

Processing of Lipoaspirate Samples for Optimal Mesenchymal Stem Cells Isolation

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1. Introduction

Over the last 10 years, huge advances have been made worldwide in the adult stem cell field. Several donor sites can be used for harvesting mesenchymal stem cells (MSC), bone marrow and adipose tissue being the most frequent. The latter is easily harvested by liposuction and, in most patients, a large quantity of MSC can be obtained without harm to the donor (Casteilla et al., 2004). In 2001, Zuk and co-workers showed that a human lipoaspirate contains multipotent cells and may represent an alternative stem cell source to bone marrow-derived MSC. Adipose-derived MSC are capable of proliferation in monolayer culture and multilineage differentiation in response to inductive conditions, and thus have potential clinical application (Bailey et al., 2010; Fraser et al., 2008; Rigotti et al., 2009;).

However, research and clinical groups have distinct protocols to isolate and manipulate these cells, differing in the type and concentration of the enzyme used, time and conditions of incubation for adipose tissue digestion and methods of cell culture. These methodological differences result in diverse characteristics of the cells isolated and varied functional results. Therefore, the development of a standardized and reproducible method of isolating MSC as well as standard techniques for functional characterization is fundamental to validate cells for its use in therapeutic protocols.

A tentative for functional characterization of MSC was recently proposed by the International Society for Cellular Therapy (ISCT) (Dominici et al., 2006). According to this statement, MSC must: (i) be plastic-adherent in standard cultures; (ii) express a mesenchymal set of surface molecules; (iii) differentiate into osteoblasts, adipocytes and chondroblasts *in vitro*. The detection of these mesenchymal set of surface molecules as well as the differentiation assays can be performed on adipose-tissue MSC population after their isolation in laboratory and will be carefully described in this chapter.

2. Cell physiology of adipose tissue and mesenchymal stem cells

Only recently, the white adipose tissue has been identified as an endocrine organ besides acting in energy storage and, in humans, it can be found mainly in two sites: visceral and subcutaneous. By secreting bioactive molecules, called adipokines, this tissue plays an active role in the regulation of several functions in the organism (Gregoire, 2001; Trayhurn, 2005).

The white adipose tissue is composed by connective tissue, nerve endings and a rich vascular network (Figure 1). Cellular content includes specialized cells, the adipocytes, and a stromal vascular fraction composed by pre-adipocytes (which differentiate into adipocytes), fibroblastic cells, endothelial cells, macrophages (Casteilla et al., 2004) and lymphocytes (Caspar-Bauguil et al., 2005). A subset of cells that is associated with blood vessels, called MSC, exhibits developmental potential beyond angiogenesis. MSC are found throughout fetal and adult tissues. They are members of the pericyte cell family and may be defined as progenitor cells capable of giving rise to a number of differentiated mesenchymal cells and also contribute to tissue homeostasis (Baptista et al., 2007; da Silva Meirelles et al., 2008).

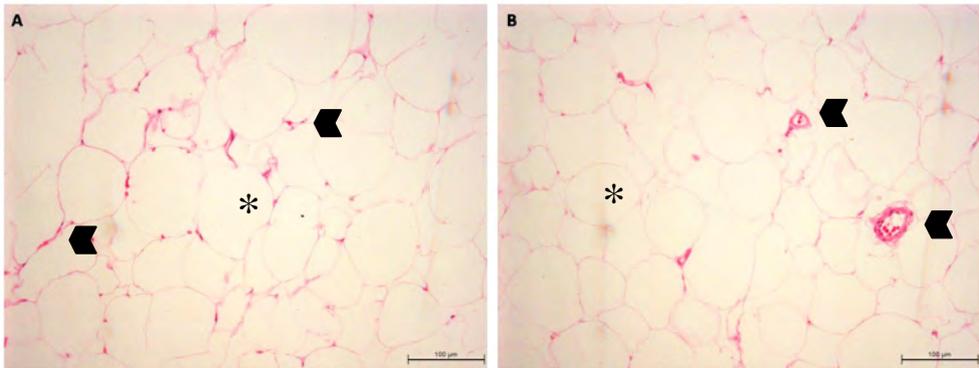


Fig. 1. Histological analysis of subcutaneous adipose tissue. Adipose tissue fragments were fixed in formaldehyde and processed for paraffin embed. Histological sections were stained by hematoxylin and eosin (H/E). Because histological process involves the use of xylol, the fat inside adipocytes are dissolved and the inclusions appear as its negative image, that is the area occupied by fat inside adipocytes (asterisks). Note the significant network of blood vessels (arrowheads) composed by small (A) and large ones, surrounded by multiple layers of cells (B), where the population of MSC dwells. Bar size=100 μ m.

The term “mesenchymal stem cell” was introduced by Caplan (1991), after the studies of Friedenstein and co-workers, who isolated and characterized these cells from bone marrow (Friedenstein et al., 1968, 1974). MSC were distinguished from hematopoietic cells by plastic adherence and fibroblastic morphology. Besides that, when cultivated at a clonal density (few cells per cm^2) these cells adhere to the plastic and discrete colonies are established, initiated by a single proliferative cell, termed the Colony Forming Unit Fibroblast (CFU-F). These fibroblastic colonies, under adequate experimental conditions, give rise to differentiated cells of distinct types of connective tissue, like adipocytes, osteoblasts and chondroblasts (Friedenstein et al., 1974). These cells have also the potential to differentiate into myoblasts (Wakitani, Saito & Caplan 1995; Ferrari et al., 1998, Zuk et al., 2001, Mizuno et al., 2002, Crisan et al., 2008), as well as into fibroblasts, and possibly, tendon (Caplan, 2007; Chamberlain et al., 2007).

Although bone marrow MSC-like cells can be isolated from different tissues, adipose tissue have been proposed to be an alternative to bone marrow, since fat tissue is abundant, easily harvested by liposuction and adipose tissue MSC, like bone marrow MSC, can differentiate towards mesenchymal lineages (Zuk et al., 2001).

Long-term cultured MSC maintain their differentiation capacity towards osteo-, chondro-, adipo- and myogenic lineages, also expressing MSC markers. Many reports have described stable phenotype after extensive expansion (Zuk, et al., 2001, Crisan et al., 2008, Khoo et al., 2008, Poloni et al, 2010). However, there is a growing body of literature demonstrating murine MSC transformation after long-term culture (Qin et al., 2009, Miura et al, 2010, Ahmadbeigi et al, 2011). To our knowledge, no *in vitro* spontaneous transformation of human MSC has been reported under usual conditions of culture. Although Rubio and co-workers (2005), have described this event, this group recently reported the contamination of MSC with tumor cells in his laboratory (Garcia et al, 2010).

Recent studies showed that MSC actively migrate to and proliferate in tumor progression. Moreover, MSC could undergo transformation into malignant cells and tumor formation *in vivo* (Muehlberg et al, 2009, Karnoub et al, 2007). Others suggest that MSC should not affect the status of dormant cancer cells (Zimmerlin et al, 2011). The possibility of tumor growth and metastasis induced by MSC has an effect on the safety of their use for clinical applications. Nevertheless, three research groups have now found contamination of the MSC with tumor cells used for other projects in their laboratories. In addition, over 1,000 patients were transplanted with MSC, and no tumor formation related to MSC has been reported (for a review, see Klopp et al, 2010)

There are no irrefutable studies about the role of MSC in stimulating or inhibiting tumor progression and metastasis. Discrepant results obtained by investigators are probably due to variations in MSC origin (humans or animals), MSC tissue source, individual donor variability, timing of MSC injection as many other factors. Studies considering the role of these factors are necessary to lead to new insights to resolve this important issue.

3. Adipose tissue harvesting and preparation for isolating mesenchymal stem cells

Surgeons have distinct techniques for harvesting adipose tissue and also to prepare them in the operating room. Liposuction can be performed using standard or vibro-assisted techniques. Vibro-assisted liposuction has already been reported to reduce the duration of surgery because of its large rate of aspiration (Viterbo & Ochoa, 2002). We were able to isolate MSC from lipoaspirates harvested by standard or vibro-assisted techniques, using a mechanic method (see topic 4). The total number of MSC obtained from vibro-assisted lipoaspirate samples was superior to that obtained from the standard one, but no differences in adhesion or proliferation in culture was observed (Baptista et al., 2009).

When taking into account the isolation of MSC with high cell quality, the use of a defined method of tissue harvesting and preparation in the operating room is crucial. Centrifugation of the adipose tissue harvested by liposuction has been used for nearly three decades. It is one of the preferred methods of fat processing for soft tissue augmentation.

We then investigated how the manipulation of lipoaspirate samples influences in the yield and quality of MSC subsequently isolated in the laboratory (Condé-Green et al., 2010). We have examined lipoaspirates prepared by centrifugation (1228g for 3 minutes) and decantation (30 minutes under the action of gravity). Centrifuged lipoaspirates had a lower yield of isolated MSC. Moreover, they were less capable to proliferate *in vitro*, probably due to the centrifugation forces suffered by cells in lipoaspirates. Also, centrifuged samples showed a fraction of cells in the bottom of the syringe, in the pellet, which was not identified in decanted samples. This fraction had a significant quantity of MSC.

In the majority of published reports, adipocyte viability analysis is qualitative, and only a few groups showed a more reliable analysis using quantitative methods.

Possible architectural alterations of adipose tissue caused by these methods was quantified based on the degeneration of adipocytes, staining tissue histological sections with Hematoxylin & Eosin (Condé-Green et al., 2010) or with antibodies against perilipin, an abundant protein in the adipocyte cytoplasmic membrane (Figure 2). Another histological quantification was performed by Rose and colleagues (Rose et al., 2006), showing that decanted samples had twice the quantity of intact adipocytes as compared to centrifuged and washed samples. Our study showed similar histological results. We observed that the adipose tissue resulted from centrifuged lipoaspirate samples contained most of adipocytes with disrupted membranes and general extracellular matrix disruption, whereas decantation maintained the adipocytes' integrity (Figure 2; Condé-Green et al., 2010).

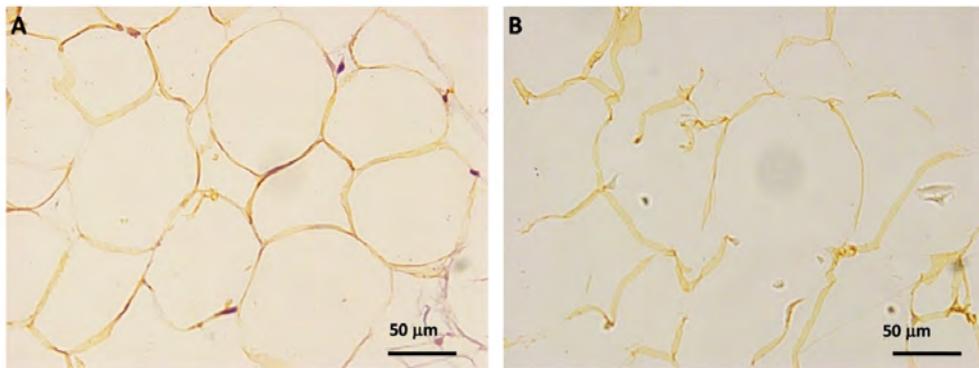


Fig. 2. Microscopic aspect of adipose tissue obtained from lipoaspirate samples. Adipocytes were specifically identified in histological sections of decanted (A) or centrifuged (B) lipoaspirates by staining with the commercially available polyclonal antibody Perilipin to evaluate the impact of both methods on tissue architecture. Decanted lipoaspirate shows relatively intact, nucleated adipocytes with minor trauma and overall normal morphology (A). Centrifuged lipoaspirate clearly shows a reduced number of intact, nucleated adipocytes with more extensive trauma (B). Bar size 100 μm .

We cannot exclude the fact that reducing centrifugation forces will improve MSC recovery on centrifuged lipoaspirates samples, as already described (Kurita et al., 2008). The authors tentatively recommend 1200 g as an optimized centrifugal force, lower than used in our study, for obtaining good short- and long-term results in adipose transplantation. However, MSC content by surface marker expression was not evaluated on centrifuged adipose tissue samples of this study. Various speeds and time intervals for centrifugation have been recommended, but some reports demonstrated histologically a destruction of the most living fat (Chajchir et al., 1993; Rose et al., 2006).

These observations demonstrate that the centrifugation of adipose tissue harvested by liposuction have a negative effect on tissue architecture and morphology, losing its stem cell content, as MSC are lost in the pellet, as well as on the yield and quality of MSC subsequently isolated from the resulted tissue. On the other hand, decantation resulted in no harms to tissue structure and allows a substantial quantity of cells isolated, with a good proliferation rate and morphology.

The future of autologous fat transplantation seems to lie in stem cell research, specifically in adipose MSC. However, the use of adipose MSC raises numerous concerns, including the choice of harvesting and processing, cell isolation and culture. Scientific research is emerging to address these issues.

Membranes of adipocytes were disrupted when adipose tissue was submitted to forces generated during centrifugation, probably due the fact that adipocytes are very fragile cells with only a thin cytoplasmic envelope surrounding large fat droplets. On the other hand, MSC are smaller and more resilient, make them more practical to work than adipocytes (Suga et al., 2008).

4. Human adipose tissue mesenchymal stem cells: author's protocol

4.1 Mesenchymal stem cell isolation

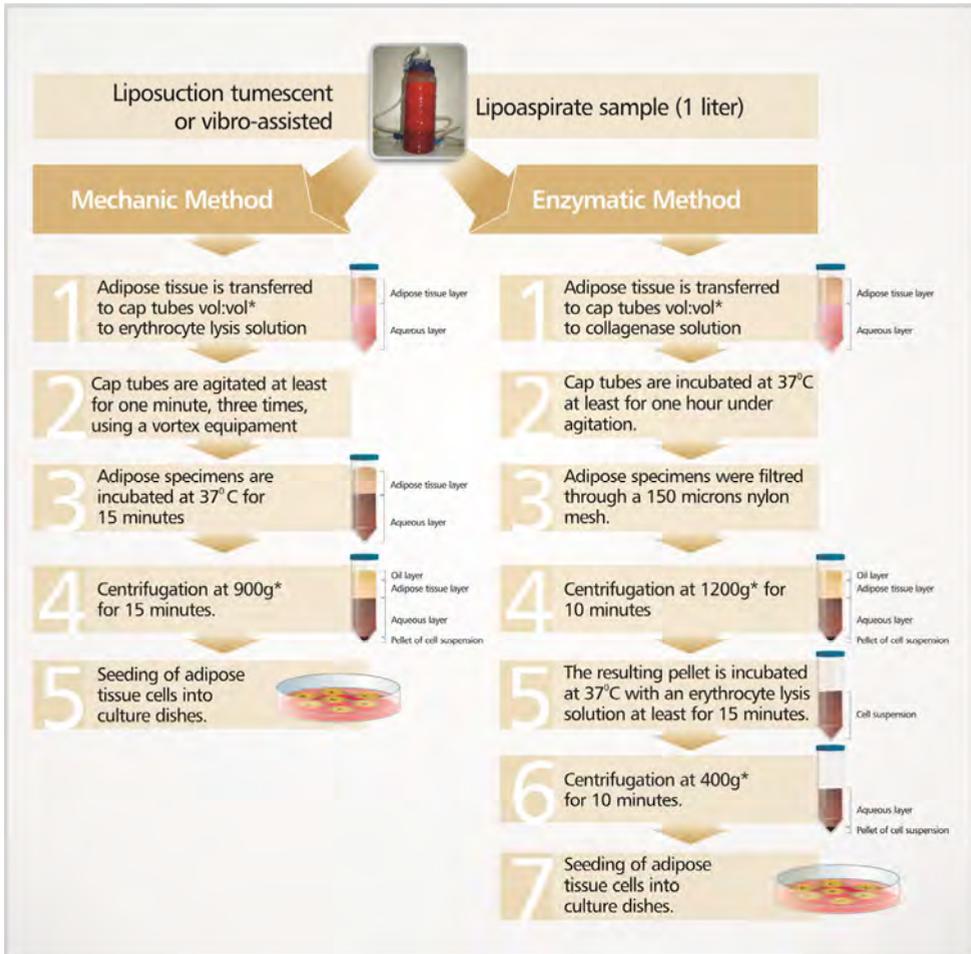
The commonly used method of isolating MSC from adipose tissue is enzymatic digestion (Gimble & Guilak, 2003; Jing et al., 2007; Bunnell et al., 2008), that consists of at least four main steps: digestion, washing, centrifugation and red blood cell lysis. Adipose tissue from lipoaspirate samples is incubated with collagenase for up to 1 hour. Then, the digests are washed, and centrifuged to separate the floating adipocytes from the pelleted stromal cells. The pelleted stromal cells are finally incubated with red blood cell lysis solution and centrifuged one more time. This enzymatic procedure generates tissue fragments that should be removed before cell plating through a 100–150 μm nylon mesh. Irrespective of the source of tissue, enzymatic digestion is time consuming and expensive, especially when applied to large volumes of tissue (Baptista et al., 2009); decreased cell viability due to lytic activity is also a problem with this method (Ishige et al., 2009).

We have described a novel method of isolating MSC from lipoaspirate samples, based on mechanical tissue dissociation. Despite the major differences between the enzymatic and mechanic methods, similar populations of MSC have been isolated. The population of cells derived from mechanic process was positive for mesenchymal surface markers such as CD90 and CD105. They were also positive for CD34, which is reported only in adipose tissue-derived mesenchymal cells (Planat-Benard et al., 2004). They also were able to accumulate lipid droplets, deposit extracellular calcium and cartilage extracellular matrix, under specific stimuli for each differentiation event (Baptista et al., 2009). Their proprieties support their use for diverse therapeutic applications. Techniques used on these assays will be detailed below (See topic 4.2).

MSC derived from mechanic process can be isolated easily from lipoaspirate samples and provide a significant quantity of cells with minor time and costs for the procedure. As commented above, the enzymatic procedure for adipose tissue consists of at least four main steps. Conversely, mechanical dissociation consists basically of two steps: dissociation of adipose tissue concomitantly with red blood cell lysis, followed by centrifugation. There are no visible tissue fragments, and it is not necessary to the filter cell suspension. The ease of mechanical digestion reduces considerably both time and cost, and does not interfere with cell viability (Figure 3). Furthermore, MSC culture derived from mechanic process gave higher yield of cells than digestion method after primary culture.

Besides taking advantages in time and cost when using mechanic process, their reproducibility makes it a preferred method for larger volumes of samples. We observed a large standard derivation among cell numbers isolated with the enzymatic digestion process, in opposition to mechanic process (Baptista et al., 2009). However, the most

advantage of this innovative process is the possibility of cryopreservation of freshly isolated MSC cells. Interestingly, another study has investigated a method for cryopreserving human adipose-derived stem cells isolated by an enzymatic process. Fresh human cells were cryopreserved using Me_2SO as the cryoprotective agent at a density of 10^6 cells/mL (Liu et al., 2008), 10 times lower than the cell quantity cryopreserved in our study.



*vol:vol - volume to volume - the adipose tissue volume added should correspond to the same volume of solution.

*g = (gravities, the standard unit of centrifugation speed).

Fig. 3. Comparing mesenchymal stem cell isolation by mechanic and enzymatic methods.

Note that the enzymatic method consists of at least six main steps and the mechanic method basically of four. The centrifugation step (four in both methods) is used to separate cells from adipose tissue fragments, oil and debris. Resulting pellets - step four in mechanic and six in enzymatic - must be resuspended and seeded into culture dishes in suitable cell

culture medium containing at least fetal bovine serum and antibiotics. Only the pellets obtained by mechanic method can be alternatively resuspended in fetal bovine serum supplemented with 10% dimethylsulfoxide (DMSO) for cryopreservation and storage at -196°C until thawing (see topic 4.2.1 Cell Culture).

Cryopreservation is interesting because it reduces labor costs and avoids possible loss of cell viability and senescence after long-term cultures (Serakinci et al., 2004; Rubio et al., 2005).

Another approach to isolate cells is based on primary explants culture (Klingbeil et al., 2009; Vunjak-Novakovic et al., 2006; Zhang et al., 2005). A simple small fragment of any tissue that adheres to the growth surface will usually give rise to an outgrowth of cells. Since the 1970s most studies of adipose tissue metabolism were carried out by explants methods (Smith, 1974). In the 1990s was documented the first evidence for preadipocyte proliferation during culture of adipose tissue explants (May, 1994), but there are few reports on the scientific literature regarding this method for isolation of adipose tissue stem cells.

Recently, Jing and co-workers (2010) described the explants culture as a time-saving and cost-effective method for isolation of adipose tissue MSC. They showed that adipose tissue fragments could adhere onto the growth surface of culture flasks after plating and MSC migrated from the explants reaching confluence after a while. Following *in vitro* expansion, this population of MSC was successfully induced into adipogenic, osteogenic, and chondrogenic lineages which demonstrated their multipotency. Despite the cost advantages of explants method, the techniques that are employed to adhere successfully adipose tissue fragments onto surface of flasks depend exclusively on the manual skills of the laboratory technician, which makes it a non reproducible method.

The Celution System is a medical device marked for processing adult adipose tissue stem cells for autologous re-implantation or reinfusion, and is currently being used in cosmetic & reconstructive surgery in Europe and Japan, but is not yet available in the United States because U.S. Food & Drug Administration rules. This system enables beside access to adipose stem cells by automating the extraction, washing, and concentration of a patient's own cells for immediate use.

The suctioned adipose tissue is introduced into the Celution cell-processing device and being enzymatically digested into a single cell suspension, which contains a combination of MSC, endothelial progenitor cells and other adipose tissue stromal cells. The cell suspension is washed and all lipid-laden adipocytes and matrix fragments are separated from it. The whole procedure is in a closed circuit and this reduces the chance of cell suspension contamination by fungus and bacteria (Duckers et al., 2006).

This automated closed circuit system would facilitate translational of bench research ideas and results to technologies for bedside use. However, this system has as a disadvantage the use of an enzymatic procedure to obtain a cell suspension. There is a concern over immunological reactions caused by enzyme-derived animal proteins (Spees et al., 2004).

4.2 Mesenchymal stem cell characterization for quality control

4.2.1 Cell culture

The ability to isolate, expand, criopreserve and differentiate MSC is an important step in the development of cell therapy approaches for therapeutical proposes of chronic-degenerative diseases, as well as for their application in plastic or reconstructive surgery. It was suspected that inconsistent data about therapeutical potentials of mesenchymal MSC is a result from different cell culture practices.

Based on our experience, we had set up a standardized protocol for the culture of human adipose tissue MSC and microbiological quality control procedures. Standards for the culture system included the use of alphaMEM (without nucleotides) containing 10% of fetal bovine serum selected for cell growth and 100U/mL penicillin and 100µg/mL streptomycin. After isolation, cultures of cells (10^5 cells/cm²) were maintained at 37°C in a humid atmosphere containing 5% CO₂. A subset of plated cells could adhere to the culture dish, developing a spindle shape morphology (Figure 4A). Non-adherent cells were removed by washing 24 hours after plating. The medium was changed every 3–5 days for proliferation until cells reach pre-confluence, which means 90% of the culture area covered by cells (10 to 15 days after plating – Figure 4B). Adherent cells were detached with 0.78mm EDTA and 0.125% trypsin and cell suspension was centrifugated 400 g (gravities) for 7 min. For cell expansion, cells were re-plated into culture dishes (10^4 cells/cm²). This procedure was considered to be ‘one passage’. Typical morphology of proliferating cells can be visualized during cell expansion when the cultivation conditions are adequate (Figure 4B – arrows). Cells isolated by the mechanic method maintained the ability to proliferate and the fibroblastic morphology even after 10 passages. No stress actin bundles were visualized (Baptista et al., 2009).

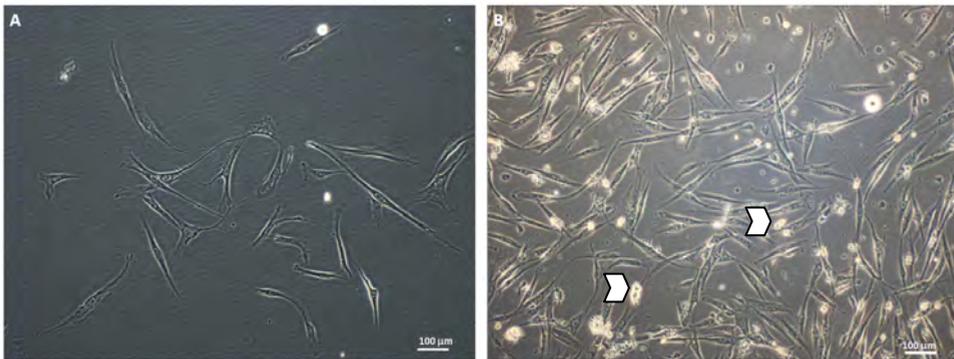


Fig. 4. Culture of human adipose tissue mesenchymal stem cells isolated by mechanic method. Immediately after isolation, cells were resuspended in alphaMEM (without nucleotides) containing 10% of fetal bovine serum and 100 U/mL penicillin and 100 µg/mL streptomycin and seeding at 10^5 cells/cm² into culture dishes. Cultures were maintained at 37°C in a humid atmosphere with 5% CO₂, and the medium was changed every 3–5 days until cells reach pre-confluence. (A) After 5 days of culture, the monolayer of cells showed typical fibroblast morphology, and after 15 days (B) proliferation events can be observed (arrowheads). Bar size 100 µm.

For cell cryopreservation, cell suspension was centrifugated 400 g for 7 min and pellet was resuspended in cryopreservation medium consisting of 90% fetal bovine serum and 10%DMSO (dimethyl-sulfoxide). This cell suspension was distributed in cryotubes in the ratio of 10^6 to 2×10^6 cell/tube. Cell freezing was performed for 24 hours in -70°C freezer, the cryotubes were then transferred to the gas phase of liquid nitrogen (-196°C) for long term storage. Analyses carried out after thawing showed that cells maintain their typical fibroblastic morphology and high viability. The ability to differentiate into mesodermal (adipogenic, osteogenic and chondrogenic) lineages was also attested.

Cells used both for clinical or experimental purposes must be free of microbiological contamination. Standards to monitor this type of contamination includes the use of hemoculture like tests and molecular biology. Cultures are daily observed under an optical microscope as for the presence of structures similar to microorganisms. Before detaching cells with trypsin for cell expansion or cryopreservation, an aliquot of the culture medium is collected to perform hemoculture like tests, which can detect the presence of both bacteria and fungi. However, mycoplasma is a not visible bacteria and also not detectable by this test. Its identification in the culture medium can only be performed using RT-PCR (Reverse Transcriptase-Polimerase Chain Reaction - Figure 5). For details about the basis of PCR, see Alberts et al., 2002. Detection kits are commercially available to identify several species of mycoplasma. If tests show the presence of a microorganism, an antibiogram is done to indicate the best antibiotic or antifungal to be used for decontamination.

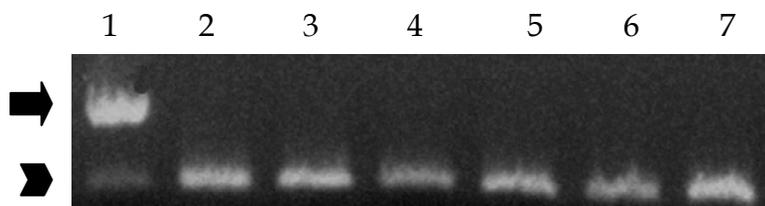


Fig. 5. RT-PCR to detect mycoplasma. RT-PCR was performed to monitor the presence of mycoplasma in the supernatant of mesenchymal stem cell cultures. We used a commercially available Detection Kit, which contains positive and negative samples used as reaction controls and reference. It is able to identify a range of mycoplasma species. Image was captured from a agarose gel with controls and experimental samples. Line 1: Positive control. First band is the detection of mycoplasma (arrow). Second band is the detection of a mRNA which serves as an internal control of the reaction (arrowhead), meaning that no interferences have occurred during sample preparation for analysis. Line 2: Negative control, absent of the mycoplasma band, but with internal control band presented (arrowhead). Lines 3 to 7, five different samples of the supernatant of cultured cells, free of mycoplasma. Note that the internal control band is present.

4.2.2 Flow cytometry

Standardized methods are necessary to assess the presence, viability and functional quality of MSC on the cell preparation obtained after the isolation procedure and after *in vitro* cell expansion. Fluorescence-activated flow cytometry is a very interesting tool to be used for this purpose. This is a technology based on the use of laser radiation, hydrodynamic fluid, optics, fluorochromes and computing resources. It is used to determine some structural and functional characteristics of biological particles, like cells. It is the most used technique to detect cellular antigens, called cluster of differentiation (CD) antigens, having a broad field of application in hematology, pharmacology, immunology, oncology, microbiology, genetics and stem cell research.

CD antigens are proteins expressed on cell membranes. They are commonly used as cell markers, allowing cells to be defined based on which molecules are present on their surface. However, CD antigens are not merely markers of cells. They usually act as receptors or ligands which initiate a signal cascade, being responsible for different cell behaviors. Besides cell signaling, some of CD antigens have different functions, like cell adhesion. A

nomenclature is used to describe different monoclonal antibodies against specific antigens, using the term CD plus a number. For example, an antibody that detects a specific glycoprotein on the surface of T helper lymphocytes is named CD4.

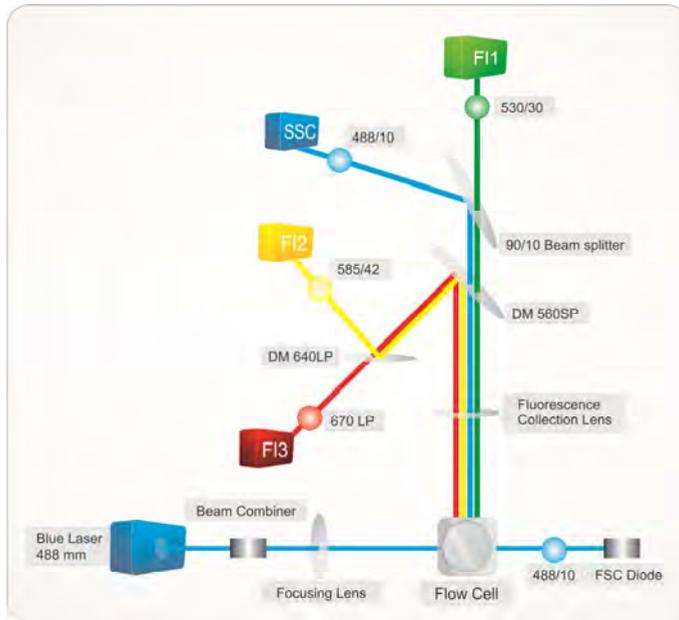


Fig. 6. Schematic representation of a flow cytometer. Flow cytometers aspirate cells from a suspension and force them to pass by the flow cell, using a system of pressurization. A laser intercept cells individually. The modifications that occur in this light beam due to cell characteristics are detected and measured by sensors (detectors) disposed adequately. Dispersed light is collected by an optical system which allows to identify cells by their size and complexity (The FSC and SSC Detectors). Fluorescence emitted by fluorochromes are also collected. To select these luminous signals emitted by fluorochromes, optical filters are used to block certain incident light wavelengths and let pass only the desired one. Each fluorescence emission is identified by different detectors (FL1, FL2, FL3), which convert luminous signals in electrical pulses and amplify this signal.

Flow cytometer, the equipment used to this end, is prepared to aspirate cells or particles in a previously prepared suspension and force them to go through a special chamber, centralized in a continuous flow of liquid (sheath fluid) and leaving this chamber one after another so that a single cell is intercepted by a laser. After laser interception, physical phenomena occurs, giving information about cells: First, part of the light is scattered according to structural and morphological cell characteristics. The Forward Scatter (FSC) is related to cell size and the Side Scatter (SSC) is related to cell granularity/complexity. Second, cells previously stained with fluorochromes coupled with antibodies are excited by the laser and a light emission occurs according to their fluorescent characteristics. Different fluorochromes absorb the light and emit it in a higher and specific wavelength. Each fluorochrome has a spectral pattern of absorption and emission, allowing up to three light

colors to be optically separated by selectively filters in common cytometers. Lenses are placed in series closed to the area of laser interception. They collect the light scattered and send it to photomultipliers that convert luminous signal in electrical pulses, which are proportional to the quantity of light dispersed or fluorescence captured by the photomultipliers (Figure 6).

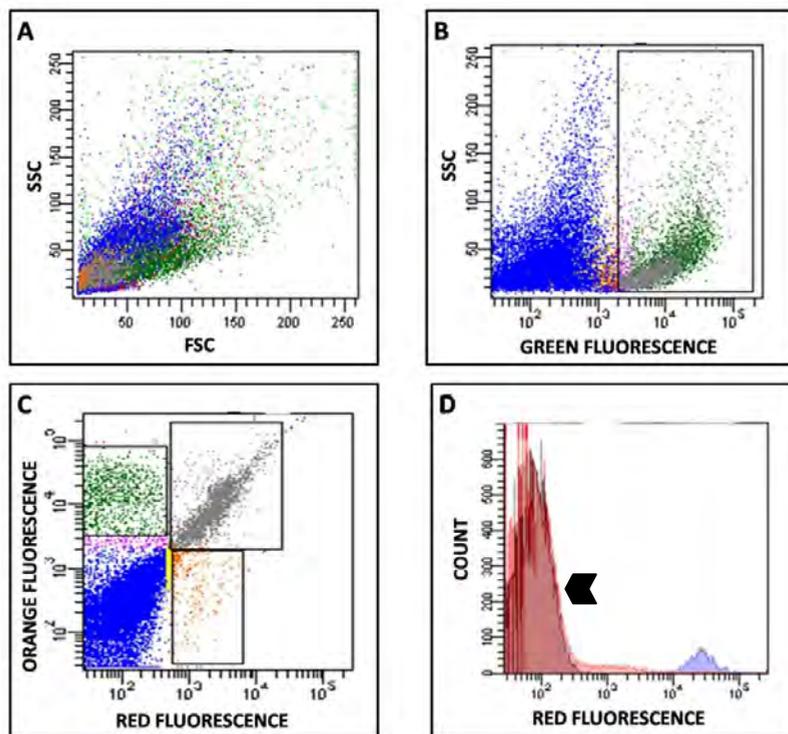


Fig. 7. Usual representation of data generated by the flow cytometer. Dot-plots (A – C) and Histogram (D) graphs are showed and represent the number of events or cells acquired. (A – C) Each dot is representative of one cell, (D) the number of cells is represented in y axis (counts). (A) Graph represents cell complexity in y axis (Side Scatter - SSC) and cell size in x axis (Forward Scatter - FSC). Parameters are presented in a linear scale. Cells with different degrees of complexity and size can be observed (A). Three-color immunofluorescence (green, red and orange) data were also collected from this sample and are presented in a log scale. (B) Side Scatter - SSC - is represented in y axis and green fluorescence channel in x axis. Only part of cells is positive for this green fluorescence parameter (box). (C) Graph represents two fluorescence channels (orange and red) simultaneously. We can identify at least four different cell populations. Cells exclusively positive for orange fluorescence parameter (box upper left), positive for both fluorescence parameters (box upper right), positive only for red fluorescence parameter (box lower right), and negative for both (cells outside boxes) (D) In this histogram it is possible to separate three populations of cells: Negative cells for red fluorescence parameter, which is overlaid with experimental negative control (arrowhead), positive cells with an intermediate staining (in red) and positive cells with a high staining (in blue).

To select and capture these luminous signals, optical filters are used to block specific incident light wavelengths and let pass only the desired wavelength. The electrical signs generated by the photomultipliers are amplified, converted to digital sign and sent to a computer. The data sampling, analysis and interpretation can then be performed using a specific software. Data generated by the flow cytometer can be represented in the form of mono- or biparametric histograms (Figure 7). By this way, it is possible to detect 10000 cells (called events) per second. Five parameters are considered basic and can be measured simultaneously: cell size, cell complexity, green fluorescence, red fluorescence and orange fluorescence.

To be detected by flow cytometry, cellular antigens must be labeled by immunofluorescence techniques. The antibodies against the antigens must be conjugated to fluorochromes. There are many fluorochromes, each of them with different ranges of excitation and emission light wavelengths. The most used are those that can be excited by the emission wavelength of the lasers available in flow cytometers. Some of them are Fluorescein, Phycoerithrin, Rodamin, Texas red, Cianins etc. There are fluorochromes with properties to attach directly to biological molecules, like the DNA stains: Propide Iodide (PI) and Ethidium Bromide (EB). PI is not able to cross a healthy cytoplasmic membrane. So, only dead cells stains with PI. EB can cross the cytoplasmic membrane, but only attach to DNA when cells are dead, because a transport system that expels the stain is off (Midgley, 1987). By using these or other DNA markers, it is possible to ascertain the viability of cells in a cell preparation.

It is possible to combine, in the same sample, two or more fluorescent stains if they emit light in different wavelengths and if the system is able to excite all of them and discriminate each emission. The most used multiple dyeing technique is green-red, by applying the fluorescein with maximum emission at 520nm, and phycoerithrin which emits at 576nm. Both can be excited by an Argon laser at 488nm, the most used laser in flow cytometers. Nowadays, studies are undertaken using 4 and 5 antibodies conjugated to different fluorochromes with different light wavelength emission. The most sophisticated cytometers can discriminate the information of up to 17 fluorescent markers, allowing the analysis of multiple possibilities of cell characteristics (Perfetto et al, 2004). This versatility is called multiparametric analysis. In cell biology, this property allows selective discrimination of subpopulations, based on the combination of many fluorochromes.

Cells isolated by the mechanic method contained two different major mononuclear cell subpopulations, CD45 positive and CD45 negative cells. Cells positive for CD45, a pan-hematopoietic marker, were also positive for CD16, CD14, CD31, surface markers of granulocytes, monocytes-macrophages and endothelial cells, respectively (Baptista et al., 2009). MSC do not have a hematopoietic origin, but a stromal one. So these cells are essentially CD45 negatives (Figure 8A). In this fraction, we could identify MSC, which are CD146⁺ (Figure 8B).

After seeding the initial cell suspension, culture dishes were washed with saline solution, removing the CD45 positive peripheral blood contaminant cells, remaining only a fraction of adherent cells. MSC that must be plastic-adherent into culture dishes, are a part of this fraction. Not surprisingly, this adherent cells were negative for CD45 and positive for CD44, CD90, CD105 (Baptista et al., 2009) and CD73 (Figure 8D), surface markers described in MSC populations of different origins (Dominici et al., 2006). They were also positive for CD34, (Figure 8C), a glycoprotein reported to be present only in adipose tissue MSC (Planat-Bernard et al., 2004). After expansion *in vitro*, we and others detected a progressive increase of mesenchymal markers expression like CD73 and CD90, while the expression of CD34

decreases until being completely loss (McIntosh et al., 2006; Mitchell et al., 2006; Baptista et al., 2009; Bernardo et al., 2009).

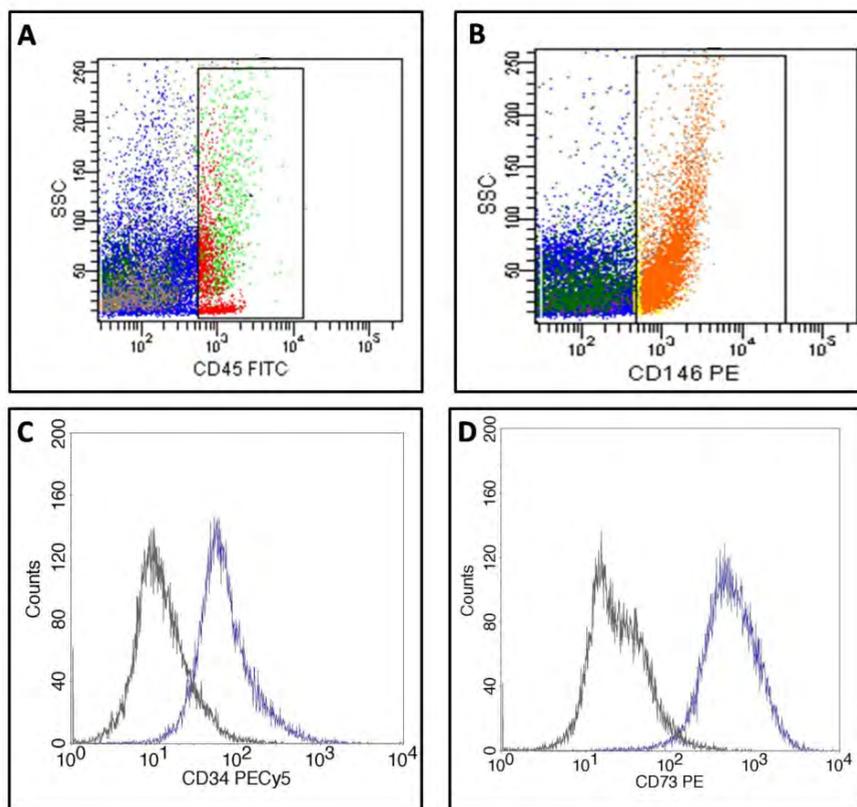


Fig. 8. Phenotypic characterization of subcutaneous adipose tissue cells. Cells isolated by the mechanic method were monitored for surface marker expression at the moment they were isolated (A, B) and at first passage *in vitro* (C, D) using flow cytometry. Cells were stained with monoclonal antibodies conjugated with fluorescent dyes: CD45–fluorescein isothiocyanate (FITC), CD146–phycoerythrin (PE), CD73-PE and CD34- PECy5 (PECyanin-5). For each profile, 200.000 events were acquired in freshly isolated samples and 50.000 events for cultured cells. Flow cytometry analysis were performed using a FACSCanto (A, B) or FACSCalibur (C, D) - BD Biosciences. (A, B) Dot-plots graphs. (A) Hematopoietic cells (CD45 positive) are gated (box). Cells outside the box (non-hematopoietic cells) in (A) are distributed in (B) and positive for the perivascular stem cell marker - CD146 (box). At first passage, cultured mesenchymal stem cells maintained the pre-adipocyte (C) and the mesenchymal stem cell marker (D), CD34 and CD73, respectively. (C, D) Gray lines on histograms graphs represents isotype controls.

No unique single marker has been described yet to distinguish MSC from other cells in the tissue of origin (Mosna et al, 2010). Instead of it, a combination of markers is used for an

adequate detection of these cells. Thereby, flow cytometry represents an important tool to make a detailed immunophenotypic analysis of these cells, providing information of many fluorescent markers in the same cell, reading millions of cells in few minutes (Perfetto et al., 2004). It allows a rapid qualitative and quantitative multiparametric analysis of cells, making measurements on single cells as they travel in suspension one by one.

With this understanding, we propose the use of flow cytometry to characterize the cell preparation obtained after the isolation procedure and after *in vitro* cell expansion, as an efficient standard method to identify the presence and viability of adipose-derived MSC in these preparations, to assure the quality of cells that will be used in therapeutic approaches.

4.2.3 Differentiation assays

To assure the multilineage differentiation capacity of expanded MSC, standard methods of *in vitro* cell differentiation are used. These functional assays allow testing the ability of MSC to differentiate to the adipogenic, osteogenic and chondrogenic lineages. To test this capacity, specific stimuli are used for each lineage differentiation, like growth factors, hormones and drugs. These molecules can act in specific cell receptors, which transduce signals of growth and differentiation through cascades of intracellular events (Gregoire et al., 1998).

For induction to the adipogenic lineage, cells are cultivated in monolayer and incubated for at least two weeks with culture medium containing 10% of fetal bovine serum, insulin 10 μ M, IBMX (isobutylmethylxantine) 0,5mM, dexamethasone 10⁻⁶M and indomethacin 200 μ M. To test the osteogenic differentiation capacity, cells are cultivated in monolayer and incubated for three weeks with medium containing 10% fetal bovine serum, ascorbic acid 5 x 10⁻⁶M, dexametason 10⁻⁸M and β -glicerophosphate 10⁻²M.

To promote chondrogenic differentiation, we used three-dimensional cell culture methodology. Cells, expanded until third passage, were enzymatically detached from culture dishes and cell suspension was centrifuged 400g for 7 min. Pellet was resuspended in chondrogenic medium containing insulin 6,25 μ g/mL, transferrin 6,25 μ g/mL, ascorbic acid 50 μ g/mL, albumin 1,25 μ g/mL, dexamethasone 10⁻⁷M and TGF- β 3 (*Transforming Growth Factor- β 3*).

Cell suspension containing 2x 10⁵ cells were distributed in polypropylene conical tubes with capacity of 15ml, centrifuged 300g for 10 min and maintained at 37°C in a humid atmosphere with 5% CO₂ for four weeks. Chondrogenic medium was renewed every 3 or 4 days, taking care not to damage the cell pellet. After 4 days, the pellet appeared like a sphere, with around 0,9 mm of diameter.

The inductive media must be renewed twice in a week and after the appropriate time period, cells are fixed and evaluated for lipid accumulation, extracellular calcium deposition and cartilage tissue extracellular matrix (ECM) production, to assess adipogenic, osteogenic and chondrogenic differentiation respectively (Baptista et al., 2009). The presence of lipid droplets can be detected by staining induced cells with specific hydrophobic stains, being Oil Red O the most used (Figure 9A, B). Mineral depots are revealed by Alizarin Red staining (Figure 9C, D). Both stains can be eluted from cells and quantified by spectrophotometry, giving a quantitative analysis about the level of differentiation. Production of sulfated glycosaminoglycans (GAGs) and type II collagen - the main molecules of cartilaginous tissue ECM - can be assessed by Alcian Blue (pH 1.0) and Safranin O-Fast Green (Figure 9E, F) stainings or by immunofluorescence techniques, respectively.

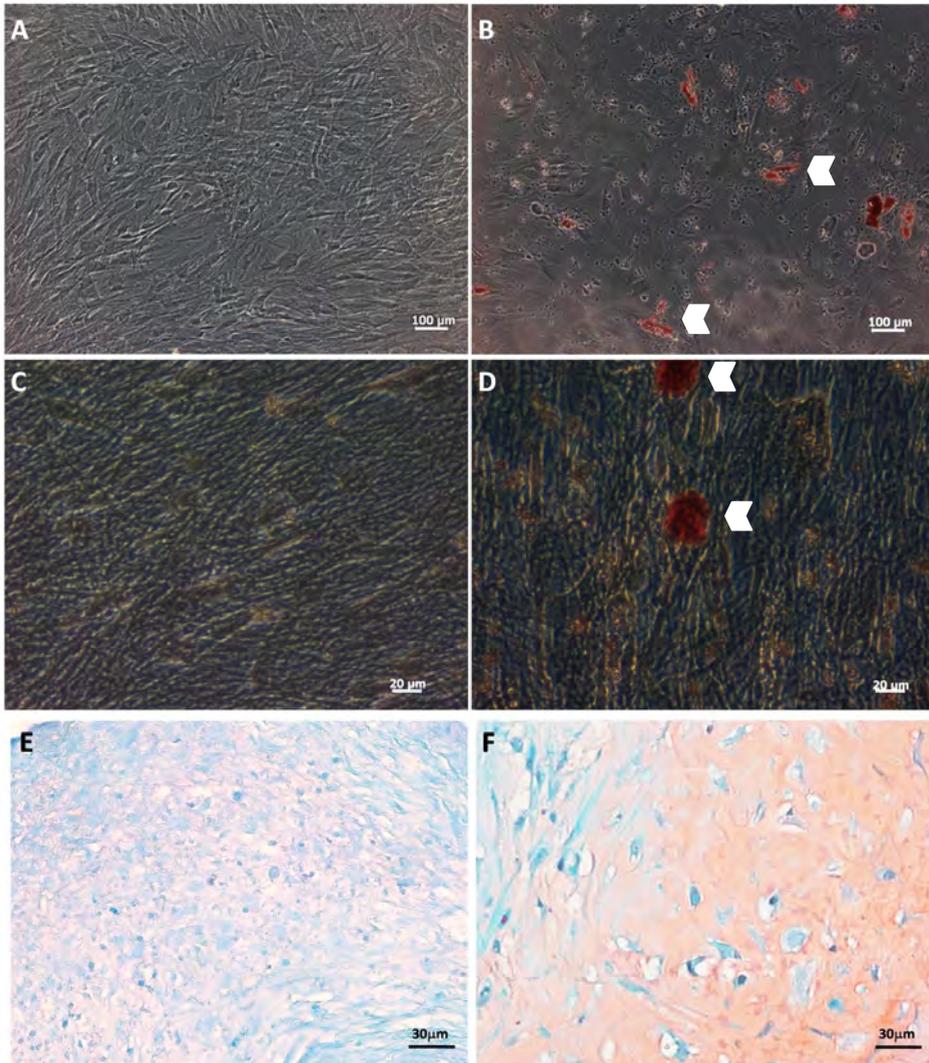


Fig. 9. *In vitro* multipotentiality of adipose tissue mesenchymal stem cells. Cultured mesenchymal stem cells were able to differentiate into the three mesodermic lineages (adipogenic, osteogenic and chondrogenic). Cultures of cells were maintained under adipogenic (B) or osteogenic (D) inducing media for 14 days. After this period, they were fixed and stained with Oil Red O to identify the lipid droplets (B - arrowheads), and with Alizarin Red to reveal extracellular calcium deposits (D - arrowheads). Media without (A, C) and with (B, D) appropriate inducing factors. (A, B) Bar size, 100 µm. (C, D) Bar size, 20 µm. Pellet cultures formed by mesenchymal stem cells under media without (E), and with (F) chondrogenic inducing factors for 28 days. (E, F) Safranin O staining. Matrix accumulation is typical of cartilage only in induced cells (F). Bar size, 30 µm.

5. Future research

The use of adipocytes and MSC for tissue repair and regeneration can follow two different procedures. The liposculpture uses the freshly harvested adipose tissue, generally obtained by liposuction. The tissue is frequently harvested, processed and re injected during the same surgical procedure. MSC can be introduced simultaneously, and the tissue processing should be concerned by their viability and their capacity to functionally integrate into the tissue where they are implanted.

Alternatively, MSC can be harvested and expanded *in vitro*, in order to reach the required cell number, and used subsequently for filling or repair of different tissues such as dermis, connective tissue, bone and associated tissues, as well as blood vessels in repair of both peripheral or cardiac tissue ischemia (Hicok et al., 2004; Casteilla et al., 2005; Hanson et al., 2010). In these cell therapies, the implantation of cells lags behind the harvesting for several days or weeks. The viability of harvested cells and their proliferative capacity *in vitro* are critical for such therapeutic approaches.

5.1 Mesenchymal stem cell bank

Autologous MSC, such as those derived from bone marrow or adipose tissue, can be used clinically for regenerative cell therapy or for tissue engineering only when isolated in a reproducible manner and in sufficient quantities. The expansion and differentiation steps may provide increased cell number, purity, and maturity, but they do so at a cost. This cost can include one or more of: loss of cell function due to cell aging, increased monetary cost, and increased risk of contamination of cells with environmental microorganisms during culture.

Liposuction surgery often generates large volumes of samples to be processed, so it is important not to waste them. There is need for alternative methods in which a population of active cells with increased yield can be prepared rapidly and reliably, and whereby the need for post-extraction manipulation of the cells can be reduced or eliminated. We developed an innovative method based on mechanic dissociation of adipose tissue in order to release MSC population from it and that attends all these needs described (Baptista et al., 2009). MSC population can be isolated in a manner that is suitable for their direct placement into a recipient or for their direct cryopreservation in a laboratory.

The possibility of cryopreservation of freshly isolated MSC abrogates culture-associated changes found in cells after prolonged expansion, and provides the possibility of generating extemporaneously a large stock of cells (MSC bank) using a relatively simple method. Once cryopreserved, MSC can be thawed as the need of use, without loss of cell viability and functionality.

5.2 Autologous fat grafts

Autologous fat grafts are becoming a major procedure for soft-tissue filling. However, resorption of fat transplanted has been reported (Sommer & Sattler, 2000; Masuda et al., 2004; Kaufman et al., 2007) and current efforts focus on identifying methods that may minimize this undesirable result. There is no universal agreement on what constitutes an ideal methodology to obtain better graft takes and results.

Our results showed that centrifugation, although cleaning adipose tissue of potentially harmful substances yields adipose tissue which is not only devoid of viable adipocytes but also has a diminished percentage of MSC (Condé-Green et al., 2010). Taken together, the

long term graft of the implanted centrifuged adipose tissue is less probable, and the implant resorption naturally occurs with clearance of non-viable organic components introduced into the receptor site. In contrast, decantation acceptably separated the supernatant layer of adipose tissue from the oily and sero-sanguinous liquids (infranant layer), besides preserving the integrity of adipose tissue, number and viability of adipocytes and MSC.

5.3 Fat graft enriched with mesenchymal stem cell

Adipose tissue harvesting and processing techniques employed in the surgery room play an important role on the viability and integrity of adipose tissue, and according our study, also on the percentage of MSC (Condé-Green et al., 2010). A long-term graft is reached mainly by angiogenesis and MSC enhance local angiogenesis by differentiation events, and secretion of angiogenic factors (Hanson et al., 2010).

To overcome drawbacks of autologous lipoinjection, Yoshimura et al., have developed a novel strategy which is based on MSC association with autologous fat working as scaffold. This novel strategy resulted on long-term retention of fat graft (Yoshimura et al., 2008). Recently, our group was responsible for the development of an innovative method to isolate adipose tissue MSC on lipoaspirate samples (Baptista et al., 2009). Our method is based on mechanic dissociation of adipose tissue instead of enzymatic, and generates a cell suspension devoid of both: tissue debris and enzyme waste. It is possible performed mechanical dissociation on operating room then, cell suspension enriched with MSC can be injected simultaneously with fresh adipose tissue scaffold. This association (cell suspension enriched with MSC and fresh adipose tissue from decanted lipoaspirate sample) could be used to volume restoration of facial depressions caused by sequelae of trauma and tumors.

6. Conclusion

The scientific community is working on ways to standardize processes so that it is safe and effective, no matter what the application. The major advantages of adipose tissue as a source of regenerative cells, which distinguish it from other alternative cell sources, include: 1) Yield: A therapeutic dose of regenerative cells can be isolated in approximately one hour without cell culture when using our mechanic method of isolation; 2) Safety: Patients receive their own cells (autologous-use) so there is no risk of immune rejection or disease transmission; 3) Versatility: Stem cells from adipose tissue impart benefit from multiple mechanisms of action.

The use of these cells as a product for cell therapies in humans implies the development of standard methods to ensure high cell quality. We described methods of harvesting and preparation of the human adipose tissue and isolation, cultivation, expansion and characterization of adipose derived MSC, developed to achieve this cell quality and to monitor MSC potential for clinical application.

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