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Evaluation of The Viability and Phenotipe of Adipose Derived Cells Harvested Using Different Harvesting and Processing Procedures: A Pilot Study

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Abstract

Background: Clinical studies demonstrated the efficacy of therapies based on the autologous grafting of adult mesenchymal stem cells to accelerate the healing and regenerative processes of the skin and mesenchymal tissues therefore, this is considered a valuable approach in the aesthetic rejuvenation treatment to give volume restoration and skin regeneration effects.

Objective: The aim of this project consists of the evaluation of the cell viability of adipose tissue (AT) harvested using the Superficial Enhanced Fluid Fat Injection (SEFFI) procedure standardized by the Authors (AG, FPB). The harvesting procedure was performed using two different cannulas having 0.8 mm and 1 mm side-port holes, respectively. Cells phenotype and ability to adhere to the plastic surface have been analysed. The results have been compared to those recorded in adipose tissue harvested using a liposuction system and processed with enzymatic digestion (collagenase).

Methods: This study was performed on adipose tissues harvested from 7 patients (6 females and 1 male) with an average age of 48.5 years with two different techniques and three different cannulas. We compared the cell vitality of every sample at T0 and T72. Moreover, the samples were analysed to determine the phenotype: cells were incubated with antibodies anti human against CD90-FITC, CD73-PeCy7, CD44-PE, CD31-PECy5, CD235a- PECy7, CD34-FITC, CD45-FITC and CD146-FITC and read using the flow cytometer s3e Cell Sorter, BioRad. Results were analysed using the software Flow Jo.

Results: Lipoaspirate tissue-derived by 0.8 and 1 mm cannula from all samples proved to be vital and to possess viable cells the average absorbance was similar immediately after plating (T0) and 72 hours after (T72) for both the two cannulas. The two systems proved to equally harvest vital tissue and were comparable to the cell harvested with liposuction system (harvesting cannula of 3 mm, 2 oval: width 2.5 mm height 5 mm side port holes), and isolated from the stromal vascular fraction (SVF) by enzymatic digestion, which is the gold standard procedure to obtain adipose tissue derived cells. An increase in cell viability was observed in all samples for each condition after 72 hours (T72) (0.8, 1 mm and enzymatic digestion). Cells from all samples were able to adhere to a plastic surface and could be expanded to obtain the right number of cells for the staining. Morphologically, cells harvested by different cannula or isolated by enzymatic digestion showed differences in their morphology they appeared as fibroblasts, proving their mesenchymal phenotype.

The adipose derived mesenchymal stem cells (MSCs) proved their heterogeneity among individuals, because differences in surface antigens were noted between 0.8 mm and 1 mm derived cells from different donors.

Conclusion: This study proved the vitality of adipose tissue harvested using the guided SEFFI procedure using both 0.8 and 1 mm side port holes cannula. Tissue was vital with both cannulas and the values were comparable to the isolation of SVF by enzymatic digestion, the gold standard procedure for adipose tissue derived cells. To be defined as MSCs, cells need to adhere to a plastic surface and express specific surface antigens, especially CD44, CD90 and CD73. Cell viability was confirmed by the ability to adhere to a plastic surface. Cells derived from each individual were able to attach and proliferate for at least three passages in culture and showed the typical fibroblastic morphology.

Keywords: Adipose-Derived Stem Cells, Autologous Fat Transfer, Stromal-Vascular Fraction, Clinical Regeneration Applications, Adipose Tissue Cell Viability, Adipose Tissue Manipulation, Adipose Tissue Processing, Adipose Tissue Harvesting

Abbreviations List:

AT: Adipose Tissue

SEFFI: Superficial Enhanced Fluid Fat Injection

SVF: Stromal Vascular Fraction

MSCs: Mesenchymal Stem Cells

ADSCs: Adipose Derived Stem Cells

ECM: Extra-Cellular Matrix

AST: Superficial Adipose Tissue

DAT: Deep Adipose Tissue

PZ: Patient

Introduction

Regenerative therapy based on the injection of micro fragmented adipose tissue is a promising treatment for degenerative diseases or disorders that cannot yet be successfully managed through conventional care moreover, it is also a promising treatment in antiaging therapy [1].

It exploits the properties of the cells of stromal vascular fraction (SVF), naturally present in the adipose tissue: 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 [2,3]. Adipose tissue is a promising source of MSCs similar to the one from bone marrow as described by Zuk et al. in 2001 [4-7].

Adipose Derived Stem Cells (ADSCs) share similar characteristics with bone marrow mesenchymal cells still, they have some advantages, including their easy availability and harvesting through a less invasive surgical procedure [8]. They can be isolated through enzymatical digestion from the SVF, which contains a large number of cells composed of interrelated cell populations: adipocyte progenitors, pericytes, endothelial progenitor cells, and transit-amplifying cells [9]. ADSCs have been shown to possess differentiation potential towards different lineages like osteogenic, chondrogenic, myogenic, hepatogenic and endothelial cells, both in vitro and in vivo [10,11]. Moreover, like all MSCs, they exhibit antifibrotic and immunomodulatory characteristics, and they stimulate angiogenesis and revascularization of fat grafts [12,13].

Thanks to these characteristics, adipose tissue implantation has been used to improve skin trophism, accelerate the closure of complex wounds or ulcers, and to enhance skin appearance after damage from radiotherapy [14,15]. Recently Mantovani et al. proved the injection of micro fragmented adipose tissue as a promising therapy in genitourinary syndrome of menopause in gynaecology [16]. Therefore, the micro fragmented adipose tissue graft, naturally rich in cells from SVF and ADSCs, is considered a valuable approach in the aesthetic rejuvenation treatment to give volumization and skin regeneration effects [17].

To obtain efficient engraftment and regenerative effect, superficial (subdermal plane) injections of smaller adipose tissue clusters are suggested [18,19].

Once cells have been harvested, two techniques are available nowadays to isolate SVF: enzymatic and mechanical. The enzymatic method is particularly indicated in SVF isolation since it disrupts the extra-cellular matrix (ECM) and the binding of adipocytes and other cells however, its use is restricted by regulatory issues related to enzymatic procedures, especially within the European Community. Alternative mechanical methods were proposed and proved to be capable to provide cell viability without adipose tissue manipulation [20-23].

Using SEFFI (Superficial Enhanced Fluid Fat Injection) techniques, Authors proved that it is possible to obtain a good potential regenerative tissue with a good amount of viable cells with mechanical procedure, without any substantial manipulation. Using micro-cannulas with very small side port holes (0.8 mm and 1 mm), Authors selected the clusters dimension during the guided harvesting procedure, without any substantial manipulation in order to thin the tissue [21-23].

In the light of available evidence, Authors tested cell viability and phenotype of adipose tissue (AT) harvested using two types of cannula (0.8 and 1 mm) and minimal manipulation (cases) compared to the one harvested usign enzymatic digestion (controls).

Materials and Methods

In October 2021, Authors harvested adipose tissue from 7 consecutive patients (6 females and 1 male) with an average age of 48.5 years the procedures were performed in AA's medical facilities. To be included in the study, patients need to have a body mass index > 25 kg/m^2, but < 30 kg/m^2, and to be < 65 years old. Patients meeting one the following characteristics were excluded from the study:

- Type 1 or type 2 diabetes mellitus
- Cardiovascular or neurologic disorders
- Patients taking chronic drug therapy
- Smoke
- Previous abdominal surgery (laparotomy).

The procedures were performed under local anaesthesia using the following cannulas (Figure 1):

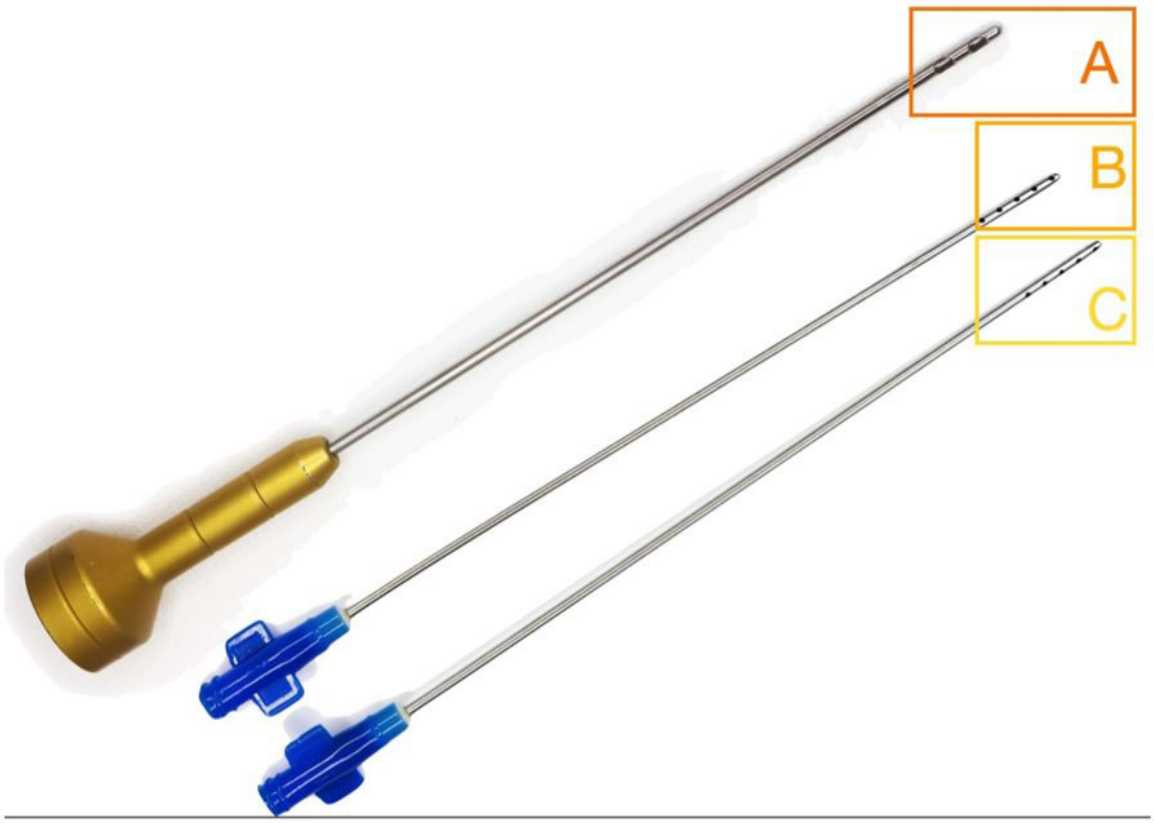

Figure 1: A: Cannula of 3 mm, 2 oval (width 2.5 mm, height 5 mm) side port holes, liposuction cannula, Coleman cannula **B:** Cannula of 2 mm, 15 round side port holes, 1 mm diameter **C:** Cannula of 2 mm, 15 round side port holes, 0.8 mm diameter

The guided harvesting procedures with a cannula C and B with side port holes of 0.8 mm and 1 mm were performed with the medical devices SEFFILLER and SEFFICARE (Seffiline Srl, Via delle Lame 98 Bologna Italy), respectively, and the procedure was performed according to the producer instructions (Figure 2).

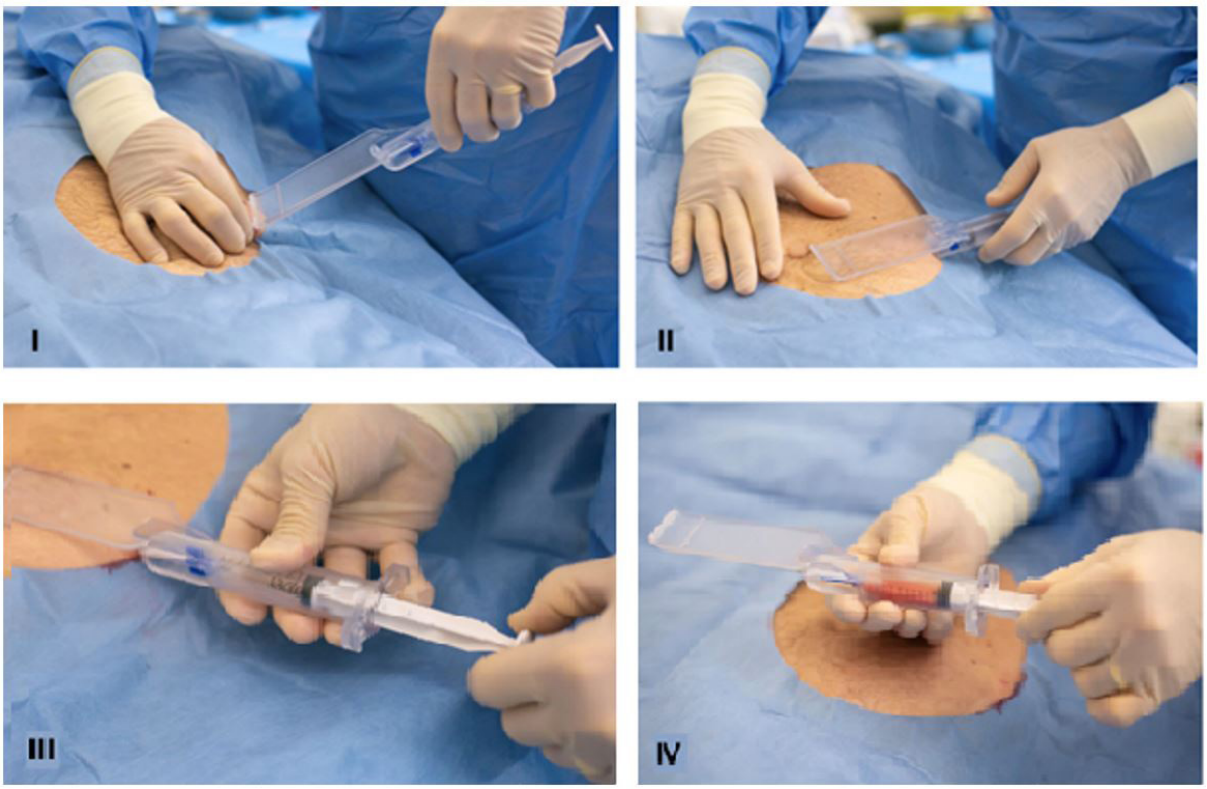

Figure 2: The harvesting procedures with cannulas with side port holes 0.8 mm and 1 mm were performed under local anesthesia. The guided procedure: I) Penetration of the tip of the cannula until the stop of the guide II) Rotation and insertion of the cannula in the subcutaneous tissue. During the procedure, the blade of the guide touches the skin III) The harvesting syringe with plunger lock IV) The fluid harvested tissue inside the syringe

The guide included in these devices was addressed to standardize the procedure and guarantee that the harvesting of tissue was performed in the subcutaneous plane adjacent to the dermis. Previous studies proved that the superficial adipose tissue (AST) is richer in SVF cells comparing with deep adipose tissue (DAT) [24,25].

The tissue harvested with the liposuction technique was performed with a cannula of 3 mm diameter it was administrated with Klein's tumescent solution and proceeded with aspiration after the infiltration. The harvesting procedure with liposuction cannula was performed with a standard no guided liposuction.

The tissue harvested with the first two cannulas was processed without any enzymatic digestion the tissue harvested with standard liposuction cannula was processed with a standard procedure consisting of enzymatic digestion (collagenase). The results of cell viability and phenotype of the cells harvested by the SEFFI methods were presented along with their comparison with the ones recorded after enzymatic digestion as a control.

Cell Viability

Authors Analysed Seven Samples: AT obtained using a 0.8 and 1 mm cannula was monitored for its cell viability using Presto Blue assay (Thermo Fisher scientific). 100 µl of cell suspension were plated in a 96 well plate and 10 µl of Presto blue was added to each well. For each individual and the two types of cannula, triplicate was run. The cell viability alterations were analysed via absorbance

spectroscopy and Authors reported the absorbance 570 nm after 10 min incubation (T0) at 37°C, through multi-mode microplate readers (VICTOR Multilabel plate reader, PerkinElemer). Cell viability was evaluated at T0 and after 72 h (T72), and graphed.

Adipose tissue harvested using Coleman cannula was processed by enzymatic treatment as quality control of tissue. The lipoaspirates were digested at 37°C in DMEM with a 0.25% weight per volume percent (w/v) collagenase type I (Sigma-Aldrich) and 1% fetal bovine serum (FBS) for 180 min at 37°C. Following digestion, Authors filtered the resulting suspension through a sterile 100 μm nylon mesh to remove undigested parts and centrifuged the remaining suspension at 1200 g for 10 min, to extract a high-density pellet, composed of the SVF. The SVF thus obtained was re-suspended in 1 ml DMEM-low glucose supplemented with 10% FBS and 1%penicillin-streptomycin (all Gibco, Thermo Fisher Scientific) and cells were counted by Crystal violet to exclude a-nucleated cells. Cells were then plated at a density of 10,000 cells/cm^2 in a 96 well plate in growth medium with the addition of 10% of Presto blue and stored in a humidified incubator at 37°C with 5% CO_2. Cell viability was assessed after 10 minutes (T0) and after 72 hours (T72).

Phenotypic Analysis

Five samples were analysed to determine the phenotype of adipose derived cells. Adipose tissue was processed as follow:

The oily part of lipoaspirate tissue from 0.8 and 1 mm derived AT was aspirated and discarded. Mechanically digested tissue was resuspended and plated in a culture tissue flask at a density of 0.5 ml tissue/10 cm^2 in an expansion medium consisted of DMEM low glucose, 10% FBS and 1% penicillin and streptomycin (all Gibco, Thermo Fisher Scientific)

Adipose tissue harvested using Coleman cannula was processed by enzymatic treatment as quality control of tissue. After digestion, the SVF obtained was re-suspended in 1 ml of an expansion medium and cells were counted by Crystal violet to exclude a-nucleated cells. Cells were then plated in a tissue culture dish at a density of 5000 cells/cm^2.

For both conditions, directly plated and enzymatic digested, once cells were attached to the plate, fresh medium was replaced. When cells reached confluence, cells were expanded at a cell density of 5,000 cells/cm^2 and the medium was replaced. Cells were expanded until they reached at least one million before staining. Cells were incubated with antibodies anti human against CD90-FITC, CD73-PeCy7, CD44-PE, CD31-PECy5, CD235a- PECy7, CD34-FITC, CD45-FITC and CD146-FITC and read using the flow cytometer s3e Cell Sorter, BioRad. Results were analysed using the software Flow Jo.

Results

Cell Viability

Lipoaspirate tissue derived from all samples (0.8, 1 mm and enzymatic digestion) proved to be vital and to present viable cells. Average absorbance was similar immediately after plating (T0) and after 72 hours for the two cannulas, 0.8 and 1 mm cannula. The tissue harvested with the two systems proved to have the same vitality rate. An increase of the cell viability was observed in all samples, for each condition (0.8, 1 mm and enzymatic digestion). The increase in the absorbance signal was related to an increase in metabolic activity of cells, meaning that the adipose tissue and released cells (mesenchymal, pericytes and cell from the immuno system) were vital and proliferate during the 72 hours of incubation.

The cell viability of adipose tissue harvested using a cannula of 0.8 mm side port holes, after 72 h of incubation, resulted to be statistically more vital, meaning that released cells from the harvesting procedure and cells entrapped in the extracellular matrix were vital and metabolically active (t-test student, *** $p<0.001$) (Figure 3).

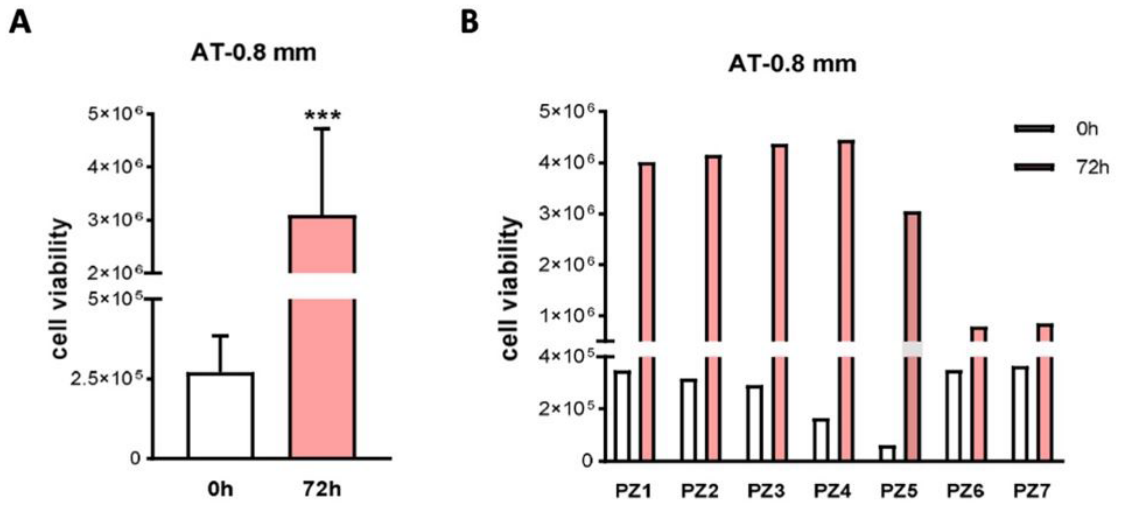

Figure 3: Cell viability of adipose tissue harvested using cannula of 0.8 mm side port holes. (A) Average absorbance value was represented as an average for time point 0, and after 72 hours of incubation. After 72 hours, adipose tissue resulted statistically more vital, meaning that released cells from the harvesting procedure and cells entrapped in the extracellular matrix were vital and metabolically active (t-test student, *** $p<0.001$). (B) Results for each patient (PZ) (from patient 1 to patient 7). Patient 6 and 7 showed a lower level of cell viability after 72 hours compared to the other five individuals

The cell viability of adipose tissue harvested using cannula of 1 mm side port holes after 72 hours of incubation, resulted statistically more vital, meaning that released cells from the harvesting procedure and cells entrapped in the extracellular matrix were vital and metabolically active (t-test student, ** $p<0.01$) (Figure 4).

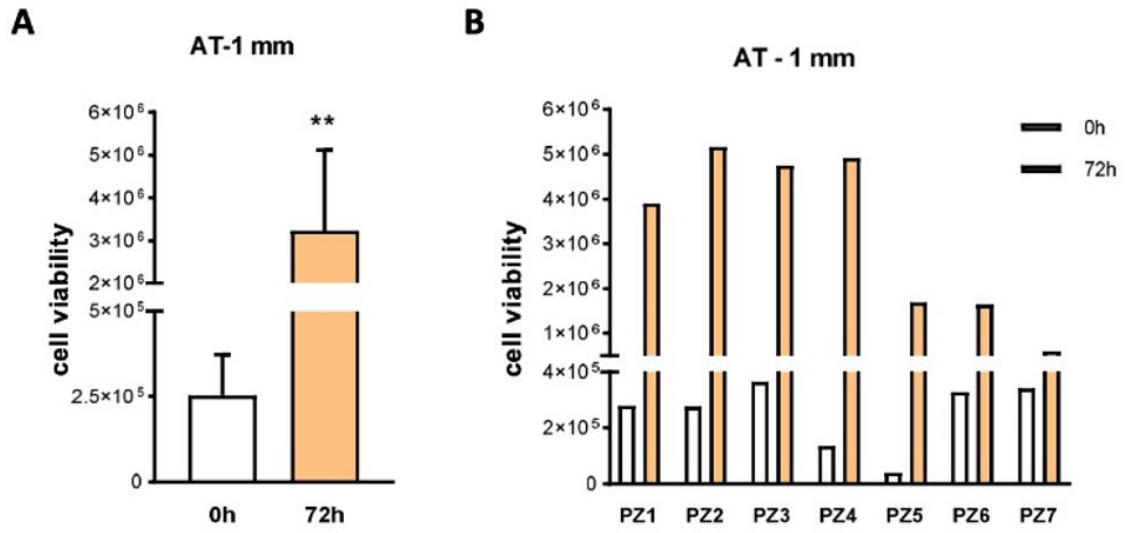

Figure 4: Cell viability of adipose tissue harvested using cannula of 1 mm side port holes. (A) Average absorbance value is represented as an average for time point 0 and after 72 hours of incubation. After 72 hours, adipose tissue resulted statistically more vital, meaning that released cells from the harvesting procedure and cells entrapped in the extracellular matrix were vital and metabolically active (t-test student, ** $p<0.01$). (B) Results for each patient (PZ) (from patient 1 to patient 7). Patient 5, 6 and 7 showed a lower level of cell viability after 72 hours compared to the other 4 individuals

Regarding the cell viability of AT harvested using liposuction cannula and enzymatically digested (SVF), after 72 hours, absorbance of SVF cells resulted statistically more metabolically active and proliferative compared to time 0 (t-test student, **** p<0.0001) (Figure 5).

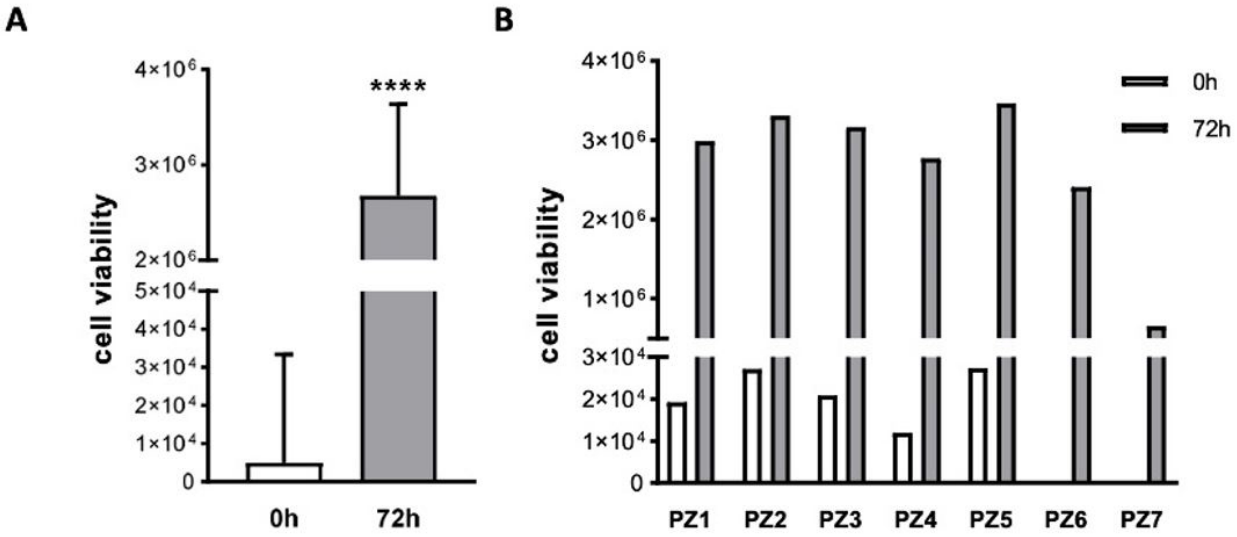

Figure 5: Cell viability of SVF. SVF was derived by enzymatic treatment of adipose tissue harvested using a Coleman cannula and 10,000 cells/cm^2 were plated in a 96 well plate for analysis. (A) Average absorbance value is represented as an average for time point 0, and after 72 hours of incubation. (B) Results for each patient (PZ) (from patient 1 to patient 7). Only PZ7 resulted to express a lower level of cell viability after 72 hours compared to the other four individuals

Absorbance at time 0 was higher in tissue compared to isolated SVF. However, the absorbance after 72 hours was similar between the two cannulas and the enzymatic digestion method. Statistics were run compared to the time 0 of each group (t test) (Figure 6)

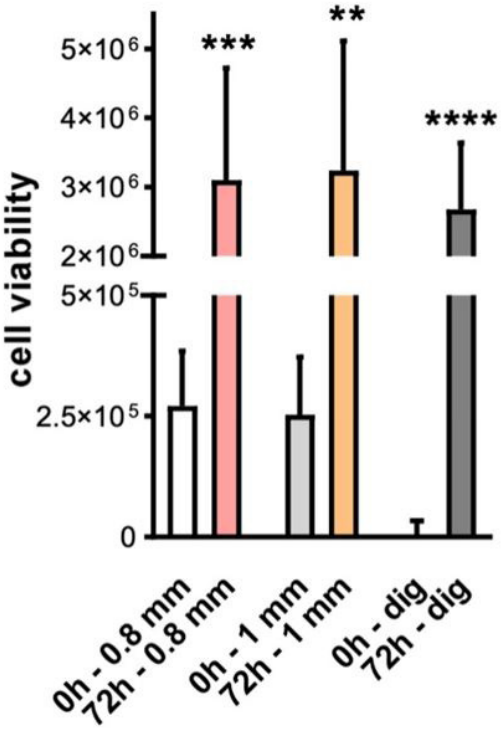

Figure 6: Cell viability of adipose tissue and stromal vascular fraction (SVF) from seven individuals for time point 0, and after 72 hours of incubation adipose tissue harvested with the three different cannulas

Mesenchymal Properties

Cells from all samples were able to adhere to a plastic surface and could be expanded to obtain the right number of cells for the staining. Morphologically, cells harvested by different cannula or isolated by enzymatic digestion showed differences in their morphology. They appeared as fibroblast proving their mesenchymal phenotype. Moreover, the adipose derived MSCs proved their heterogeneity among individuals because differences were noted between 0.8 mm and 1 mm derived cells from different donors (Figure 7)

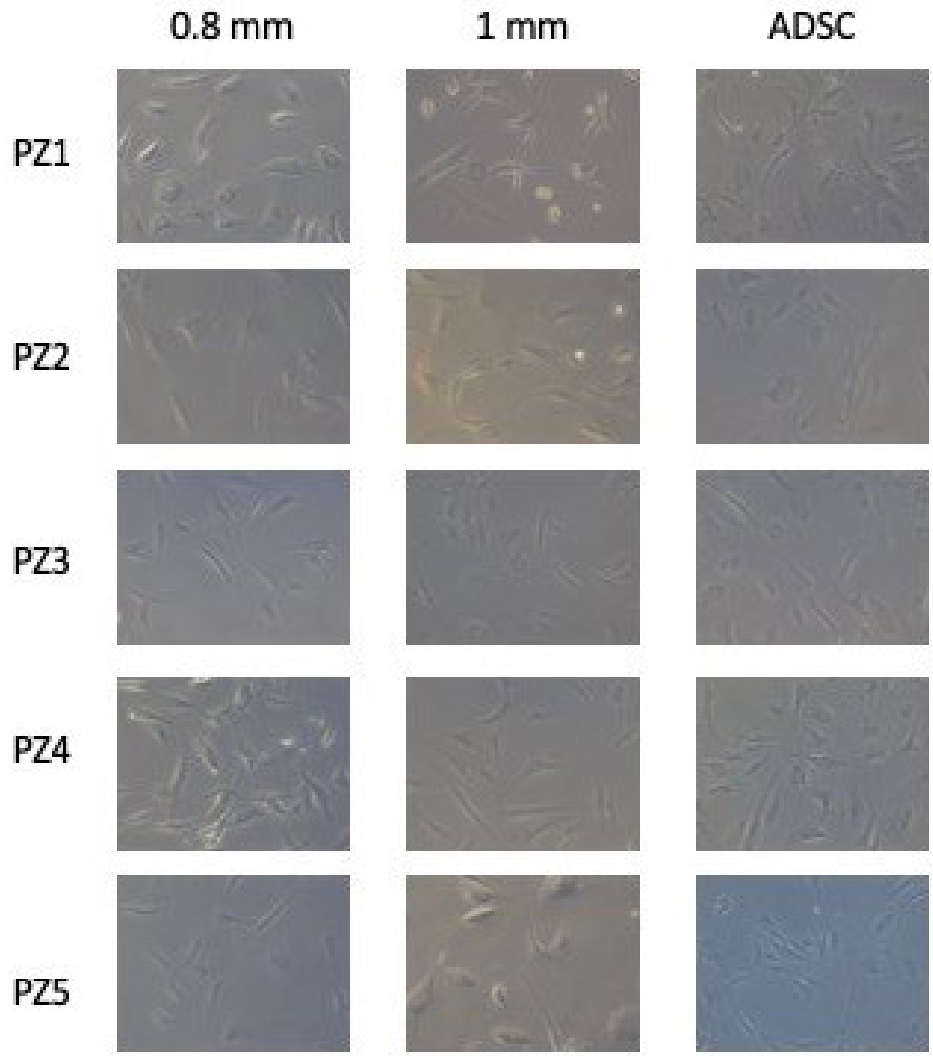

Figure 7: Morphology of adipose tissue derived cells harvested using 0.8 and 1 mm cannula and by enzymatic digestion (ADSC) for each patient (PZ) (PZ1-PZ5). Images were captured using a Leica light microscope and a 10X magnification

Flow cytometry analysis proved that cells are mesenchymal cells as they resulted positive for mesenchymal markers CD90 and CD44, poorly positive for CD73 and negative for CD146 and for the endothelial markers CD31 and hematopoietic markers CD34 and CD45. CD44 was highly expressed, around 100%, in all samples from the two cannulas and in enzymatically digested tissue (ADSC). CD73 was poorly expressed and interestingly, there was the difference of CD90 expression in cells derived by the three methods: enzymatically digested cells (ADSCs) were strongly positive followed by the 1 mm derived cells and the 0.8 mm derived cells showing the lower expression of CD90. The difference between the three groups was significantly different (Two-way ANOVA). The negativity for the other markers means the absence of hematopoietic and endothelial progenitors' cells in the cell culture. (Figure 8).

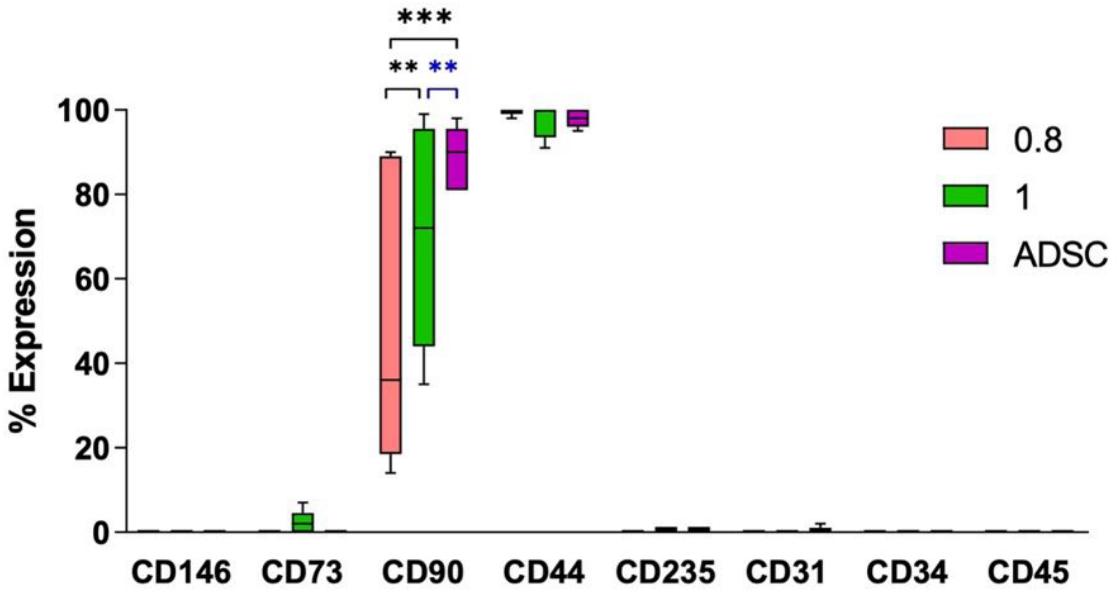

Figure 8: Phenotype of cells derived by adipose tissue harvested using 0.8 and 1 mm cannula and by enzymatic digestion. Two-way ANOVA statistical analysis was performed (** p<0.01 *** p<0.001)

This result showed a great variety between donors and how this is depending on the harvesting procedure and culture methods. The following graphs showed the diversity of expression for each individual. This is in agreement with the knowledge of cells variability between individual, age, sex and anatomical locations (Figure 9).

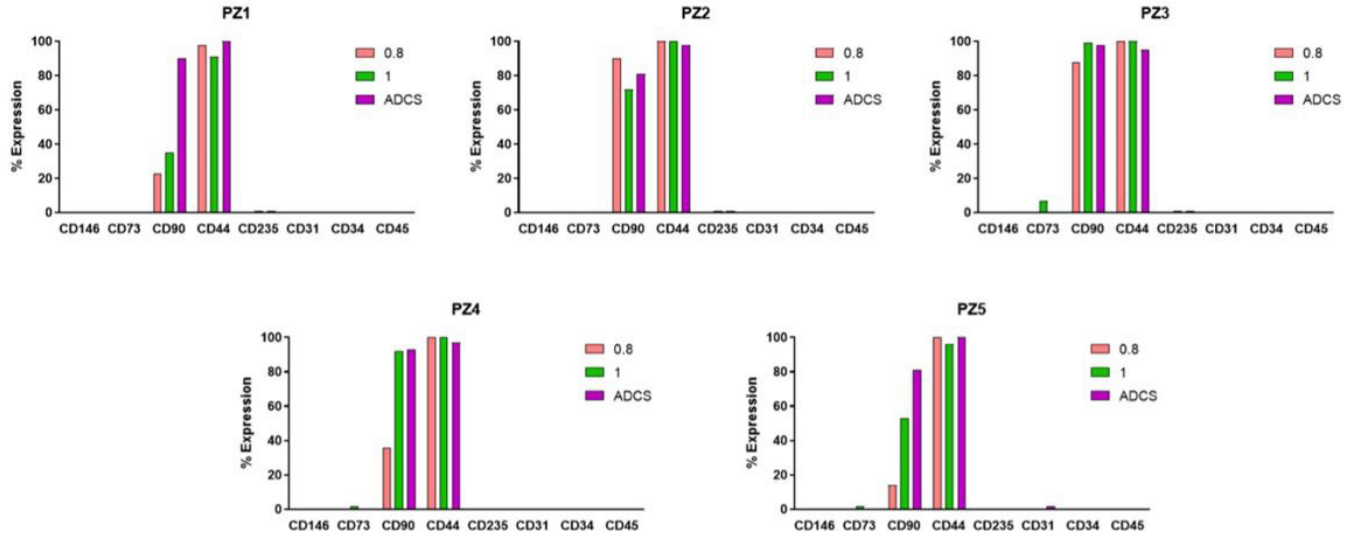

Figure 9: Expression of markers by flow cytometry for each individual. Samples were analysed to determine the phenotype. Cells were incubated with antibodies anti human against CD90-FITC, CD73-PeCy7, CD44-PE, CD31-PECy5, CD235a-PECy7, CD34-FITC, CD45-FITC and CD146-FITC and read using the flow cytometer s3e Cell Sorter, BioRad

Discussion

The results proved the vitality of adipose tissue harvested using the 0.8 and 1 mm side port holes cannulas, as the harvested cells showed to be vital and the values were comparable to the cell isolated from the SVF by enzymatic digestion, which is the gold standard procedure to obtain adipose tissue derived cells. In order to be defined as mesenchymal stem cells, they need to adhere to a plastic surface and to express specific surface antigens, especially CD44, CD90 and CD73 [26]. Cell viability was confirmed by the ability of plastic adhesion. Cells derived from each individual were able to attach and proliferate for at least three passages in culture and show the typical fibroblastic morphology. The differences in morphology and markers expression among cannulas agree with the selection of different size clusters and release of cells entrapped in the tissue based on cannula's port-holes size. Moreover, inter-individuality is a variable that need to be taken into account. SVF is a very heterogenous population, and it contains MSCs, fibroblasts, endothelial cells, pericytes, adipocytes and cells from the immune system [27] Therefore, in the attachment, differences can be seen. Differences were seen also on the surface markers expression. Cells were all positive for the CD44 which binds several ligands including hyaluronic acid, osteopontin, chondroitin, collagen, and fibronectin. CD44 is involved in cell proliferation, adhesion, migration, haematopoiesis, and lymphocyte activation. CD90 expression was lower in two individuals. It was shown that CD90 marks a rare adventitial population and human adventitial $CD90^{+}$ cells fulfilled standard MSC criteria, including plastic adherence, spindle morphology, passage ability, colony formation, and differentiation into adipocytes, osteoblasts, and chondrocytes [28]. The other mesenchymal marker tested, CD73, a glycoprotein that acts as an enzyme involved in signal transduction and modulate a variety of biological effects [29]. CD73 also has non-hydrolase function, which is also a signal and adhesion molecule that regulates cell- extracellular matrix interaction [30]. However, the role of CD73 in regeneration therapy of MSCs is rather limited. It was shown how the morphological subset of flat cells lacked CD73 [31] and its expression can be significant different in MSCs from different sources [32].

This observational study was conducted under the Declaration of Helsinki's guidelines and its amendements. Before entering the study, all patients received detailed information regarding the procedure, purpose, and investigation's objective and provided written consent for participation and publication of data obtained.

Conclusion

The presented data shows that adipose tissue harvested with small diameter cannulas with small diameter side port holes are vital and viable cells can be derived from it. These cells are an heterogenous population and they show mesenchymal stem cells properties. This study proved the quality in terms of vitality and stemness of the Superficial Adipose Tissue (SAT) guided-harvested with small cannulas (2 mm diameter) and small side port holes (0.8 and 1 mm) without any substantial manipulation comparing with the SAT and Deep Adipose Tissue (DAT) harvested with a liposuction cannula and enzymatically processed the evidences of this study is promising for minimally invasive guided regenerative procedures with minimally manipulation of the adipose tissue. Of course, the main limitation of this study is the small number of patients enrolled, further larger trials will be needed to confirm the results of this pilot study.

Conflict of Interest

Alessandro Gennai is scientific director of Seffiline Academy , founder and CEO of Seffiline other Authors declare no conflict of interest.

Appendix

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