

Isolation and Characterization of Different Cell Types from Lipoaspirates Used for Treating Alopecia

Ana Sofia Galrinho Borges¹

¹Instituto Superior Técnico, Universidade de Lisboa, Lisboa, Portugal

Abstract

Alopecia is a frequent hair loss disorder and the injection of fat into the scalp has been investigated as a possible therapy. However, the retention of transplanted fat is quite variable, sometimes presenting low survival rates. Recent studies have focused on the enrichment of fat grafts with stromal vascular fraction (SVF) to increase cell viability and graft retention. The optimization of the fat harvest, processing and injection procedures is essential for the improvement of this therapy. Therefore, it is necessary to analyse parameters such as the surgical technique and the width of the cannulas used. In this work, nine conditions involving variations on these parameters were studied in order to assess their influence on SVF cells. Small cannula widths were focused on as they are necessary for ambulatory therapies. The harvested fat was processed by an enzymatic isolation protocol.

Cell yield presented high donor dependence, but average results revealed a decrease with increasing fat manipulation and narrower cannulas. Such conditions were also associated with increased adipocyte rupture and consequent release of oil droplets as shown through flow cytometry. Additionally, a decrease on the mesenchymal stem cell (MSC) population present in the SVF was verified. Nonetheless, some conditions exhibited stromal progenitors with clonogenic capacity. The identity of the isolated MSCs obtained upon culture of SVF was confirmed in terms of immunophenotype, clonogenicity and differentiation potential. Further studies should be performed to find a compromise between wider cannulas and minimally-invasive procedures for the treatment of alopecia.

Keywords: Alopecia, Autologous Fat Grafting, Stromal Vascular Fraction, Flow Cytometry, Mesenchymal Stem Cells.

1. Introduction

Alopecia is a hair loss disorder which results in visible hairless areas. Up to 40% of women and 70% of men are affected by androgenetic alopecia (AGA), the most common type. Although medicines have been developed, they are associated with unpleasant side effects. Autologous hair transplantation is considered to be the gold standard treatment, but it is less effective than the aforementioned medicines in preventing disease progression [1].

One of the key factors affecting hair growth is the surrounding adipose tissue (AT). It is composed of adipocytes and the SVF, which includes around 25 – 45% of haematopoietic-lineage cells, 10 – 20% of endothelial cells, 3 – 5% of pericytes and 15 – 30% of stromal cells including MSCs [2]. The cellular composition of the AT is not fully known yet because of the lack of a reliable *in vitro* method to analyse cell yield in this tissue. The main difficulty is the assessment of the number of viable adipocytes due to their fragility and to possible overestimation by confusion with lipid droplets, which are derived from ruptured adipocytes [3].

MSCs are defined by the following criteria: plastic adherence, expression of the cell surface markers

Cluster of Differentiation (CD) 73, CD90, CD105, lack of the expression of CD11b or CD14, CD19 or CD79 α , CD34, CD45, and Human Leukocyte Antigen (HLA) – DR, and the ability to differentiate towards the adipogenic, osteogenic and chondrogenic lineages [4]. There are different sources of MSCs such as the bone marrow, which is associated with painful harvesting and low cell yield upon harvest. AT is another possible source which is characterized by less painful harvesting and higher initial cell numbers. Therefore, the AT seems quite promising as an alternative source of MSCs [5]–[7].

The hair growth cycle and the thickness of the intradermal adipocyte layer are synchronized. Immature adipocytes are necessary and sufficient to lead to the activation of hair follicle stem cells and to the initiation of the hair growth cycle [8]. Cells from the adipogenic lineage secrete factors that can regulate the hair growth cycle and Adipose Tissue – Derived Mesenchymal Stem Cells (AT-MSCs) participate in the activation of skin stem cells by secreted factors [9], [10].

There are surgical reports stating that fat transplantation leads to improvements in skin and hair [11]. Therefore, the injection of autologous fat

in the scalp has been studied as a possible treatment for alopecia. Autologous fat grafting is a technique with more than 100 years that uses fat removed during plastic surgeries for the volumization of other areas. Autologous fat is less expensive than other fillings, easily harvested and biocompatible [11]–[13]. However, the long-term retention of fat grafts is variable, ranging from 20% to 80% estimated loss, which leads to the need for repeated fat transfer procedures [11]. Fat resorption may be due to lack of proper vascularization, dedifferentiation of adipocytes or the presence of contaminants. The high variability is most likely due to the lack of standardized methodologies for fat harvest, processing and injection. Nevertheless, ever since the discovery of AT-MSCs in 2001, there has been a remarkable increase in the usage and clinical experience regarding autologous fat transfer [11], [12], [14]. Several recent studies have been focusing on autologous fat transfer enriched with SVF to enhance adipocyte viability and graft retention [10]. In 2017, the first pilot study on the injection of enriched fat in the scalp for the treatment of alopecia was published, presenting promising results and demonstrating the safety of such treatment [11]. More clinical trials are being conducted nowadays to assess the tolerability and efficiency of such therapy [15]. Since neither autologous fat transfer nor the enrichment in SVF would involve modification of the cells and their function, their approval by regulatory agencies would be relatively faster than for Advanced Therapy Medicinal Products [16]. Furthermore, this therapy could be minimally invasive and possibly ambulatory if narrow cannulas were used and the size and number of incisions was limited, avoiding the need for general anaesthesia [17]. Hence, this therapy seems promising not only due to scientific evidence but also to its easier acceptance by regulatory agencies and patients. Cells can lose their viability or be influenced by several different parameters, causing the aforementioned loss of volume of fat grafts. Some of these are the surgical techniques used for fat harvesting and injection, as well as the width, length and shape of the cannulas used and the applied pressure [18]–[20]. Patient factors and the choice of donor site for harvesting may also have an impact on cell yield and viability [21]. Moreover, there is still no standardized procedure for fat processing. Protocols may rely on enzymatic or mechanical methodologies, different centrifugation

parameters, and be carried out either in the laboratory or at the point of care in closed systems that have been developed recently and that are still being improved [22]–[24]. Such differences influence the isolated cells and so it is imperative to establish a standard procedure for cell isolation. Closed, sterile and automatized systems would be the ideal choice as they minimize protocol variations, contamination risks and unpredictability of the isolated cells, while complying with regulatory requirements and good manufacturing practice (GMP) guidelines [22], [25], [26]. The aim of this work was to demonstrate the impact of different cannula widths and surgical procedures on the different cell types contained in the SVF upon enzymatic isolation. Small cannula widths were focused on due to the requirements for a minimally-invasive and ambulatory procedure. The following conditions were studied: 1) fat harvesting with a 0.8 mm cannula followed by decantation; 2) fat harvesting with a 0.8 mm cannula followed by decantation and emulsification with a 0.8 mm cannula; 3) fat harvesting with a 0.8 mm cannula followed by decantation, emulsification with a 0.8 mm cannula and simulated injection with a 25G or 23G cannula; 4) fat harvesting with a 2.4 mm cannula followed by decantation; 5) fat harvesting with a 2.4 mm cannula followed by decantation and simulated injection with a 0.9 mm cannula; 6) automatized fat harvesting with a 4 mm cannula with a pressure of 0.8 bar; 7) automatized fat harvesting with a 4 mm cannula with a pressure of 0.8 bar followed by decantation; 8) fat excision with a 0.5 mm cannula post-surgery without purification; and 9) fat excision with a 2.2 mm cannula post-surgery without purification. It is important to note that the conditions can be divided into four clusters since some of the techniques were applied sequentially: 1 to 3, 4 and 5, 6 and 7, and 8 and 9. Samples from different conditions were analysed in terms of cell yield per mL of processed fat. AdipoRed and Hoechst 33342 staining was performed on samples of fresh fat for the distinction of adipocytes and oil droplets. The SVF cells were characterized by flow cytometry to determine their immunophenotype, and colony forming unit – fibroblast (CFU-F) assays were performed to evaluate their clonogenicity. SVF cells were cultured and, after some passages, the isolated AT-MSCs were characterized in terms of clonogenic ability and differentiation potential using CFU-F assays and multilineage

differentiation studies, respectively. The expression of stromal surface markers was analysed by immunophenotypic analysis.

2. Materials and Methods

2.1. Isolation of Human AT-MSCs

AT samples were provided by Dr. Tiago Baptista Fernandes from Hospital St. Louis, Portugal. The samples were collected from healthy donors after written informed consent according to the Directive 2004/23/EC of the European Parliament and of the Council of 31 March 2004 on setting standards of quality and safety for the donation, procurement, testing, processing, preservation, storage and distribution of human tissues and cells (Portuguese Law 22/2007, June 29).

AT samples were washed with an equal volume of Phosphate Buffered Saline (PBS, Gibco®) with 1% Antibiotic-Antimycotic (Gibco®). After gentle shaking, AT was allowed to settle and two phases were formed, an AT supernatant and an aqueous infranatant that was discarded. This washing step was repeated 2 times more to remove erythrocytes. Afterwards, the AT was mixed with an equal volume of 0.1% Collagenase Type II solution in Hank's Buffered Salt Solution (HBSS, both from Gibco®). The enzymatic digestion was performed at 37°C for 30 min at 500 rpm in a thermomixer (Eppendorf). The resulting digested product was filtered through a Steriflip filter unit (Merck Millipore®), followed by centrifugation at 1250 rpm for 7 min. The supernatant was discarded and Ammonium Chloride was added in a 4:5 proportion. The samples were left at 4°C for 10 min to lyse the remaining erythrocytes. Afterwards, the samples were centrifuged at 1750 rpm for 10 min. The SVF pellet was washed once with Dulbecco's Modified Eagle's Medium (DMEM, Gibco®) supplemented with 10% Foetal Bovine Serum (FBS, Gibco®) and 1% Antibiotic-Antimycotic and then centrifuged at 1250 rpm for 7 min. The pellet was resuspended in DMEM supplemented with 10% MSC qualified FBS (Hyclone®) and 1% Antibiotic-Antimycotic (hereinafter referred to as culture medium). The cell number was then determined under the microscope (Olympus) by using the Trypan Blue (Gibco®) dye exclusion method in a 1:2 proportion. Cells were plated at 2.5×10^4 cells/cm² in 24-well plates (Sigma®) in culture medium and incubated at 37°C and 5% Carbon Dioxide (CO₂) in a humidified atmosphere. After 24h, the supernatant was discarded and replaced by fresh culture

medium, which was changed every 3 days. If confluency was not achieved within 16 days, the samples were discarded. If cells were 80% to 90% confluent, cells were either passaged and plated in culture medium at a cell density of 3×10^3 cells/cm², or prepared for flow cytometry or cryopreserved depending on the cell number obtained.

2.2. Immunophenotypic Analysis

SVF cells were analysed by flow cytometry using a panel of mouse anti-human monoclonal antibodies for the expression of CD34 (Fluorescein Isothiocyanate (FITC)-conjugated, BioLegend®), CD31 (Phycoerythrin (PE)-conjugated, BioLegend®), CD45 (Peridinin-chlorophyll proteins – Cyanine dye 5.5 (PerCP-Cy5.5)-conjugated, BioLegend®). The viability stain LIVE/DEAD™ Fixable Far-Red Dead Cell Stain Kit (LIVE/DEAD, Thermo Scientific™) was also used. Before preparing SVF cells for flow cytometry, it was necessary to verify if there were enough dead cells using the LIVE/DEAD stain. If that was not the case, then the volume of cell suspension that corresponded to half of the volume for the single staining of the LIVE/DEAD marker was put aside in order to apply the death protocol, which consisted of subjecting the cells to 70°C for 30 min in a thermomixer. Then, cells were centrifuged at 1250 rpm for 7 min, resuspended in PBS and centrifuged again at 1250 rpm for 7 min. Afterwards, the cells were resuspended in 100 µL of PBS per Fluorescence-activated Cell Sorting (FACS) tube (BD Biosciences) and distributed throughout the FACS tubes using a filter (Thermo Scientific™) to avoid the presence of fat and oil residues. The cells were then stained with 10 µL of diluted LIVE/DEAD staining per 1×10^5 cells and incubated at room temperature for 30 min in the dark. PBS was added and cells were centrifuged at 500 g for 5 min. The PBS was discarded and cells were resuspended in 50 µL of PBS per FACS tube. Then the cells were stained with 5-20 µL of the desired antibody(ies) and incubated at room temperature for 15 min in the dark. PBS was added and cells were centrifuged at 500 g for 5 min. If flow cytometry was not going to be performed right away, the cells were resuspended in 2% Paraformaldehyde (PFA, Sigma®) and stored at 4°C to preserve the cells up to 7 days. Isolated MSCs were analysed by flow cytometry using a panel of mouse anti-human monoclonal antibodies for the expression of CD73 (FITC-conjugated, BD Biosciences), CD105 (FITC-

conjugated, BD Biosciences), CD34 (PE-conjugated, BioLegend®), HLA-DR (PE-conjugated, BioLegend®), CD45 (PerCP-Cy5.5-conjugated, BioLegend®), CD90 (PerCP-Cy5.5-conjugated, BioLegend®), CD11b (Allophycocyanin (APC)-conjugated, BD Biosciences) and CD19 (APC-conjugated, BD Biosciences). MSC preparation was similar to the above described protocol for SVF cells, with the exception that the death protocol, filtration and LIVE/DEAD staining were not performed.

After cell preparation, each sample was analysed by flow cytometry (FACSCalibur cytometer, Becton Dickinson®) using the CellQuest™ software (Becton Dickinson®). The results were analysed using the FlowJo X 10.0.7r2 software.

2.3. AdipoRed and Hoechst 33342 Staining

AdipoRed™ Assay Reagent (AdipoRed, Lonza) and Hoechst 33342 (trihydrochloride, trihydrate, 10mg/mL, Thermo Scientific™) staining was performed on clean fat, which corresponds to AT after the washing step. If a particle was positive for both stains, it was considered an adipocyte. If the particle was positive only for AdipoRed, it was defined as a lipid droplet, whereas if it was positive only for Hoechst 33342, it was considered a cell other than an adipocyte [3].

15 µL of AdipoRed and 5 µL of Hoechst 33342 (0.1 mg/mL) were added to 500 µL of cell suspension, followed by incubation at room temperature for 15 min in the dark. Then the cells were observed under the microscope.

2.4. CFU-F Assay

The clonogenicity of both MSCs contained within the SVF and isolated MSCs was tested, though with different initial densities to account for the fact that not all SVF cells are MSCs. Firstly, the cells were plated either on a T-flask 25 (T25, BD Falcon™) at 10 cells/cm² in the case of isolated MSCs, and on a well of a 6-well plate (Sigma®) at both 50 cells/cm² and 100 cells/cm² for SVF cells. Cells were incubated at 37°C and 5% CO₂ in a humidified atmosphere for 14 days for MSCs and 27 to 29 days for SVF cells. The culture medium was changed every 3 days. Afterwards, the cells were washed once with PBS and incubated with 0.5% Crystal Violet (Sigma®) solution in Methanol (Sigma®) for 30 min at room temperature. The stained colonies were rinsed four times with PBS and once with distilled water. Finally, the colonies were counted under the microscope.

2.5. Multilineage Differentiation Studies

Multilineage differentiation assays (osteogenic, adipogenic and chondrogenic lineages) were performed for isolated MSCs.

2.5.1. Osteogenic Differentiation Potential

The cells were cultured at 3×10³ cells/cm² in 24-well plates with culture medium. Once they were 80% to 90% confluent, osteogenesis was induced using StemPro® Osteogenic Differentiation Kit (Gibco®). The medium was changed twice a week for 14 days and then the Alkaline Phosphatase (ALP) and von Kossa staining was performed to evaluate osteogenic commitment and the presence of deposits of calcium, respectively. Firstly, the medium was removed and cells were rinsed once with PBS. The cells were then fixed with 4% PFA for 30 min at room temperature and rinsed twice with PBS. Staining with Naphtol (Sigma®) mixed with Fast Violet (Sigma®) in a 1:26 proportion was performed, followed by incubation at room temperature for 40 min in the dark. Then, the cells were rinsed three times with distilled water and Silver Nitrate (Sigma®) was added to stain the cells upon incubation at room temperature for 30 min. The cells were rinsed once with distilled water and left in PBS for observation under the microscope.

2.5.2. Adipogenic Differentiation Potential

The cells were cultured at 3×10³ cells/cm² in 24-well plates with culture medium. Once they were 80% to 90% confluent, adipogenesis was induced with StemPro® Adipogenic Differentiation Kit (Gibco®). The medium was changed twice a week for 14 days and then the Oil Red-O staining was performed to assess the accumulation of lipids, characteristic of the differentiation process towards the adipogenic lineage. Firstly, the medium was removed and the cells were rinsed once with PBS. Then, fixation was performed at room temperature during 30 min using 4% PFA. Afterwards, cells were rinsed once with distilled water and incubated with 60% Isopropanol (Sigma®) for 5 min at room temperature. Subsequently, the cells were incubated with the Oil Red-O (Sigma®) staining solution and rinsed three times with distilled water. Cells were left in distilled water for observation under the microscope.

2.5.3. Chondrogenic Differentiation Potential

This assay was performed using the hanging drop method. First, 5×10⁴ cells per droplet were centrifuged at 1250 rpm for 7 min. The supernatant

was removed and cells were resuspended in about 30 μ L of culture medium per droplet. 6 – 7 mL of PBS were placed on the bottom of a Petri dish (Thermo Scientific™) to prevent the droplets from drying. On the undersurface of the inverted lid, 30 μ L droplets were placed sufficiently apart from each other. The lid was inverted carefully and the Petri dish was closed. After overnight incubation at 37°C and 5% CO₂ in a humidified atmosphere, the lid was turned upwards and the cell aggregates were transferred into ultra-low binding (ULB) 24-well plates (Corning Inc.) together with StemPro® Chondrogenic Differentiation Kit (Gibco®). The aggregates were then incubated at 37°C and 5% CO₂ in a humidified atmosphere for 14 days and the medium was changed twice a week. After that, the Alcian Blue (Sigma®) staining was performed to evaluate the synthesis of proteoglycans by chondrocytes. Firstly, the medium was removed and the aggregates were rinsed once with PBS. Then, the cell aggregates were fixed for 30 min at room temperature using 4% PFA, after which they were rinsed once with PBS. Next, incubation with 1% Alcian Blue in 0.1 N Hydrogen Chloride (HCl, Sigma®) was performed for 1h, followed by three rinses with 0.1 N HCl. The cell aggregates were left in distilled water for observation under the microscope.

2.6. Statistical Analysis

Relationships between variables were analysed with different statistical tests that do not assume normality as it was not verified for the tested variables. Depending on the variables' natures, Spearman's correlation, logistic regression, Fisher's exact test, Mann Whitney test and Kruskal Wallis test were used. The independent variables were body mass index, age, donor site, days until processing and conditions. The dependent variables were the number of isolated SVF cells per mL of AT, being able to achieve enough confluency to be passaged, and if so the number of days until passaging. The cell number per mL of AT was also regarded as an independent variable when compared with the other two dependent variables. Gender was not included as all donors were females. A p-value inferior to 0.05 was considered as statistically significant. All statistical analyses were completed using the SPSS Statistics V22.0 software (IBM Corporation, USA).

3. Results and Discussion

3.1. Cell Yield

There was a great dispersion regarding the number of cells per mL of processed AT for each condition between different donors. High donor dependence has been reported previously by Kilinc *et al.*, whose study verified that SVF cell yield depends on the patient's characteristics [27]. The results may have been influenced by the inability to determine exact initial volumes, use of a not so adequate collagenase type, undigested lumps that could not be filtered, and subjective manual cell counts after cell isolation that might have overestimated the number of cells by confusing them with oil droplets or debris.

When clustering conditions according to their respective groups, there were no tendencies common to all donors besides a decrease from condition 4 to condition 5, which is natural since it was the most drastic decrease in cannula width. However, the average cell number per mL for each condition showed that overall there is a decrease in cell yield when the harvesting conditions involve more manipulation of the cells and a progressive decrease in cannula width. This tendency could be observed from conditions 1 to 2, 2 to 3 and 4 to 5. Fat from conditions 6 and 7 presented similar cell yields per mL. From condition 8 to 9 there was an increase in cell yield with increasing cannula width, but only one donor was analysed.

It was expected that between conditions 1 and 4, in which the only varying parameter was the cannula width, there would be a lower cell number per mL of AT for condition 1, which involves a much narrower cannula. However, that was not verified and the cell yield obtained was actually quite similar. This might have been due to the subjectivity associated to cell counting.

It is also interesting to compare conditions 1 and 4 with condition 7, which differ in terms of cannula width and use of a robot for fat harvest. Although condition 7 involved the largest cannula, the average cell number per mL obtained was much lower than for the other two conditions. Conditions 6 and 7 always presented many more lumps of dense fat, either due to the use of a robot or to the larger cannula used, which would not grind fat as much as narrower cannulas. Such lumps were hard to digest and the posterior filtration step would sometimes retain them, which probably led to cell loss. However, the lower cell counts verified may have been caused by the harvesting method itself instead of fat processing and respective

limitations. The use of a robot with the specified pressure might have had a harsher impact on cell number than the usual manual harvesting process.

3.2. Cell Characterization

3.2.1. AdipoRed and Hoechst 33342 Staining

AdipoRed and Hoechst 33342 staining was performed on clean fat from conditions 1 to 7 from one donor. Its observation under the microscope led to the notion that most particles containing lipid droplets were oil droplets (yellow arrow on Figure 1) and not adipocytes (white arrow on Figure 1) due to lack of Hoechst 33342 staining on top of the AdipoRed staining.

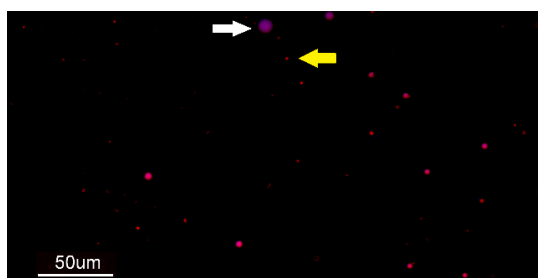


Figure 1 - Merged image of AdipoRed and Hoechst 33342 staining on clean fat.

3.2.2. Days Until Passaging

Within 16 days of cell culture, in some cases AT-MSCs could achieve the necessary confluency level for passaging, but the majority of the cultures could not. Table 1 shows the number of cultures that could be passaged or not.

The initial cell density is definitely one factor that influences the ability to become confluent. On one donor, cells were plated at an initial cell density of 1×10^5 cells/cm² and it was possible to isolate MSCs from most conditions. However, such high cell density hindered the determination of confluency and thus an initial cell density of 2.5×10^4 cells/cm² was used instead afterwards,

which led to an evident decrease in the amount of cultures that became confluent.

The MSC isolation efficiency for all the conditions tested was low. However, it is evident that in conditions 2 and 3, which involved more intensive manipulation, it was not possible to isolate MSCs from a single donor. These results seem to defy the usage of intense manipulation and small cannula widths for the treatment of alopecia if the goal is to obtain viable AT-MSCs and to profit from their beneficial effects. Conditions 1 and 4 were clearly the ones associated with more proliferation from a qualitative perspective, as even in non-confluent wells there were always more replicating progenitors comparing with other conditions. Conditions 6 and 7 have less evidence, but their overall aspect always involved many more small particles than the other conditions, which could be adherent small non-stromal cells or debris. For conditions 8 and 9 the results have much less confidence since they are based on a single donor. However, it is important to note that overall, in every group of conditions, if more manipulation and narrower cannulas were involved in the harvesting process, there would be a decrease in the MSC isolation efficiency.

Isolated AT-MSCs could be grown at least until passage number 5 (P5), with progressive loss of contaminants.

The statistical analysis performed between donor site and the ability to become sufficiently confluent to be passaged or not revealed a statistically significant relationship with a p-value of 0.005. 66.7% and 93.8% of the abdominal fat samples and of the mixture of abdominal and flank fat samples were not passaged to P1. Therefore, it can be stated that abdominal fat alone has led to the isolation of cells with greater proliferative

Table 1 – Days until passaging from P0 to P1 for each condition. The number of cultures passaged on each day and the interval within which the cultures were passaged are highlighted in grey. The number of non-confluent cultures is presented on the last column against the total number of donors for each condition.

	Days Until Passaging from P0 to P1																Not Passaged
	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	
1								1		1							n=10/12
2																	n=12/12
3																	n=11/11
4						1		1			1						n=9/12
5									1							1	n=10/12
6										1		1					n=3/5
7						1											n=5/6
8																	n=1/1
9			1														n=0/1

abilities than the mixture of different types of fat. This is in accordance with the tendency verified in the literature regarding cells isolated from the abdominal region [21], [28]–[30].

3.2.3. Immunophenotypic Analysis

3.2.3.1. SVF Cells

Firstly, cryopreserved SVF that was processed without a lysis step was analysed. The first step of the gating strategy consisted of identifying the SVF population and excluding debris using an unstained sample. Next, live cells were gated out of the SVF population and from a sample only stained with the LIVE/DEAD staining. Out of the population of live cells, CD45⁻ cells were selected from a full stained sample in order to exclude haematopoietic cells. Then, four quadrants were designed to analyse all possible combinations between the CD31 and the CD34 surface markers. According to Suga *et al.*, CD45⁺ cells are blood-derived while CD45⁻ are from adipose origin. Out of these, CD34⁺ CD31⁻ cells correspond to AT-MSCs, CD34⁺ CD31⁺ to endothelial progenitor cells and CD34⁻ to other cells such as pericytes. Suga and colleagues reported 37% of blood-derived cells, 37% of AT-MSCs, 15% of endothelial progenitor cells and 11% of other cells [3]. In the present study, cryopreserved SVF presented 18.6% of haematopoietic cells, 63.7% of AT-MSCs, 4.3% of endothelial cells and 13.4% other cells. The percentage of haematopoietic cells was clearly lower in this work, even though no lysis step was involved. This was probably due to cryopreservation, which has already been described to cause depletion of haematopoietic cell populations [31]. The percentage of endothelial progenitor cells was also lower in this study, and an enrichment in AT-MSCs was verified. It is important to note that Suga *et al.* did not use any staining to distinguish live from dead cells and did not include a lysis step in their cell isolation protocol [3]. Furthermore, the authors determined all CD34⁺ CD31⁻ CD45⁻ cells to be AT-MSCs, but other cells such as preadipocytes and adipocytes could be included. Although adipocytes are very large, the diameter of the fluidics of cytometers allows the passage of at least part of the fat cells, which are characterized by a great deformability that would also help in this matter [32]. To effectively distinguish AT-MSCs from these two other cell types, CD105 could be used as the later cells are negative for it whereas AT-MSCs are positive [33], [34].

On another donor, fresh cells from conditions 1 to 5 were analysed, leading to different results from what was obtained with cryopreserved SVF. Only in condition 4 a distinct AT-MSC population was found, though it was rather small (3.2% of the total SVF live cells). All other conditions presented even fewer AT-MSCs, and the number of endothelial progenitor cells was almost null in all conditions. Although it may be just a coincidence, it was interesting to find that the condition involving a much larger cannula presented better results in terms of the population of interest. The most interesting aspect of this flow cytometric analysis was the confirmation of an increase in oil droplets with decreasing cannula size and increasing fat manipulation, which is most likely due to a higher percentage of adipocytes rupturing and consequently releasing their inner lipidic contents to the surrounding environment [3]. Oil droplets typically create a S-shaped diagram due to the oscillations in the intensity of the forward scatter (FSC), while there is a monotonic increase of the intensity of the side scatter (SSC) due to the size of the droplets [35]. An increase of the proportion of events presenting this S-shape with low FSC values was observed from condition 1 to 2 (observable in Figure 2), 2 to 3 and 4 to 5, which correspond precisely to decreases in cannula width and/or more fat manipulation.

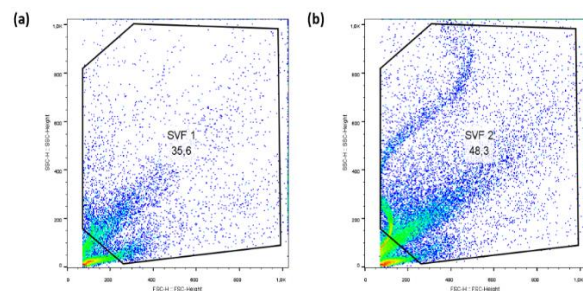


Figure 2 – Total cell populations for conditions (a) 1 and (b) 2, showing an increase in oil content.

Flow cytometry was also applied to conditions 6 and 7 from fresh SVF of a different donor. In condition 6, 59.9% of the cells were blood-derived, while 1.6% were AT-MSCs, 0% endothelial progenitor cells and 38.5% were other cells. The distribution of cells in condition 7 was as follows: 41.5% haematopoietic cells, 2.5% AT-MSCs, 0% endothelial progenitor cells and 56.0% other cells. Although the cell distribution for the two conditions was slightly different, they were similar in the fact that the cells of interest were almost non-existent. Nevertheless, it is important to notice that there

was a clear population of AT-MSCs even though it was very small. The expression levels of each individual expression marker were quite similar, being the haematopoietic marker CD45 the only one with a greater difference, which is coherent with the fact that condition 7 corresponds to condition 6 after decantation. Furthermore, these results corroborate the ones presented previously in terms of haematopoietic populations on the multicolour analysis.

3.2.3.2. Isolated AT-MSCs

Immunophenotypic analysis of isolated MSCs was performed on three different donors, whose results are presented in Figure 3.

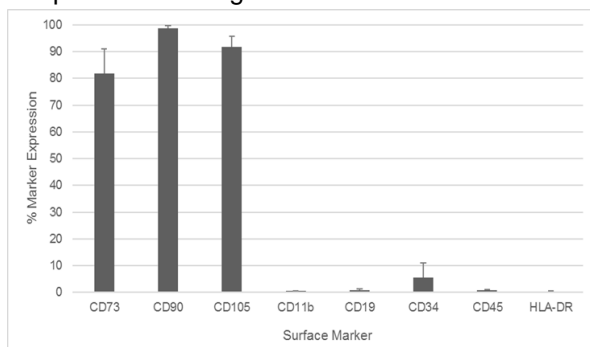


Figure 3 - Isolated AT-MSCs surface marker expression (n=3).

CD90 presented an expression over 95%, while less than 2% of the cells analysed expressed CD11b, CD19, CD45 and HLA-DR, which is in agreement with the minimal criteria determined by the International Society for Cellular Therapy (ISCT) [4]. CD105 expression level was lower than 95%, but still higher than 90%, which corresponds to the limit established by the joint statement of the International Federation of Adipose Therapeutics and Sciences (IFATS) and the ISCT [2].

Although CD73 expression was still quite high and completely distinct from the expression levels of the markers that are usually negative on MSCs, it was below the 90% limit. A possible reason for this is that freshly isolated MSCs may present low initial expression of CD73, CD90 and CD105, which increases throughout cell culturing [36]. Although these markers are implied in the minimal criteria for defining MSCs, those requirements were mainly determined based on adherent cultures and not fresh samples. Since the samples analysed were at low passage number (either P1 or P2), the expression of these markers could have still been increasing [37].

CD34 also showed a slightly deviant profile regarding the 2% threshold determined by the

ISCT. However, this was determined based on MSCs growing on plastic surfaces. Freshly isolated MSCs from various tissues have been found to express CD34 in several studies (reviewed in [38]). Therefore, it has been proposed that CD34 is expressed when MSCs are in resident tissues and it becomes negative as a consequence of cell culturing [2], [38].

3.2.3.3. Isolated AT-MSCs with Slower Growth

Some of the isolated cells from SVF presented a slower growth, not achieving confluency after 16 days of culture. However, the MSCs were clearly visible and formed large colonies that were highly concentrated in a small area of the well. To understand the difference between the cells that formed such colonies and the usual MSCs, immunophenotypic analysis was performed on two samples from these cells using the same panel of antibodies. The results are presented in Figure 4.

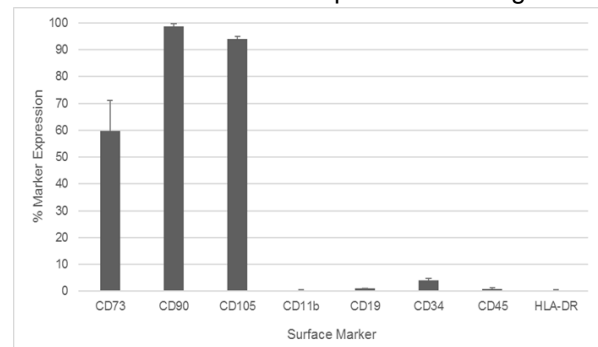


Figure 4 - Surface marker expression of isolated AT-MSCs with slower growth (n=2).

The expression profile of the isolated MSCs with slower growth is quite similar to the one of MSCs with a usual growth rate. CD90 is above 95%, and CD11b, CD19, CD45 and HLA-DR are below 2% expression levels, meeting the minimal criteria for MSC definition [4]. CD105 expression is slightly lower than the 95% limit but still above 90%, resembling the isolated MSCs with normal growth rate. CD34 was expressed in 4.15% of cells, which also represents a very slight difference regarding the 2% limit established by the ISCT, similarly to the MSCs with usual growth rate [2].

The greatest difference between the two sets of MSCs was the expression of CD73, which was present in only 59.65% of the cells of the latter group. Although this could have been caused by still growing expression levels, it is not common to find MSCs with such low expression of CD73. However, in a study by Ode *et al.* from 2011 in which bone marrow MSCs were characterized under mechanical loading, it was demonstrated

that both CD73 and CD29 have a diminished expression level after applying a loading stimulus. This stress also led to a decrease in migratory properties of MSCs. Consequently, the authors proposed that CD73 and CD29 are simultaneously involved in the mediation of the decrease in migratory capacity caused by mechanical stress [39]. In the present study, diminished levels of CD73 were found in certain populations of MSCs that were unable to achieve confluency and remained highly concentrated in a single colony. This might be due to a decrease in migratory ability. Therefore, it is hypothesised that these slower growth MSCs corresponded to MSCs that were under mechanical stress in the resident tissues or that suffered more mechanical loading during the harvesting and/or isolation process than the other MSC populations, which caused a decrease in the expression of the surface marker CD73 and in their migratory capacity. Nonetheless, these differences might also be an inherent characteristic of these cells as MSC population are highly heterogeneous, not only between different sources but also MSCs from the same source and clonal MSCs [37].

3.2.4. CFU-F Assays

CFU-F assays were performed on SVF cells from conditions 1 and 4 from three different donors straight after the isolation process. Since each colony is supposed to be originated from one progenitor, the frequency of stromal progenitors was 0% to 1% in the present study, which is below the 1% to 10% interval relative to the total percentage of nucleated cells as specified by the joint statement of the IFATS and the ISCT [2]. This may be due to loss of viability of the progenitor cells during the harvesting process of the specified conditions. It is important to note that cell cultures lasted for 27 to 29 days instead of 11 to 14 days so as to obtain more developed and visible colonies, which may have influenced the results. Furthermore, it should be noticed that CFU-F assays were only conducted on conditions 1 and 4, which probably had an impact on the results as these were the conditions in which cells presented a greater proliferative potential. Nonetheless, the proliferation of cells cultured at such a low initial density confirms the presence of stromal progenitors within the heterogeneous SVF populations and proves their clonogenic ability. CFU-F assay was also performed on isolated AT-MSCs from one donor, leading to a final density of

approximately 5 colonies/cm². Although the colonies were not very dense, maybe due to being already at P5 while progenitor frequency was evaluated at P1 in the joint statement of the IFATS and the ISCT [2], the proliferation of cells cultured at such a low initial density proves the clonogenic abilities of the tested AT-MSCs. About 50% of the initial nucleated cells were stromal progenitors, which is much higher than the threshold presented in the joint statement of the IFATS and the ISCT and within the established values (frequency of more than 5% [2]).

3.2.5. Multilineage Differentiation Studies

AT-MSCs from two donors were characterized in terms of multilineage differentiation potential into three different lineages: osteogenic, adipogenic and chondrogenic. Both ALP and von Kossa staining was strong, reflecting the presence of immature osteoblasts and calcium deposits, respectively. Oil Red-O staining revealed the presence of lipid vacuoles within the cells, which is attributed to adipogenic commitment. Differentiation of chondrocytes was made visible through Alcian Blue staining, identifying proteoglycans within cell aggregates. The multipotency and differentiation potential of AT-MSCs were thus demonstrated and they are in agreement with the joint statement of the IFATS and ISCT [2].

4. Conclusions and Future Trends

The cell yield per condition upon cell isolation was quite divergent depending on the donor. There were no tendencies common to all donors, except for a decrease from condition 4 to 5. This is consistent with the fact that this cluster involved the most remarkable decrease in cannula width. However, the average cell number showed that more fat manipulation and the use of progressively narrower cannulas lead to a decrease in cell yield. Interestingly, automatized conditions involving larger cannulas led to lower cell counts than manually harvested fat with narrower cannulas. One possible explanation is that the use of a robot with the specified pressure may influence the cells. The cell numbers obtained would be much more reliable if an automatic cell counter had been used, which is highly recommended for future studies, as well as the use of clinical-grade closed, sterile and automatized isolation systems.

Staining with AdipoRed and Hoechst 33342 revealed that most particles including lipids did not

exhibit a nucleus, hence being classified as oil droplets instead of adipocytes. It would be valuable to perform this experiment on more donors together with a quantitative analysis to confirm these results.

SVF cells were cultured and the MSC isolation efficiency was low for all harvesting conditions. However, the cells of conditions 2 and 3 never reached sufficient confluency to be passaged, which suggests that smaller cannulas and more manipulation of AT during fat harvesting leads to a decrease in the proliferative potential of the AT-MSCs contained within it.

Most immunophenotypic analyses involving fresh SVF cells were not successful, particularly due to insufficient cell numbers. Therefore, it is advisable to collect larger amounts of fat in future studies.

Fresh cells from conditions 1 to 5 from one donor were analysed, leading to the identification of a clear small AT-MSC population only on condition 4. Since this was the condition involving a larger cannula, it is suggested that smaller cannulas and more manipulation may be harmful for AT-MSCs, leading to the loss of these cells during the harvesting process. Interestingly, increasing amounts of oil droplets were found within the clusters of conditions, indicating that narrower cannulas and greater manipulation may lead to more adipocytes rupturing and consequent release of lipid droplets.

On another donor, small AT-MSC populations were identified within fresh cells from conditions 6 and 7. The main difference between the two conditions was that the haematopoietic population was greater for condition 6, which is consistent with the fact that decantation was only performed for condition 7. Immunophenotypic analysis was also performed for cryopreserved SVF, which led to much more satisfactory results, with an evident AT-MSC population within the SVF live cells.

AT-MSCs were also characterized by immunophenotypic analysis, which revealed that the stromal markers CD73, CD90 and CD105 were highly expressed by these cells whereas CD11b, CD19, CD45 and HLA-DR were expressed by less than 2% of the cell population. Although still low, CD34 had slightly higher expression levels, which have been described to decrease throughout cell culturing. These results were predominantly in accordance with the joint statement of the IFATS and ISCT [2]. During cell culturing, it was noticed that some AT-MSCs grew at a slower pace in localized colonies that did not achieve confluency

except for their limited area. Such cells were also analysed by flow cytometry, which exposed that only CD73 expression level was decreased comparing with MSCs with the usual growth rate. It is suggested that these cells were subjected to greater mechanical stress than most MSCs of the processed AT, either during fat harvesting or cell isolation. In the future, more studies should be performed on mechanically stressed AT-MSCs in order to confirm these findings and highlight the role of the CD73 surface marker.

Although the progenitor frequency of MSCs contained within SVF cells was below the interval established by the literature, namely 0 – 1% instead of 1 – 10%, the result obtained for isolated MSCs was in agreement with the literature, specifically 50% versus more than 5% [2]. Since only conditions 1 and 4 of SVF were tested, it would be interesting to ascertain whether other conditions associated with lower proliferative ability would behave differently in terms of clonogenicity.

Multilineage differentiation studies performed on isolated MSCs were successful, as the cells differentiated into the osteogenic, adipogenic and chondrogenic lineages.

Overall, narrower cannulas and more fat manipulation seem to affect the cell content and viability of SVF cells. However, larger cannulas do not comply with a minimally invasive protocol. Therefore, it is imperative that more studies comparing wide and narrow cannulas are performed in the future so as to identify the ideal compromise between higher cell numbers and viability and minimally-invasive procedures. Nonetheless, in this work it has already been determined that it is possible to obtain fully functional and multipotent AT-MSCs from fat harvested with cannulas as small as 0.8 mm, as they presented the expected immunophenotype, clonogenicity and differentiation potential. However, as this was not possible for all donors, better methodologies for fat harvest, processing and injection must be developed in order to obtain the desired cell populations independently from donor variations. These new methods should also be standardized in order to allow reliable comparisons throughout different studies and to create a dependable therapy for alopecia patients with the potential to be approved by regulatory agencies.

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