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In vitro Isolation and Characterization of Mesenchymal Stem Cells (MSCs) from Lipoaspirate: a new Preclinical Protocol

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Abstract

Introduction

Mesenchymal Stem Stem Cells (MSCs) could provide alternative therapeutic solutions for a number of bone and cartilage tissue diseases, playing an important role in regenerative medicine. Several sources of stem cells are currently available. In recent years, researchers attention was focused on the isolation of Adipose Stem Cells (ASCs) because they are easily obtainable in large quantity trough a minimally invasive procedure. Furthermore, Adipose Derived Stem Cells (ADSCs) have multiple properties, including self-renewal, differentiation potential, secretion of active factors that might influence surrounding cells and immune modulatory effects useful in the treatment of several degenerative diseases. The aim of this study was to present a new "in vitro" ADSCs isolation and characterization protocol using a mechanical method in comparison to the enzymatic digestion that has represented the gold standard procedure to isolate adult mesenchymal stem cells.

Methods

Lipoaspirate from forty human donors were obtained by minimally invasive liposuction procedures: by definition, lipoaspirate contains a relatively high content of ASCs (1-5% of isolated nucleated cells). A minimum starting volume of 30 mL of lipoaspirate was obtained in all procedures for a sufficient yield of mesenchymal stem cells (MSCs). Lipoaspirate was stored at 2-8º C for a maximum of 24 hours before use. Tissue from each donor was split in two samples and each sample was treated by two different in vitro procedures with the goal to isolate ADSCs: enzymatic digestion and experimental mechanical isolation procedure. Tissue from both methods were first diluted with an equal volume of Phosphate-Buffered Saline (PBS), centrifuged at 500xg at 22° for 5 minutes, digested adding 1 ml of collagenase digestion solution for every 1 ml volume of tissue, incubated at 37ºC in a pre-warmed orbital shaker for 30 minutes, centrifuged at 22°C for 5 minutes at 500xg and finally suspended for expansion in alpha-MEM medium adding 10% of bovine fetal serum. Subsequently, MSCs were characterized following the guidelines of the Society for Cellular Therapy (SCT).

Results

Characterization of the processed tissue was performed after digestion and *"in vitro"* culture expansion of plastic-adherent cells. Our results showed that cells from both study groups were adherent to the plastic having fibroblast morphology. The cells were immune-characterized by Flow cytometry. Results showed a phenotypic mutation during the process of cellular expansion, from the isolation of the stromal vascular fraction to the selection of a purely population with mesenchymal stem cells properties in the subsequent passages. After the isolation of MSCs, the obtained ADSC showed a subpopulation (immuno phenotype differentiation) expressing specific markers such us CD 34, a marker associated with the state of vascular and endothelial progenitor, and CD 146, a pericytic markers. Finally, the evaluation of the cells differentiation potential showed that ADSCs, under controlled condition, were able to differentiate into adipogenic, chondrogenic and osteogenic cells. The results were similar for both differential methods.

Conclusion

Our experience suggests that ADSCs enzymatically and mechanically isolated from Lipoaspirate respond to the three criteria of the Society for Cellular Therapy (SCT) and, for this reason, can be considered as MSCs. New methods to treat fat tissue and obtain ADSCs, as the mechanical method described in

this article, can be safely developed to isolate in an easier way these promising cells. Therefore, improved characterization protocols are still necessary in order to make a final population of ADSCs as an homogeneous cellular product ready for clinical application in the field of osteogenic and chondrogenic tissue engineering. Finally, more studies are necessary to understand the immunomodulatory and anti-inflammatory properties of ADSCs before clinical applications to treat different orthopaedic pathologies.

Keywords: Mesenchymal Stem Cells; Adipose Derived Stem Cells; MSC; ADSC; Lipoaspirate; Protocol

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Introduction

Several conditions in Orthopaedics and Traumatology, such as osteoarthritis or fractures nonunion, remain a challenging clinical problem for the lack of an effective repair strategies leading to severe patient dysfunction [1, 2]. Although new techniques and biomaterials have enhanced the treatment options favoring satisfactory outcomes, bone and cartilage repair strategies are not universally recognized yet [3]. Research in different fields, such as stem cells biology, tissue engineering and regenerative medicine, have provided further insight relevant to musculoskeletal tissue management [4, 5]. The use of stem cells to repair and regenerate various tissues and organs may provide an alternative therapeutic solution for a number of diseases, due to their self renew capacity and differentiation potential when cultured under specific biochemical and mechanical stimulus [6, 7]. Furthermore, Mesenchymal Stem Cells (MSCs) seem to migrate into damaged tissue through homing or under the influence of chemokines, farther be able to secrete active factors, growth factors and angiogenic factors [8, 9]. Finally, MSCs hold immunomodulatory properties that can play a role in the pathogenesis of the uncontrolled inflammatory response to enhance bone repair and to prevent arthritis [10-12]. Bone Marrow Stromal Cells (BMSCs) have been used as popular cell source of Mesenchymal Stem Cells (MSCs) [13-15]. However, harvesting of BMSCs is a painful and invasive procedure and frequently yields a low number of stem cells. In contrast, Adipose Derived Stem Cells (ADSCs) are easy to obtain through minimally invasive procedures, like liposuction, which causes less patient discomfort and donor site morbidity [16]. The subcutaneous adipose tissue is also abundant and easily accessible and small amounts of adipose tissue yield a greater number of stem cells, approximately $5x10^3$ stem cells more than the number of MSCs available from bone marrow aspiration [17]. Besides this, ADSCs possess "stemness" properties: self renew potential, expression of stem cell surface markers and multiple cell lineage pathways in an adjustable and reproducible manner [18, 19]. ADSCs can be also safety transplanted to either autologous or allogenic hosts and can be manufactured in accordance with current good manufacturing practice guidelines [7]. For these reasons, adipose derived stem cells are considered a valid and efficient alternative source of mesenchymal stem cells in humans [20-26]. The traditional method of isolating stem cells from Lipoaspirate includes enzymatic digestion that represent, to date, the gold standard procedure. On the other hand, this technique requires the manipulation of the tissue in an available laboratory, usually outside the medical institution where the surgical procedure has been performed, in contrast with few international laws regarding the implant of the biological products in humans. Recently, new approaches have been investigated to avoid this "away processing pathway" and the aim of this study is to define an alternative perioperative protocol for *ADSCs* isolation and characterization.

Materials and Methods

Lipoaspirate from forty human donors was harvested during elective plastic surgery from subcutaneous fat through a minimally invasive procedure under local anesthesia (500 mL of saline water, 40 mL of Lidocaine 2%, 1 mL/mL Epinephrine 1:1000) after informed consent was obtained from all study subjects. The harvesting location was limited to abdomen and breast to allow for valid comparisons between tissue donors. The average donors age was 40 years (range 20 to 60 years). All donors were healthy volunteers with BMI <35. Tissues from each donor were split in two samples and treated each by two different procedures: 1) Enzymatic digestion; 2) Experimental mechanical procedure. Both samples have been characterized *in vitro* following harvesting.

Enzymatic Digestion

A portion of 10 mL of tissue from lipoaspirate was diluted with sterile Phosphate-Buffered-Saline solution (PBS-Invitrogen) containing 1% of penicillin-Streptomycin 100x (Invitrogen) and centrifuged at 500 xg at 22°C for 5 minutes. The centrifugation was repeated three times. The medium was collected with a needle, first removing the floating adipose tissue and then adding 1 mL of digestive media type IV Collagenase from Clostridium Histolyticum (Sigma) for every 1 mL of volume tissue; then it was placed in isotonic water bath for 40 minutes at 37° C; gentle shaking was applied. Enzyme activity was neutralized with alpha-MEM media (Invitrogen) supplemented with 20% of fetal bovine serum (FBS Thermo Scientific), Glutamax 100x (Invitrogen), Penicillin-Streptomycin 100x (Invitrogen) and then all cells were centrifuged at 500 xg at 22°C for 5 minutes. The digested tissue was filtered using a 50µM strainer to remove debris and larger adipocytes. The filtered cells were washed and the supernatant was aspirated without disturbing the pellet. Pellet was re-suspended in RBC lysis buffer 10x (BioLegend), placed on ice for 10 minutes in a dark environment, washed again and re-suspended in alpha-MEM (Invitrogen) containing 20% of fetal-bovine-serum (FBS-Thermo Scientific), Glutamax 100x (Invitrogen), Penicillin-Streptomycin (Invitrogen) and finally placed into T-25 flasks for culture. An aliquot of 85µL from each sample was diluted in IT-stain (1:100-Invitrogen) and 5µM of DRAQ-5 (Alexis) to perform a cell count and viability using the Accuri Cytometer. Following twenty-four hours of incubation, non-adherent cells were washed out and the cultures were maintained at 37°C with 5% CO_2. The medium was changed every three days and the cells were harvested and split when cells reach 80% confluence. The cells were finally expanded over three passages and then subjected to the characterization *in vitro*.

Mechanical System

The experimental system was composed of a sterile closed chamber containing saline water, small murbles and two different screens, one for every side of the chamber, which was connected to the saline water and collected bag on one side and waste bag on the other side. The mechanical device was placed into a Biological Safety Cabinet and 100 mL of Lipoaspirate was transferred into it passing through the first screen of the chamber. Then, gentle shaking movements were applied to wash the tissue, remove debris and red blood cells and to emulsify the oil of the adipose tissue released by the murbles. After 10 minutes of washing with saline water, the residual product was collected in the appropriate bag and pushed it through the second screen to obtain more manageable small pieces of cleaned tissue. From this product, 10 mL of sample was collected and digested following the aforementioned protocol to characterize the product obtained from the mechanical procedure *in vitro* in the same manner of the first sample. Cell counting was performed using Accury Cytometer and the cells were cultured, washed and expanded in the same manner of the cells from the Enzymatic digestion.

Characterization of Adipose Derived Stem Cells (ADSCs)

In order to demonstrate that cells isolated from both techniques, enzymatic digestion and mechanical procedure, were Adipose Derived Stem Cells (ADSCs) the Authors of the current study followed the International Society of Cells Therapy (ISCT) guidelines for definition of Mesenchymal Stromal Cells (MSCs). This classification includes three criteria: cells must be adherent to plastic, having multipotent differentiation potential and having specific surface Antigen (Ag) expression [27]. At defined steps, cells isolated from lipoaspirate treated by enzymatic digestion and mechanical system were collected and submitted to an identical analysis following the same protocol to reduce every variable that could influence the result in order to obtain comparable results.

In vitro Differentiation Assay

Cells obtained from enzymatic digestion and mechanical system, were first cultured in three different passages, then cultured one more time using three different media and finally analyzed by microscopy. To detect adipogenesis, MSCs were collected and cultured in adipogenic medium which consisted of Dulbecco‘s modified Eagle Medium High Glucose (DMEM-HG, Invitrogen) with 10% of fetal bovine serum (FBS, Invitrogen), 200 µM Indometacin (Gibco), 0,05 mg/L Human Insulin (Invitrogen), 5µM 3-isolbutyl-1-methylxanthine and 1 µM Dexamethasone (Sigma); cells were cultured for a two weeks period. The adipogenic cultures were fixed in 4% paraformaldehyde (Sigma) and then stained with fresh Oil-red-O solution (Sigma) and subsequently with the Harris Hematoxylin solution (Gibco). To detect osteogenesis, MSCs were cultured in osteogenesis medium composed of Dulbecco‘s modified Eagle Medium High Glucose (DMEM-HG, Invitrogen), 10% of fetal bovine serum (FB Thermo Scientific S-), 100 nM Dexamethasone (Sigma), 0,05 mM ascorbic acid (Sigma) and 10 mM

β- glycerolsphosphate (Sigma) for a total of 21 days. The osteogenic cultures were first fixed in 4% paraformaldehyde (Sigma) and then stained with fresh Alizarin Red (Sigma). To detect chondrogenesis, the micropellet of MSCs was cultured with chondrogenic medium which consisted of Dulbecco 's modified Eagle Medium High Glucose (DMEM-HG, Invitrogen), 10^7 M Dexamethasone (Sigma), 10^4 M Ascorbic Acid (Sigma), mM Sodium Pyruvate (Gibco), non essential amminoacids (ITS 1x-Thermo Fisher Scientific), 4,7 µg mL^{-1} Linoleic Acid (Sigma), 80,5 µg mL^{-1} BSA (Thermo Fisher Scientific) and 10 µg mL^{-1} Trasforming Growht Factors β_3 (eBioscience) for a total of 21 days. For analysis, the pellets were embedded in 4% paraformaldehyde (Sigma) and then stained with Alcian Blue (Gibco).

Immunophenotype Characterization

Flow cytometry was performed on cells obtained from both procedures immediately after the isolation of the cells and at the 1st, 3rd, 5th, 7th passage of culture. An amount of $2x10^6$ of freshly isolated cells from enzymatic and mechanical samples were washed with phosphate-buffered-saline (PBS, Invitrogen) and centrifuged for 5 minutes at 500xg with a temperature of 22°C. Pellets were collected and re-suspended in phosphate-buffered-saline (PBS, Invitrogen) with 3% of fetal-bovine-serum (FBS, Invitrogen) and stained on ice in the dark room for 60 minutes with specific antibodies and then fixed with phosphate-buffered-saline (PBS, Invitrogen) with 1% of paraphormaldeide (Sigma). The antibodies used were: CD10 + PE-Cy7, CD44 + eFluor450, CD73 + PerCP-eFluor710, CD90 +FITC CD105 + PE, CD146 + Alexa647, HCA-DR + PC7, CD31+Biotin APC eFluor780, CD34 + PECY7, CD14 + PERCPS CY5.5, CD19 + PE, CD31 + Biotin Alexa Fluor 647, CD45+APC eFluor780, divided in two panels (panel 1, panel 2) with the corresponding isotype. Sample were acquired with the use of flow cytometer and analyzed with FACS. About $4x10^6$ of MSCs from enzymatic and mechanical samples were removed at each defined passages of culture with trypsin EDTA (Gibco). Enzymes were neutralized with alpha-MEM (Invitrogen) with 3% of fetal bovine serum (FBS, Invitrogen) and stained applying the same protocol of freshly isolated cells for all samples. Samples were acquired with the use of flow cytometer and analyzed with FACS software 2005 version in the same way.

Results

Cell isolation and Culture

After 24 hours from the initial culture, cells from each sample (enzymatic and mechanic), started to be adherent and by the end of the study week almost all of them were completely attached to the flask. Analysis with optical microscopy showed the morphology of the cells, having a fusiform and spindle-shaped appearance, typical of Mesenchymal Stem Cells (MSCs); these characteristics were kept in subsequent passages. Cells grew exponentially (1:3) and reach the 80% confluence every four days.

In vitro Differentiation

Differentiation potential was tested at the 3rd passage of culture in both samples, cells obtained by enzymatic digestion as well as by mechanical procedure (Figure 1 and 2). All samples showed multipotent differentiation capacity in at least three different cell lineages.

Figure 1: Analysis of optical microscopy of culture after the isolation of the cells digested enzymatically (A), and after 7days (B)

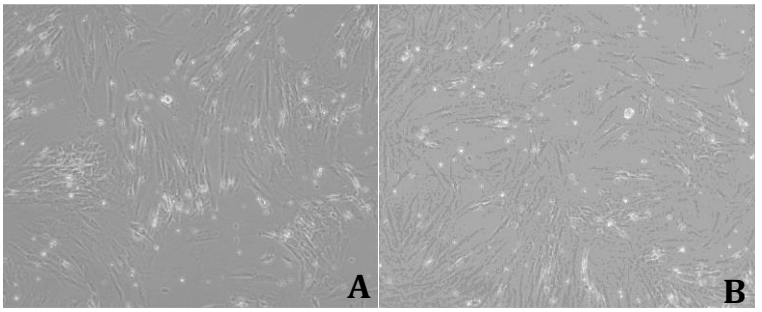
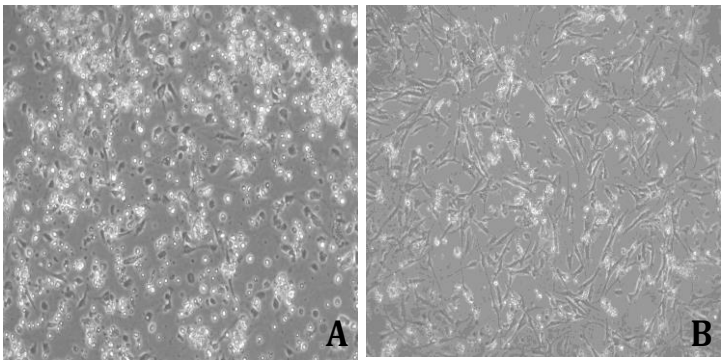


Figure 2: Analysis of optical microscopy at the 1st passage in culture of cells digested enzymatically (A), and processed by mechanical procedure (B).



Adipogenic Differentiation

Both samples were stimulated for 14 days by the use of adipogenic factors, as assessed by Oil Red O and Ematoxylin, and showed the typical intracytoplasmatic tryglicerids accumulation in comparison with the untreated cells (Figure 3 and 4).

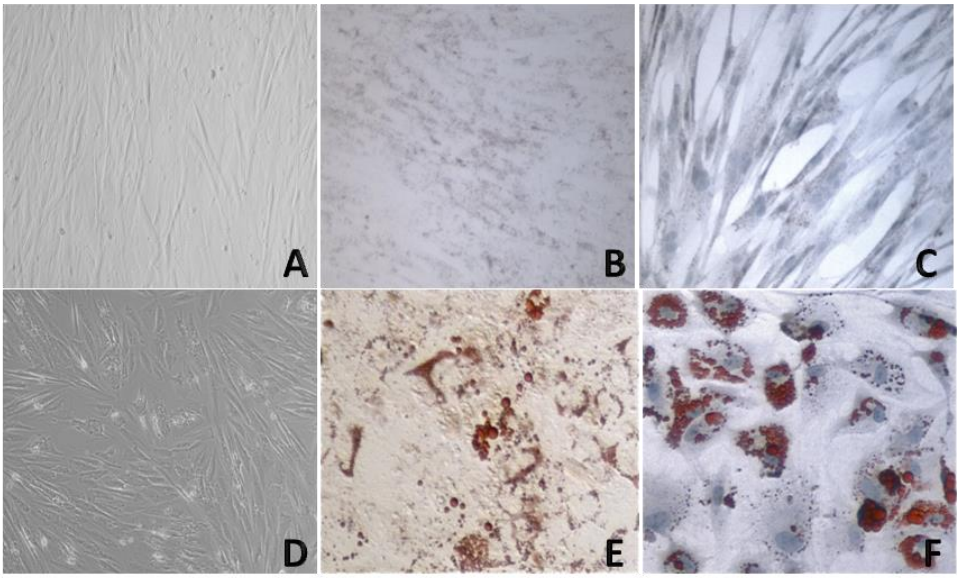


Figure 3. Cells treated **enzymatically**. **A-B-C** Negative control before the staining (A); after the staining with Oil Red O (B); and Ematoxylin (C). **Adipogenesis**. **(D-E-F)** Positive control before the staining (D); after the staining with Oil Red O (E); and Ematoxylin (F).

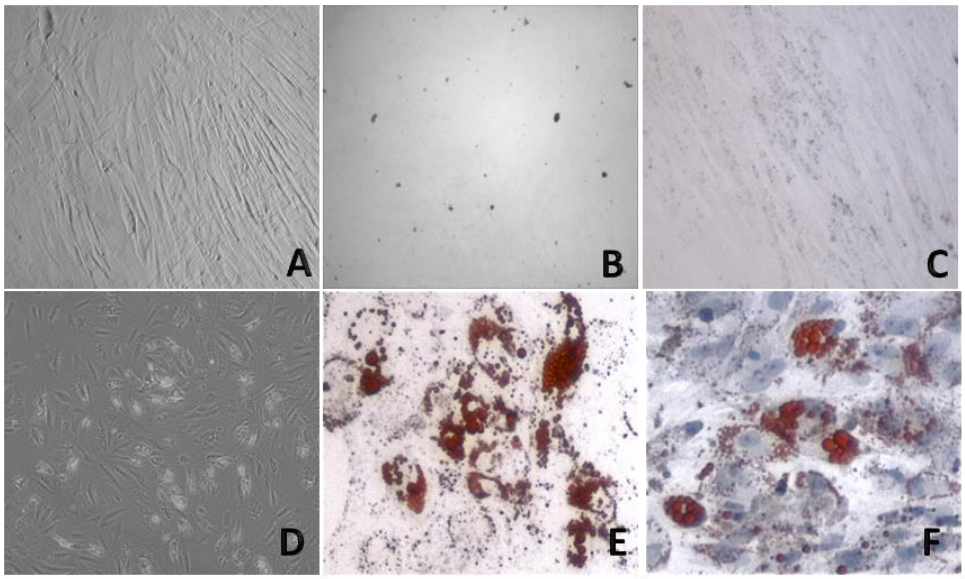


Figure 4. Cells treated **mechanically**. **A-B-C** Negative control before the staining (A); after the staining with Oil Red O (B); and Ematoxylin (C). **Adipogenesis**. **(D-E-F)** Positive control before the staining (D); after the staining with Oil Red O (E); and Ematoxylin (F).

Osteogenic Differentiation

Both samples were stimulated for 21 days by osteogenic factors, as assessed by Alizarin red S, and showed the deposition of Calcium in comparison with the untreated cells (Figure 5 and 6).

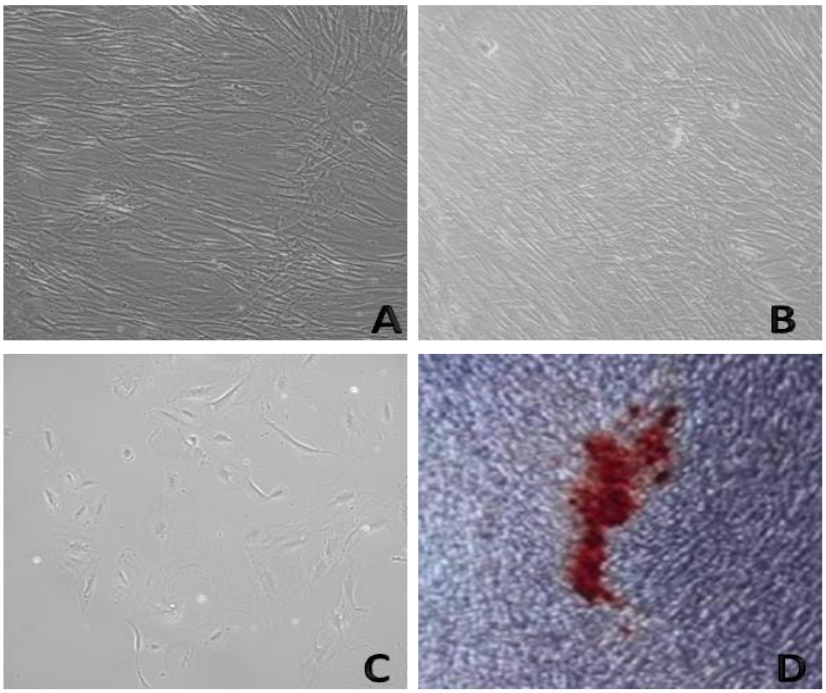


Figure 5. Cells treated **enzymatically**. **A-B** Negative control before the staining (A); after the staining withVon Kossa (B); **Osteogenesis (C-D) Positive** control before the staining (C); after the staining with Von Kossa (D).

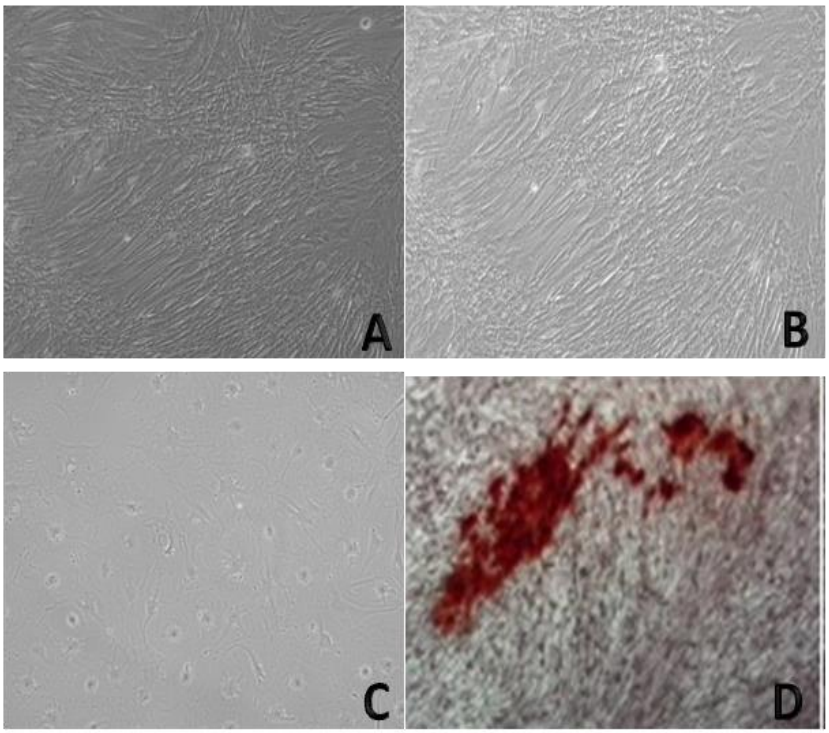


Figure 6. Cells treated **mechanically**. **A-B** Negative control before the staining (A); after the staining withVon Kossa (B); **Osteogenesis (C-D)Positive** control:before the staining (C); after the staining with Von Kossa (D).

Chondrogenic Differentiation

Both samples (enzymatic and mechanic) were stimulated for 21 days by the use of chondrogenic factors, as assessed by Alcian Blue, and showed the presence of proteoglycans in comparison with the untreated cells (Figure 7 and 8).

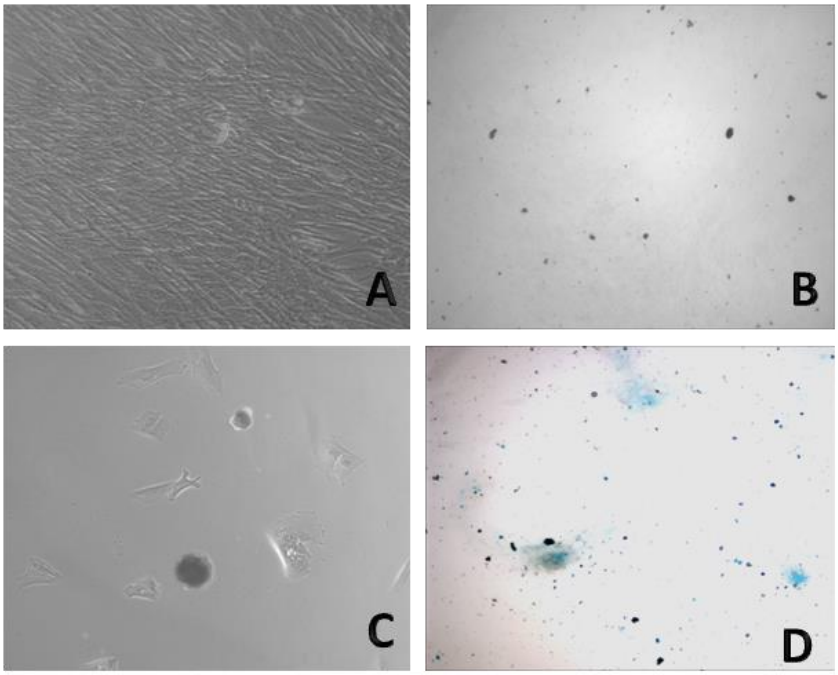


Figure 7 Cells treated **enzimatically**. **A-B** Negative control: before the staining (A); after staining with Alcian Blue (B). **Chondrogenesis (C-D)** Positivc Control: before the staining (C); after the staining with Alcian Blue (D).

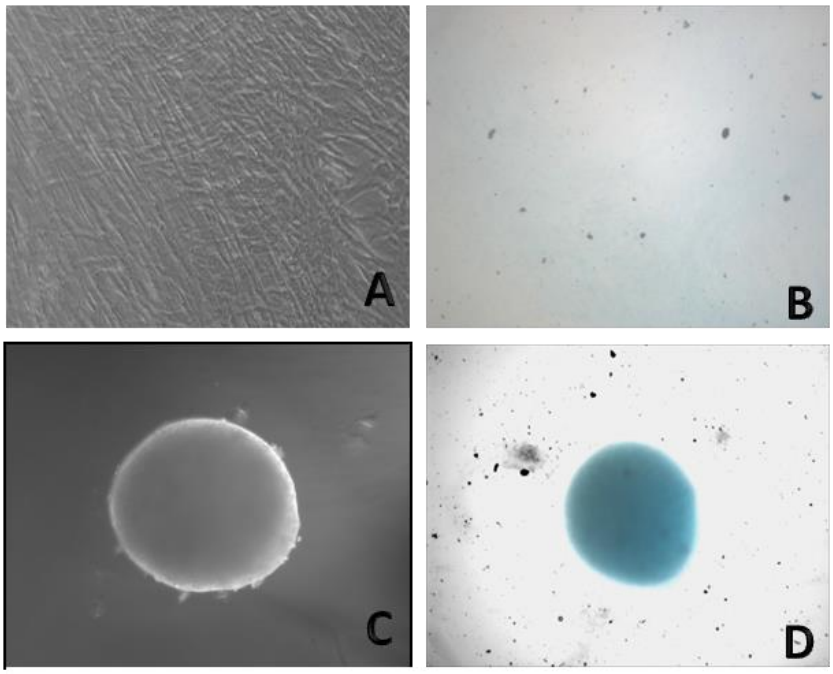


FigurE 8 Cells treated **mechanically**. **A-B** Negative control: before the staining (A); after staining with Alcian Blue (B). **Chondrogenesis (C-D)** Positivc Control: before the staining (C); after the staining with Alcian Blue (D).

Expression of Cells Surface Antigens

The cells isolated by enzymatic digestion and mechanical procedure were analyzed by flow cytometry to obtain a cell surface marker profile (Table 1). According to the Internationl Society for Cellular Therapy (ISCT), to define Mesenchymal stem cells (MSCs), cells have to express specific markers as CD90, CD75, CD44, CD73, CD146 and in the same time lack the presence of the non mesenchymal markers as CD34, CD19, CD14, CD45, CD31, HLA-DR.

Table 1: Panels of the antibodies for the charactherization of the surface markers

PANEL 1	CD 31	CD 34	CD 73	CD 90	CD 105	CD 146
PANEL 2	CD 14	CD 19	CD 45	CD 44	HLA-DR	

After the isolation of the tissue from donors, previous to the culture expansion, the cytometry of the tissue showed the prevalent expression of aspecific markers meaning the presence of an heterogeneous population composed of several different types of cells, such us machrophages, pericytes, linfocites and only a small population of stem cells. This aspecific cell population is routinally called Stromal Vascular Fraction (SVF). Instead, after the first passage in culture of the same cells, the immunophenotype showed an increase of specific mesenchymal markers tipical of MSCs, suggesting that the new cell population population was rich of mesenchymal stem cells that remained stable in all following passages (Figure 9).

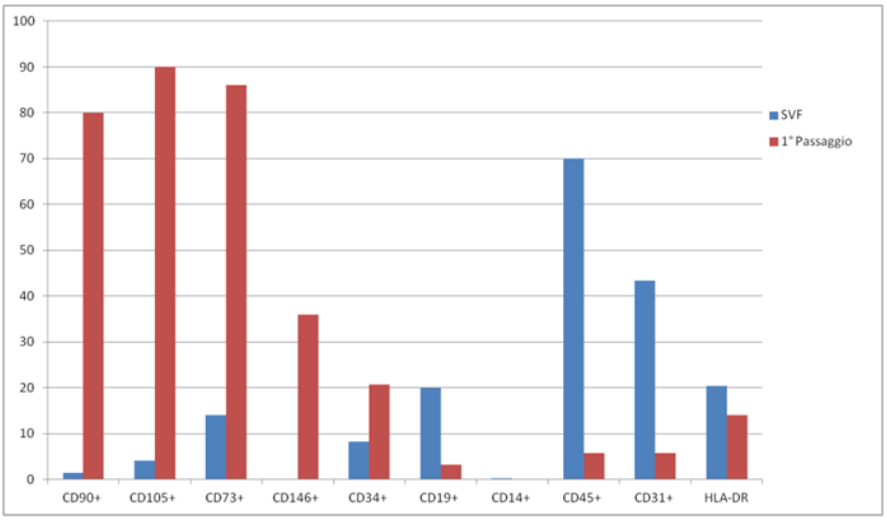


Figure 9: Surface immunophenotype. Immediately after the harvest of the tissue and the isolation of the cells without cells colture. The SVF (blue) is represented by an heterogeneous population and the mesenchymal markers are poorly exspressed. successivamente, after the first passage in colture (red) increase the expression of mesenchymal markers and decrease the expression of the restant markers.

Differences were noted between the samples from the two procedures in the Stromal Vascular Fraction (SVF), like the high expression of CD 34 in the sample treated by enzymatic digestion (Figure 10) and the low expression of the CD146 compared to the sample from the mechanical procedure (Figure 11 and 12) maybe depending to the different treatments. All the final samples have shown a co-expression of mesenchymal markers.

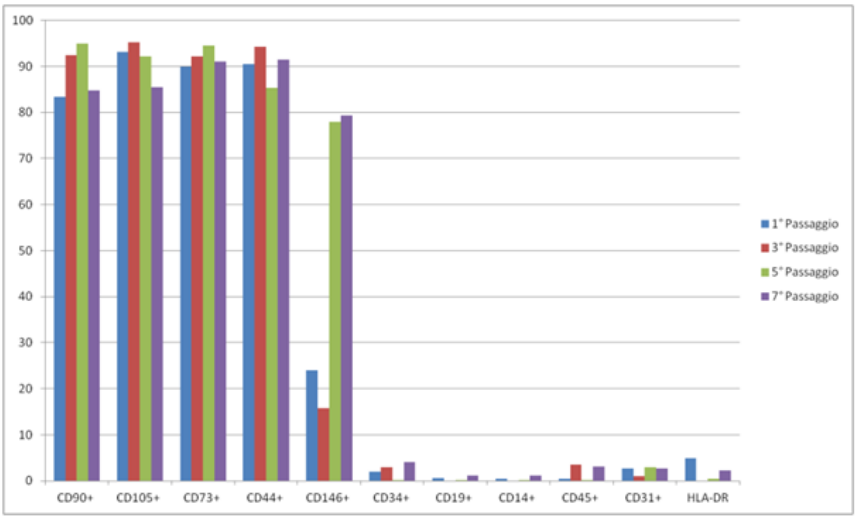


Figure 10: Surface immunophenotype of the lipoaspirate processed by enzymatic digestion. In the subsequent passages the immonophenotype showed un homogeneous mesenchymal population stable in the folowing passages.

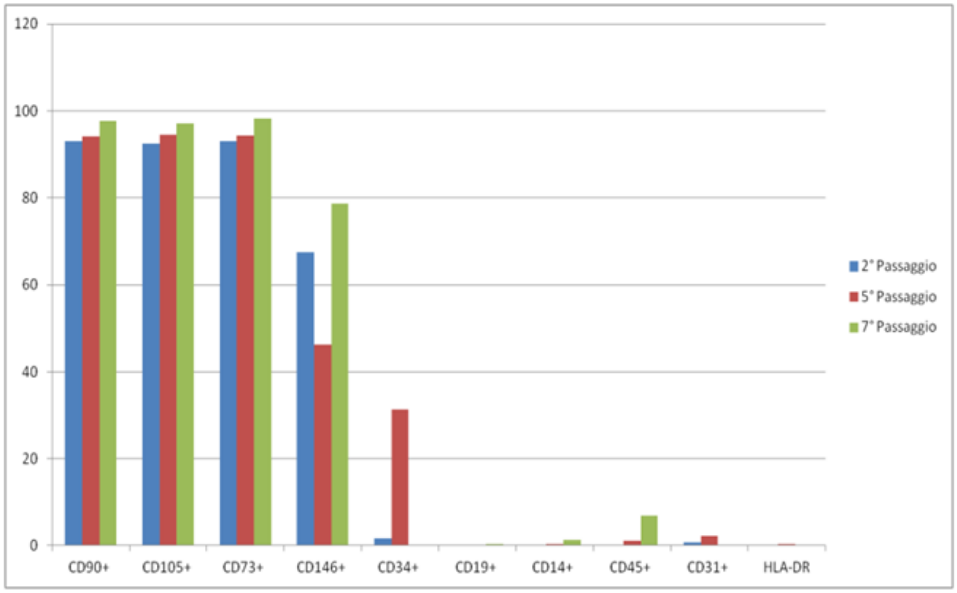


Figure 11: Surface immunophenotype of the Lipoaspirate processed through mechanical procedure. In the subsequent passages the immunophenotype showed un homogeneous mesenchymal population that stable for all the following pesages.

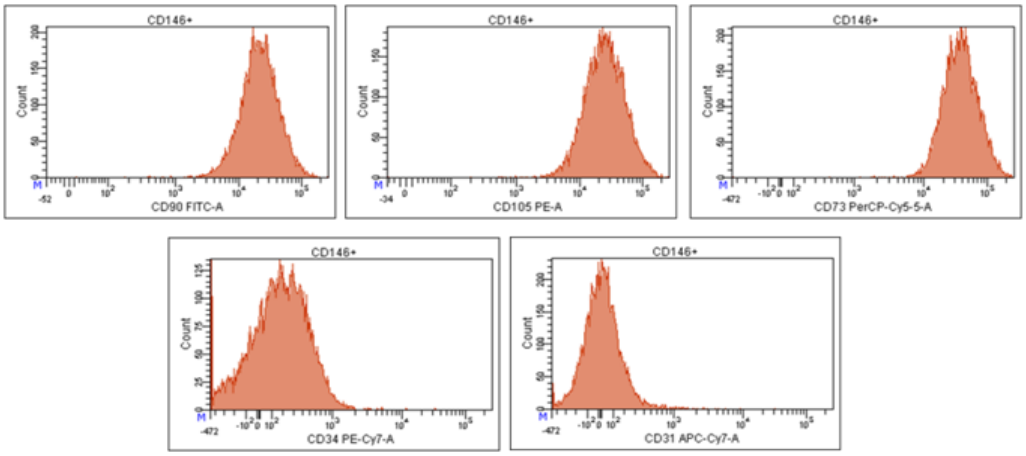


Figura 12 Sample of tissue treated mecchanically where was analyzed the positivity of co-expression of CD146 with the other mesenchymal markers.

Discussion

In this present study, we confirmed findings from previous studies showing that Adipose Tissue Derived stem cells could be considered an important alternative source of mesenchymal stem cells because easily accessible in a large quantity through mini invasive procedures, being, for this reason, practical for the surgeon and better tolerated by the patient. Moreover, according to the literature, they have all the properties of stemness: in fact, our cells responded to the three criteria of the Society for Cellular Therapy. After the isolation, cells processed by both procedures started to be adherent to the plastic of the culture tissue flask acquiring the spindle shape morphology of the mesenchymal stem cells and growing exponentially in many passages. An important finding of this study was to determine how much tissue needed to be processed, since the growth was faster with more viable cells isolated when the amount of processed tissue was superior of 5 mL. This concept suggests that, as expected, the quantity of cells isolated by both procedures could influence the clinical results: clinicians need to approximately know how many cells have been isolated and how viable are them before implantation in order to obtain clinical relevant effects. Cells obtained from the tissue processed mechanically were similar in number and characteristics to those of the enzymatic process, but a higher volume of important of adipose tissue was harvested and processed (60-100 mL) because part of the original tissue was discarded during the washing time with the debris and the oil. We directly demonstrated the plasticity of our cells stimulating them by the use of different medias obtaining, at the end, three different cell lineages: adipocytes, osteoblasts and especially chondrocytes. The cells treated by mechanical system differentiated in those three different lineages without any problems. Our findings reinforced the idea to use tissue treated without non-human enzymes and inject it directly in the donor site to stimulate the biology of the resident cells and their anti-inflammatory properties: this appeared to be a safe procedure, not in contrast with the current regulations regarding the use of human tissue.

The current Authors demonstrated that the isolated cells have mesenchymal immunophenotype, expressing surface mesenchymal markers and lack of non-specific markers for several passages as required by the guidelines of the Society for Cellular Therapy. It was interesting to observe the dynamic transformation of the immunophenotype during culture: from an heterogeneous population, the stromal vascular factor which was composed of different cells types, with only a small amount of stem cells (superior to the number of MSCs isolable from bone marrow as the current literature suggests [28], following procession with both methods, we were able to isolate an homogeneous population mainly composed of stem cells.

Regarding possible clinical applications of the mechanical isolative method, it should be considered that freshly isolated product (SVF) does not represent a pure population of mesenchymal cells, and, because of this, it could respond differently to the biological environment when injected directly or stimulated. Several studies have shown that SVF holds several other properties, such as the angiogenetic effect that could be useful in different clinical scenarios, including cases of fracture mal-union as well in every clinical scenario where the blood

support is deficient [28-30]. Furthermore, the SVF could contain several subpopulations with different properties, as researchers found in the SVF of the bone marrow [31, 32]. The authors of the current study also noticed differences in the expression of biological markers, between the tissues isolated by the two different procedures: for example, CD34 was less expressed in the tissue treated by the mechanical procedure while CD146 was more expressed in the same sample, suggesting that differences between the two techniques could influence the characteristics of the isolated mesenchymal stem cells. For this reasons, it will be mandatory for future studies, in order to obtain the desired biological effects, to consider not only the harvested tissue (in terms of quantity and quality), but also all the possible variables, including the technique, the site of isolation and the characteristics of the donor [33, 34].

In conclusion, our findings indicate that processing tissue using a mechanical protocol yields to a population of mesenchymal stem cells comparable, in terms of morphology, viability of the cells, immunophenotype and differentiation potential, to those obtained following the classical enzymatic procedure. However, further studies need to be performed to improve the quality of the final biological product and to investigate the potential properties of the adipose tissue clusters obtained using this novel method. Our study also supports the suggestion of using Adipose Derived Stem cells (ADSCs) in different treatment strategies for tissue regeneration and that this new approach could make their use more practical for the surgeon and less invasive for the patient. The development of new protocols could lead to avoid the use of non-human components, such enzymes, to obtain biologically active mesenchymal cells in a safer way and could also allow their use in the clinical treatment of several orthopaedic conditions [35-44].

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