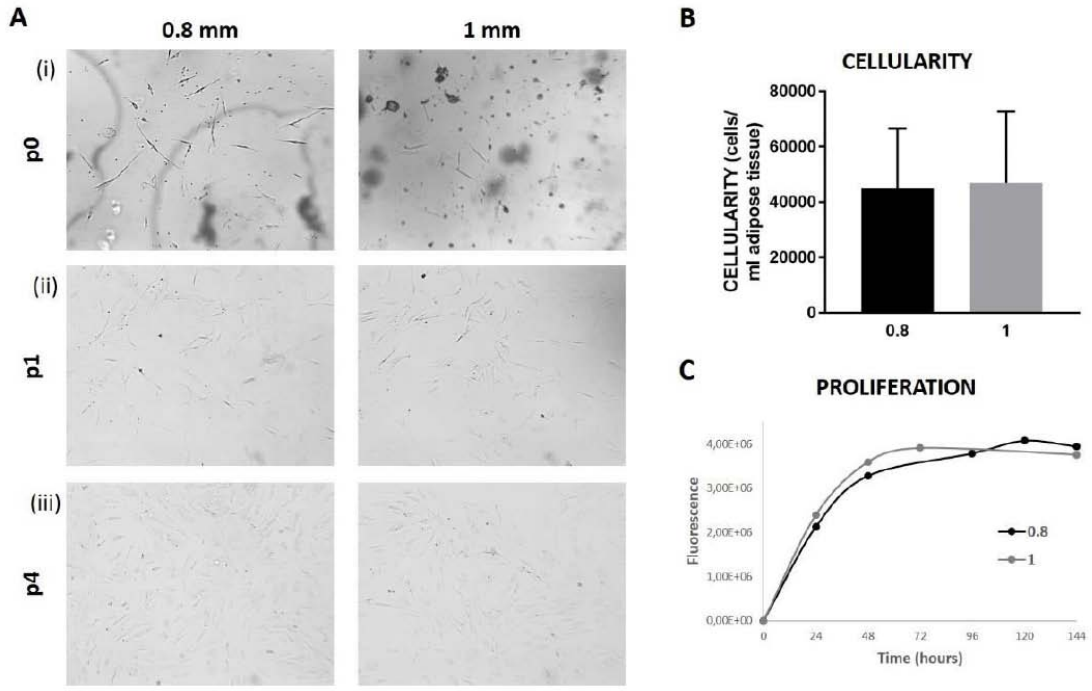

Figure 3: Isolation of adipose stem cells ADCs from lipoaspirate tissue harvested using cannulas having side port holes of 0.8 and 1 mm. (**A**) Isolated cells showed typical mesenchymal morphology with elongated cytoplasm. Size increased during *in vitro* culture from freshly isolated to passage 4. (**B**) Optimal cell recovery of proliferating cells in both cannulas. Cellularity is expressed as the number of proliferating cells attached to plastic after two weeks of culture from isolation divided millilitres of adipose tissue harvested. (**C**) ADSCs showed good proliferation rate as indicated by fluorescence activity of Alamar Blue assay.

proliferation rate, with an initial exponential phase and then the plateau after 96 hours (Figure **3B**).

Stemness Potential

Cells derived from both tissues harvested with the two systems (SEFFILLER™ and SEFFICARE™) were able to differentiate towards the mesenchymal lineages adipogenic and chondrogenic.

ASCs cultured in adipogenic medium showed the formation of lipid droplets in their cytoplasm colored in red by Oil Red O staining while in the chondrogenic medium, small micro-masses and differentiated cells releasing extracellular matrix made mostly of proteoglycan were stained in blue by Alcian blue staining. Any macroscopically difference was noted between cells derived from SEFFILLER™ and SEFFICARE™ systems (Figure **4**).

Celector® Profile

Expanded ASCs were also analyzed by Celector® to obtain their elution profile, giving an additional information about the physical properties of cells. Profiles showed that ASCs derived from both systems are quite a homogeneous population as shown by the normal distribution of the curve. SEFFILLER™-SEFFICARE™ ASCs eluted between 2 and 6 minutes, with no substantial difference between two systems (Figure **5A**). Live images of ASCs show bigger cells eluting in the ascending part of the curve (3rd minute) and smaller and more compact in the descendent one (5th minute) (Figure **5B**). The instrument is able to detect and deplete dead cells that elute always in the first minute of analysis. Thanks to this feature, we could confirm the vitality of ASCs because no dead cells were observed in the first minute of the analysis.

DISCUSSION

Many studies proved that injecting the tissue in the subdermal superficial layer we enhance the regenerative effects on the skin [40-4142-46] In order to avoid any irregularity and lumpiness injecting superficially is mandatory to have a fluid tissue; for this reason, many devices appeared on the market are addressed to manipulate the adipose tissue in order to thin the harvested adipose tissue and reduce the tissue clusters. With SEFFI (Superficial Enhanced Fluid Fat Injection) we proved that is possible to select right clusters dimension hence tissue fluidity, using special small cannulas with smaller side port holes [18-19-20-21-31-43].

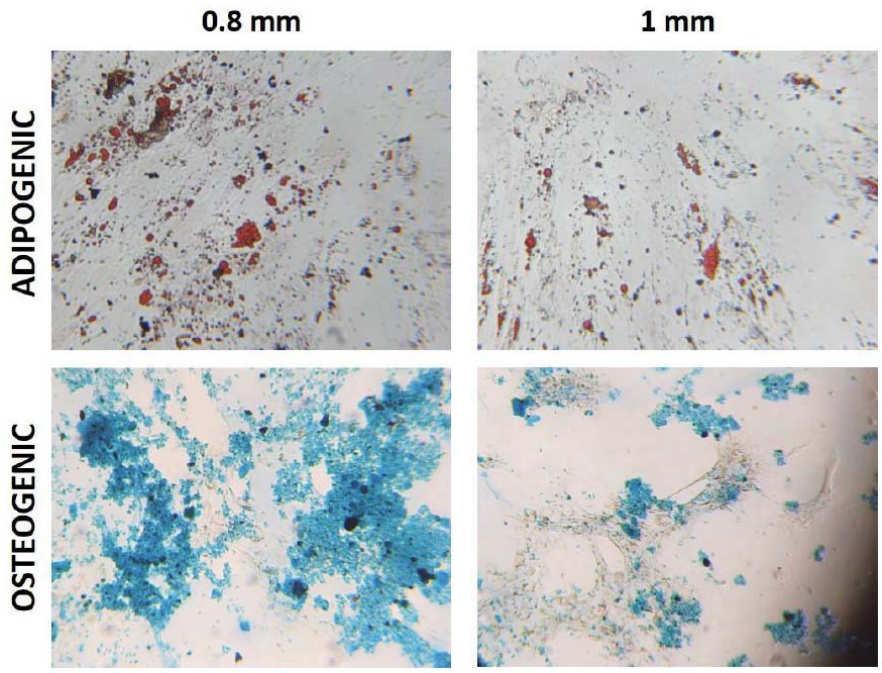

Figure 4: Differentiation potential of ASCs from 0.8- and 1-mm side port holes cannulas. Cells showed to possess staminal characteristics and to differentiate into adipogenic and chondrogenic lineage. Oil red O staining showed formation of intra-cytoplasmatic lipid droplets and Alcian blue staining colored the extracellular matrix mainly formed of proteoglycan produced by differentiated ASCs.

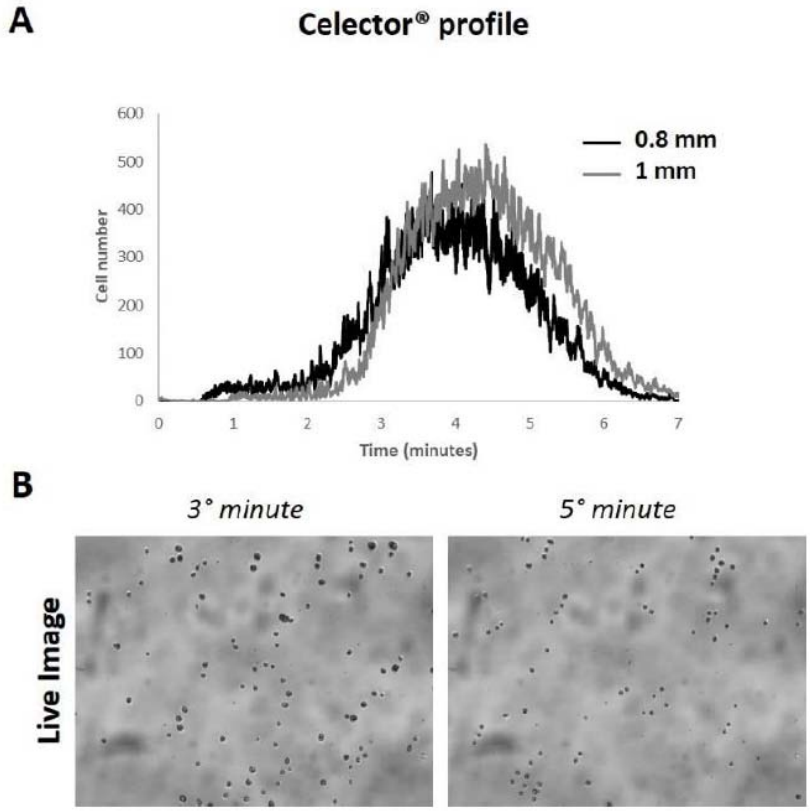

Figure 5: Quality control of expanded ASCs using Celector®. (**A**) Expanded ASCs from 0.8- and 1-mm side port holes cannulas were analysed by Celector® and their specific profiles showed identical morphology as shown by perfect overlapping of two curves. Alive cells eluted from minute 2 until minute 6, while debris and few dead cells eluted in the first minute of the analysis. (**B**) Representative screenshots of live image analysis showed cell morphology. In the ascendant part of curve (3° minute) cells are bigger while in the second part of the analysis (5° minute) cells are smaller and with more defined contours.

Anatomical location with higher density of blood vessels [34-36] is ideal to guarantees the highest concentration of ASCs, therefore subdermal layer [37] is highly suggested.

According to other AA [44-45], we consider mandatory to perform the harvesting procedure in a subdermal superficial layer in order to obtain an adipose tissue richer in SVF (Stromal Vascular Fraction) components and for this reason we used for this studies the kit SEFFILLER™ and SEFFICARE™ that provided a special guide meant to perform the harvesting procedure in a superficial, safe, effective subdermal layer and in standardized way (**Figure 1B i**,**ii**,**iii**,**iv**).

Morever depending on clinical application, the size of cannulas is the key variable and to minimize appearance of skin irregularities it is suggested to use microcannula with diameter ≤ 2 mm according to Klein's [31]. Several studies have shown the advantage to use microcannula to obtain higher number of ASCs compared to standard liposuction cannula [32] and the ability to harvest micro fragmented tissue presenting normal histological structure, with the average size of the fat lobules varied from 500 to 1000 μm appearance which facilitates fat transfer using a 1cc syringe fit with 23G and 25G cannulas [24].

In this study, we focused on the study of ASCs derived from lipoaspirate samples harvested with two different cannulas: 0.8mm diameter side port holes cannula (SEFFILLER™ kit produced by SEFFILINE srl Bologna - Italy) and 1mm diameter side port holes cannulas (SEFFICARE™ kit produced by SEFFILINE srl Bologna -Italy). These cannulas do not merely harvest adipose tissue but also select small clusters of micro fragmented tissue. Therefore, cells are already released from this procedure and we investigated the ability to isolate ASCs without need of additional enzymatic digestion.

These results proved that the micro-fragmented adipose tissue selected with small diameter microcannulas (0.8- and 1 mm side port holes cannulas) contain and release viable cells of the SVF, able to differentiate into mature cells of the mesenchymal adipogenic and chondrogenic lineages. Due to their differentiation potential, ASCs are used for regenerative applications and tissue engineering purposes, therefore quality control of expanded cells are needed. In addition to the classical methodologies, such as proliferation and microscope observation, we analyzed cells through Celector®, a new technology that gives important information about cell physical characteristics, viability, cell population heterogeneity/homogeneity and the presence of dead cells that can be depleted in case the procedure requires it [30]. Cell populations resulted homogeneous and vital with no differences between the two cannulas.

We conclude that adipose tissue harvested with these cannulas does not required any substantial manipulation to release ASCs and the use of these cannulas doesn't affect the viability and stemness capability of the cells. In our practice we use the tissue selected with 0.8mm side port holes cannula (SEFFILLER™ kit) for aesthetic purpose, injecting this micro fragmented tissue superficially with a 21-27G micro cannula or needle in such a delicate area as the face without any risk of lumpiness and irregularities and the tissue selected with 1mm side port holes cannula (SEFFICARE™ kit) for clinical regenerative treatments, injecting tissue with a 18-24G needle. Moreover, the kits SEFFILLER™ and SEFFICARE™ are all-in-one and disposable, increasing patient's and doctor's safety, and provide a special guide to facilitate and standardize the selecting tissue procedure even for physicians without any skill in liposuction.

DECLARATIONS

Authors' Contributions

Alessandro Gennai and Silvia Zia: performed procedure and data acquisition, made substantial contributions to conception and design of the study and performed data analysis and interpretation and draft of the article

Alessia Maggio: performed data acquisition

Barbara Roda: made substantial contributions to conception and design of the study

Laura Bonsi, Francesco Alviano, Andrea Zattoni, Pierluigi Reschiglian: final approval of the version to be published

Francesco P. Bernanrdini: made substantial contributions in selecting patients and performing procedure.

Availability of Data and Materials

Not applicable.

Financial Support and Sponsorship

None.

Conflicts of Interest

Andrea Zattoni, Barbara Roda and Pierluigi Reschiglian are associates of the academic spinoff company Stem Sel Srl (Bologna, Italy). The company mission includes the development and production of novel technologies and methodologies for the separation and characterization of cells and biosamples.

All the other authors report no conflict of interest since nobody have commercial associations that might create a conflict of interest in connection with submitted manuscripts.

Ethical Approval and Consent to Participate

Ethical approval from Comitato Etico Indipendente di Area Vasta Emilia Centro (CE-AVEC) (n. 3504/2018/Oss/AOUBo).

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