Characterization of adipose derived mesenchymal stem cells received via automated extraction

Author: Katarina Edbom, Bachelor of Medicine
Supervisor: Karin Johanson, PhD, Department of Laboratory Medicine, Örebro University hospital
Abstract

Introduction Stem cells are defined by their potential to differentiate into cells from all three germ layers. In 2001 Zuk et al. revolutionized stem cell research by identifying mesenchymal stem cells in adipose tissue. This enabled an easier and more ethical acceptable harvesting procedure and opened the possibility of autologous cell therapy.

Aim To analyze character, sterility and potency of the stromal vascular fraction (SVF) received by automated extraction using Sepax2® (BioSafe, Eysins, Switzerland) and cultured adipose derived stem cells (ADSCs).

Materials and Methods SVF was automatically extracted from adipose tissue from three patients using Sepax2®. ADSCs were isolated from the SVF and cultured for five passages. Sterility was tested by culturing rest fluids from the extraction procedure in blood culture flasks and by an endotoxin measurement of the final product. Cell surface markers of the SVF and cultured ADSCs at each passage were identified using flow cytometry. Morphology was documented continuously during culture and by a cytopsin analysis with May-Grünwald-Giemsa staining at each passage. A colony forming unit (CFU) assay and a cell count using trypan blue was used to measure viability. Telomerase activity was analyzed as a possible cell potency marker.

Results The SVF and cultured ADSCs had the expected cell surface markers and morphology, and the extraction procedure was sterile. Viability differed in the CFU assay (5.8 and 0 colonies per 100 cultured cells, and overgrown) and the cell count (25-38 days of culture to reach five passages). Telomerase activity in cultured ADSCs was not detected by ddPCR TRAP.

Conclusion This study suggests that automated extraction is safe and effective, but points to the need of other assays for direct identification of cell potency. Further research is needed to investigate autologous cell therapy using the SVF from adipose tissue.

Introduction Mesenchymal stem cells (MSCs) are pluripotent cells, which have the ability to differentiate into cells from all three germ layers. MSCs are defined by three criteria (I)
adherence to plastic in standard culturing conditions, (II) have the phenotype CD105+, CD73+, CD90+, CD45-, CD34-, CD1+CD11b-, CD79α-/CD19-, HLA-DR and (III) in vitro differentiation to osteoblasts, adipocytes and chondroblasts [1].

Bone marrow is used as a primary source of MSCs. The harvesting procedure for bone marrow derived stem cells (BMSCs) is painful, and requires ex vivo cell expansion because of a low stem cell density [2]. Therefore, it was a major break-through when MSCs were found in adipose tissue by Zuk et al. in 2001 [3]. This opened the possibility for easier and safer harvesting of MSCs. Another advantage for adipose derived stem cells (ADSCs) is the portion of MSCs in the end product, where MSCs from adipose tissue reach ~2% and only ~0.002% from bone marrow [4].

ADSCs are used in several clinical studies involving a wide range of medical fields: orthopedics [5,6], cardiology [7], plastic surgery [8], wound healing [9,10] and diabetes [11]. These studies show promising results and few adverse events. Many studies have small study populations, and the primary aim has so far mostly been to evaluate safety, and to analyze therapeutic effects as a secondary aim. There are different ideas of how to administrate ADSCs to receive the best therapeutic effect, and different suggestions of how the ADSCs affect the target tissue. This has been investigated in several studies, e.g. in the potential therapy of myocardial infarction where intracoronary versus transendocardial administration is discussed [12]. It seems that the choice of administration does have an impact. One possible factor influencing this is the actual action of the ADSCs. There are studies suggesting that ADSCs do not differentiate into the target tissues cell type but act on the damaged tissue by paracrine signaling [13]. However, the field is still young and needs more research.

To involve cell therapy in clinical praxis it is desirable to develop an effective and safe extraction method, considering sterility, immunological reactions and with as little discomfort for the patient as possible. To prevent immunological reactions autologous cell transplantation is the most optimal alternative, preferably without culturing the cells beforehand as this often requires products with potentially immunogenic properties, for example bovine serum. There is also a problem in containing cells at good quality during culture, because a probability of cell differentiation and chromosome damage [14].
Several instruments have been developed to extract stem cells automatically [15,16]. To avoid culturing the quantity of extracted ADSCs needs to be high. Currently, there is no consensus of the optimal cell dose, and the number of cells used varies \((3.8 \times 10^7 - 1.5 \times 10^8)\) in different studies [7,9,17,18]. This is because a variation of clinical applications. Also, results regarding the mononuclear cell yield in the SVF after automated extraction is diverse \((2.6 \times 10^5 - 1.2 \times 10^6\) cells/ml adipose tissue), both between different instruments and different studies using the same instrument [16,19]. Another aspect considering autologous cell transplantation is the interdonor variability. Viability and potency of the cells are now tested with methods requiring days of culturing. The results will therefore be provided after cell transplantation [20]. One possible factor which may correlate to cell potency is telomerase activity. Telomerase is an enzyme which expands telomere length thereby giving cells limitless cell division capacity. Telomerase is in cancer cells and embryonic stem cells [21]. However, the expression of telomerase in MSCs is debated. Studies using the standard telomeric repeat amplification protocol TRAP PCR have reported conflicting results [22,23]. Recent improvement of TRAP by using digital droplet PCR technique has demonstrated significantly higher sensitivity [24] which opens the possibility of re-evaluating telomerase activity in MSC’s.

In this study the automated instrument Sepax2® was used for extraction of ADSCs from collagenase digested adipose tissue. Sepax2® is a fully automated closed system for processing ADSCs, is CE marked and claimed by the company to be able to fulfill GMP criteria. Sterile single-use kits are used [25]. When extracting ADSCs a mix of cells called the stromal vascular fraction (SVF) is received. The SVF in this study was cultured to isolate ADSCs, and both the SVF and cultured ADSCs was studied regarding morphology, cell surface markers and viability. Viability was assessed using a colony forming unit (CFU) assay which requires approximately 14 days of culture. An improved measurement of telomerase activity was tested as an attempt to correlate telomerase activity with the potency of the ADSCs. Morphology was continuously studied, since senescence in MSCs can be detected by cell enlargement [26].

*The aim of the study* was to characterize the SVF and the cultured ADSCs to investigate if the freshly isolated SVF is a good option for autologous stem cell transplantation, and to compare the results with previously published articles. To be suitable for potential therapy we looked at the following criteria: I) sterility of the extraction procedure, II) cell surface markers CD105+, CD73+, CD90+ CD45−, CD34−, CD11b−, CD19− and HLA-DR−, III) predictability of the
viability and potency of the specific donor’s cells, IV) cells morphology, V) amount of cells extracted.

**Material and methods**

*Tissue source*

Adipose tissue was obtained from three patients undergoing abdominal plastic surgery and tumescent liposuction after given their informed consent. The adipose tissue was stored during night under sterile conditions in 4°C. The material de-identified and no medical information of the patients was collected or used in the study.

*Isolation of SVF*

100 ml adipose tissue was transferred into a 600 ml transfer bag (JMS Co. Ltd., Tokyo, Japan) and washed three times using 900 ml Ringer-Acetate solution (B. Braun, Melsungen, Germany). Collagenase (NB 4 Standard grade from *Clostridium histolyticum*, SERVA electrophoresis, Heidelberg, Germany) suspended in PBS at a concentration of 0.162 U/mg was added and the lipoaspirate was placed on a rotary shaker in 37°C for 60 min. The digested lipoaspirate was transferred into an accessory input bag RCA-100 with an integrated (300 µm) mesh filter (BioSafe, Eysins, Switzerland) and connected to the CS-900.2 processing kit (BioSafe, Eysins, Switzerland) in an ISO class 8 cleanroom. The kit was installed on the Sepax2® device according to the Adipose protocol, Opertor’s Manual. The end product of 8 ml SVF was collected manually using a sterile 20 ml Luer Lock syringe (Sigma-Aldrich®, St Louis, MO, USA).

*Sterility testing*

10+10 ml of the waste fluid from the Sepax procedure was cultured in blood culture bottles for aerobic and anaerobic bacteria (BD BACTEC™, Sparks, USA). Culture time was 14 days for patient 1 and 2, but bottles from patient 3 were accidently cultured for 7 days. The endotoxin analysis of the SVF was performed according to the clinics routine for testing endotoxin levels in hemodialysis fluids.

*Culturing*

Approximately 7 ml of the SVF was seeded in a T75 culture flask (Sarstedt, Newton, NC, USA) with α-MEM supplemented with 10% FBS (Fetal Bovine Serum Australian Origin, Lonza BioWhittaker™, Verviers, Belgium) and 1% penicillin-streptomycin (GE Healthcare
Life Science, Wien, Austria) and cultured in 37°C, 5% CO₂. Medium was changed every 3-4 day and the cells were passed when considered to be 80% confluent, approximately once a week.

**Flow cytometry**

The SVF and cultured ADSCs at every passage was analyzed considering cell surface antigens using the BD Stemflow™ Human MSC analysis kit (BD Biosciences, San Diego, CA, USA). Briefly, the cells were washed with PBS and detached using TrypleSelect (GIBCO®, Life Technologies™, Carlsbad, CA, USA) and resuspended in 2.5 ml staining buffer (PBS with 0.5% FBS). Falcon tubes (BD Falcon™, BD Biosciences, San Diego, CA, USA) were prepared with antibodies and 100 µl cell suspension and incubated for 30 minutes in the dark at room temperature. The cells were washed twice using 1 ml staining buffer and finally re-suspended in 500 µl. Analysis was performed on a FACSCanto™ II flow cytometer (BD Biosciences, San Diego, CA, USA). Compensation was made using confirmed MSCs stored in the lab.

**Colony forming assay**

Mononuclear cells (MNCs) in the SVF were counted using Sysmex XE-5000 (Sysmex corporation, Kobe, Japan), plated in duplicate in two culturing dishes with 10 cm diameter (Sarstedt, Newton, NC, USA) at a density of ~500 cells per dish corresponding to ~6 cells/cm² and cultured in α-MEM supplemented with 10% FBS and 1% penicillin-streptomycin at 37°C, 5% CO₂ with humidity for 14 days. After staining with crystal violet (Merck, Darmstadt, Germany) and washing with water the number of colonies was counted using a light microscope (Zeiss, Oberkochen, Germany). Every group of cells involving over 40 cells was considered a colony.

**Telomerase activity assay**

At every passage 1 ml cell suspension was centrifuged 5 min at 300 g and the pellet re-suspended in cryo medium (α-MEM with 10% DMSO (Sigma-Aldrich®, St Louis, MO, USA)) and stored in a nitrogen tank. Cells from passage two from all three patients were thawed and cultured for three days. ddTRAP PCR was performed as described by A. T. Ludlow et al [24]. Briefly ~200 000 cells from patient 1 and 3, 65500 cells from patient 2 (because of poor cell growth) and 200 000 cells from a positive control cell line (telomerase expressing cervix squamous carcinoma, SiHa cell line, ATCC®, Manassas, VA, USA), lysed
in 40 µl CHAPS buffer (TRAPEZE® 1xCHAPS lysis buffer, EMD Millipore Corporation, Temecula, CA, USA) on ice for 30 min. After centrifugation at full speed for 20 min in 4°C 5 µl of the supernatant was added to 45 µl extension reaction (1xTRAP buffer, 2,5 mM dNTPs (Applied Biosystems™, Warrington, UK), 200 nM TS primer (5’-AATCCGTCGAGCAGAGTT-3’) (Sigma-Aldrich®, St Louis, MO, USA), and run at 30°C for 40 min, 95°C for 5 min and 4°C∞). 4 µl was added to 21 µl MasterMix (1xEvaGreen (Bio-Rad, Hercules, CA, USA), 100 nM TS primer, 100 nM ACX primer (5´-GCGCGGCTTACCCTTACCCTTACCCTAACC-3’) (Sigma-Aldrich®, St Louis, MO, USA)). The following controls were included: heat inactivated cell lysate (HCL), no template control (NTC), no primer control (NPC) and CHAPS buffer control. Droplets were generated in a QX200 droplet generator (Bio-Rad, Hercules, CA, USA) according to standard protocol. 40 µl of the droplets was added to a PCR plate 96 (Eppendorf twin.tec®, Hamburg, Germany) and run on a Verity 96 Well ThermoCycler (Applied Biosystems, Warrington, UK) (95°C 5 min 40x(95°C 30 s, 60°C 1 min) 4°C 5 min, 90°C 5 min and held at 4°C with ramp rate 2,0°C/s). Post-PCR analysis was done using QX200™ Droplet Digital™ PCR (ddPCR) System (Bio-Rad Laboratories, Hercules, CA, USA).

Cell morphology

A cytospin analysis of the SVF and cultured ADSCs at every passage was done. Briefly, 100 µl cell suspension left from the flow cytometry was used, spun at 800 g for 5 min, fixed and stained with May-Grünwald (Sigma-Aldrich®, St Louis, MO, USA) and Giemsa (Merck KGaA, Darmstadt, Germany). Cell morphology was studied in a Olympus BX40 microscope and cellSens (Olympus, Hamburg, Germany). Cultured ADSC’s was documented with ZEN lite Digital Imaging Software (Zeiss, Oberkochen, Germany)

Results

Sterility

No bacterial growth was found in the Sepax waste after 14 (patient 1 and 2) and 7 (patient 3) days of incubation. The (<0,5 IU/ml) limit for acceptable endotoxin levels (according to the European Pharmacopoeia restrictions for medicinal products for injection) were met for all three cell preparations.
Expression of surface markers

Both SVF and cultured ADSCs were analyzed by flow cytometry considering CD105, CD73, CD90, CD45, CD34, CD11b, CD19, HLA-DR. The SVF contained cells that showed a variety of surface markers in all three patients. The positive markers for MSCs were not expressed in all cells (CD73: 43,1%, CD90: 42,6% and CD105: 4,0%) and expression of negative markers (CD45, CD34, CD11b, CD19, HLA-DR) was high (82,4%). The negative markers were all labeled with the same fluorophore, making specific markers indistinguishable. From the first passage and continuously through all five passages cells from all patients expressed CD90, CD73 and CD105 in a way that matched the expected profile of MSCs, with less than 2% of the cells expressing the negative markers (figure 1 and table 1).

**Figure 1.**

a) Flow cytometry of cultured ADSCs. CD90, CD105 and CD73 is expressed in all cells (high fluorescence), and no expression of CD34, CD11b, CD19, CD45 or HLA-DR (negative cocktail) can be seen. Representative picture of the results of all patients and passages.

b) Flow cytometry of the SVF. Expression of surface markers of SVF cells. Gates are set after estimated mean fluorescence indicating positivity in isolated ADSCs. Data is shown from patient 1, as a representative picture of the results.
Viability

To address cell viability MNCs from the SVF were plated at low density and scored for colonies of ≥40 cells after 14 days. In the cultures from patient 1 and 2, 5.8 and 0 colonies/100 plated cells were found respectively. The culture of patient 3 was overgrown and individual colonies could not be distinguished. The mean value of cells in the total volume (8 ml) of the SVF was 1.04x10^7 (range 9.4x10^6-1.2x10^7). At each passage cells were counted and analyzed considering viability using trypan blue exclusion in a TC20™ automated cell counter (Bio-Rad Laboratories, Hercules, CA, USA). Live cell number (cells/ml) was used to calculate the theoretical total cell number as if all cells were continuously cultured (figure 2). Since the cultures from all patients were continuously passed at 80% confluence the total cell number at passage five was about equal (~10^11). However, cells from different patients showed different growth rates, with the number of culturing days to reach passage five ranging from 25 to 38 (figure 2).

Telomerase activity

Cultured ADSCs from all three patients were negative for telomerase activity in the ddTRAP PCR assay, when adjusting the threshold according to fluorescence of the SiHa positive control cell line (figure 3).

Morphology

Light microscopy during culture revealed stable and homogenous cell size through culture up to five passages for all three patients. The cytospin confirmed the flow cytometry data of different cell types in the SVF where ADSCs, fibroblasts and lymphocytes could be observed. In cultured ADSCs some lymphocytes were still in passage one but not onwards in later passages (figure 4). Documentation of live cells showed a homogenous morphology considering cell length and area from all patients in all passages (data not shown).

<table>
<thead>
<tr>
<th></th>
<th>% CD90</th>
<th>%CD73</th>
<th>% CD105</th>
<th>% Negative cocktail</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pat 1</td>
<td>42,6</td>
<td>40,2</td>
<td>2,5</td>
<td>81,6</td>
</tr>
<tr>
<td>Pat 2</td>
<td>55,4</td>
<td>53,7</td>
<td>5,7</td>
<td>85,4</td>
</tr>
<tr>
<td>Pat 3</td>
<td>29,7</td>
<td>35,5</td>
<td>3,9</td>
<td>80,1</td>
</tr>
<tr>
<td>x</td>
<td>42,6</td>
<td>43,1</td>
<td>4,0</td>
<td>82,4</td>
</tr>
</tbody>
</table>

Table 1. Distribution of the different cell surface markers of the SVF cells (%) detected by flow cytometry.
Cell growth rate of cultured ADSCs. At each passage the number of viable cells was counted, presented (with range values) as if all cells were continuously cultured.

Droplet digital TRAP PCR of telomerase activity. When adjusting the threshold to the positive control (SiHa) cultured ADSCs from all three patients are negative. The reason for higher fluorescence in tube 1-8 is probably due to a poor droplet quality, since the method is not optimized.

HCL=heat inactivated cell lysate, NPC=no primer control, NTC=no template control and CHAPS=buffer control.
Discussion

The automated extraction of the SVF from digested adipose tissue via Sepax2® seems to be sterile. No bacterial growth was found in waste fluids of the extraction procedure and the endotoxin levels of the final product were below the acceptable limit. However, there are limitations to the methods used. Because of the small volume of the final product waste fluids from the extraction procedure were tested instead of the final product, which provides more uncertain results. The whole procedure from the plastic surgery to the SVF was not performed under sterile conditions in this study. Even though the results indicated sterility a more closed and controlled handling of the starting material during surgery and the product received during surgery until transplantation is desirable and very much needed to ensure patient security. To enable this suitable and sterile material for tissue transfer and optimal logistics must be available. In this study the tissue had to be handled partly outside the clean room because of lack of optimal resources.

The expression of surface markers differed in the SVF and cultured ADSCs. Earlier studies show comparable results [27]. The SVF is a mixture of cells and the high expression of MSC negative markers (CD45, CD34, CD11b, CD19, HLA-DR) is an expected result since they indicate the presence of leukocytes and endothelial cells known to exist in the SVF [1]. The
reason for the change in expression of CD105 is not understood, but is a known phenomenon [20]. The question is how it influences the therapeutic effects of the SVF compared to cultured ADSCs, and if the set of markers used to identify MSCs really is the best suited to identify MSCs in the SVF and ADSCs. This issue is addressed in several studies and other markers (e.g. CD13 and CD36) have been tested and suggested to be more specific [20]. The field of ADSC research still awaits consensus in this matter.

In this small study viability was the one factor which varied most between patients. Since only three patients were included the results are not significant, but if this difference is true it is an important input in the debate of autologous cell therapy. Inter donor variability has been addressed in other studies, and medical conditions as obesity and diabetes may correspond to a low number and a poor quality of ADSCs [28]. The medical conditions of the patients in this study are unknown and therefore possible correlations to the cell quality cannot be made. If medical conditions negatively affect cell quality, and only young healthy individuals have a high yield of therapeutically suited ADSCs, a donor bank may provide a more potent and reliable therapy rather than autologous donation. Another approach would be to identify an adequate assay to anticipate cell potency directly after extraction, without the need for culture as in the commonly used CFU assay. An attempt to set up such an assay was made in this study: by measuring telomerase activity in the cells with the most sensitive assay available, the ddTRAP PCR method and correlate the results to cell potency. In this study no telomerase activity was found, but this could be due to several limitations. The use of frozen cells could have affected the cell quality and investigation of telomerase activity in earlier passages is also needed. The method was not fully optimized, and issues concerning droplet quality have to be resolved before a definite conclusion can be drawn.

The SVF cell number (~1x10^5 MNCs/ml processed adipose tissue) did not reach the amount of cells used in most clinical trials. However, if 300-400 ml adipose tissue was harvested an acceptable cell dose could be received, and cell culture expansion could be avoided.

**Conclusion**
To conclude if autologous cell therapy with the SVF from adipose tissue is a good alternative compared to cultured ADSCs further research is needed. This study strengthens that automated extraction is a safe and effective way to receive the SVF with regard to sterility, but points to the need of other assays to be able to directly assess the quality of the cells.
Acknowledgement
A special thanks to Art Clinic (Uppsala) for providing lipoaspirate, and to Susanne Axelsson and Lorraine Eriksson for helpful assistance to operate the flow cytometry experiment and the ddTRAP PCR assay.

References


Ethical consideration
The patients donating tissue to this study were included after giving their informed consent. Information was given in written form but a represent for the study was available in person or by phone to answer potential questions. The donors were undergoing a planned abdominal plastic surgery and liposuction. The adipose tissue used in the study to extract adipose derived stem cells (ADSCs) is a surgical waste product received after the normal surgical process; therefore the participation did not cause any risks for the patients. The tissue was used de-identified, but was coded. This made it possible for patients to withdraw their participation at any time. No medical or other personal data was collected from the patients, and the tissue can therefore not be linked to a specific person in any way. During the study the material was used and stored coded in the department of laboratory medicine, Örebro University hospital, Sweden. It is a locked facility and accessible only by the staff of the department. In the end of the study all material was discarded and can therefore not be used in any other purpose than intended, and cannot be used to identify the patients.

This is a student project and the article is written without intention to be published in any journal. According to an agreement within Örebro University hospital, Sweden, and the Swedish law of ethics (etikprövningslagen, 2003:460, 2 §) no application for ethical vetting is required to perform this type of study or to written this type of article.
Letter of intent
2016-01-07

Dear Editor,

I wish to submit a new manuscript entitled “Characterization of adipose derived mesenchymal stem cells received via automated extraction” for consideration by the journal X.

In this paper we report on the diversity in the quality of adipose derived stem cells (ADSCs) received from different donors, and the need of an assay to predict the viability. ADSCs were received via Sepax2® performing an automated extraction procedure, and cultured for five passages. Character and viability was continuously followed, and the results implicate a difference in cell quality which could not be seen until after several passages. This is important when considering autologous versus allogeneic donation, where cell quality must be easy to evaluate to enable autologous donation. The paper should be of interest to readers in the area of stem cell research.

We confirm that this work is original and has not been published elsewhere and it is not under consideration for any other journal.

Please address all correspondence concerning this manuscript to me at katedv111@studentmail.oru.se.

Thank you for your consideration of this manuscript.

Best regards,

Katarina Edbom
Bachelor of Medicine
Medical School
Örebro University
Press release

Donera ditt fett för att rädda liv

Forskare världen över undersöker användning av stamceller som behandling för en mängd sjukdomar, däribland hjärtsvikt, artros och diabetes. Det bedrivs mycket forskning om stamceller funna i fettväv, där stamceller från bukfett verkar vara de mest lovande ur ett behandlingsperspektiv.

En studie har nu gjorts för att utvärdera om stamceller kan utvinnas från och ges som terapi till samma patient. Studien indikerar dock att det finns en stor variation i kvaliteten av cellerna som är svår att förutse, och föreslår att en donatorbank med celler med konstaterad god kvalitet möjligtvis är ett bättre alternativ.

- I framtiden kanske det är möjligt att donera fett för att hjälpa behövande patienter, ungefär på samma sätt som man nu kan donera blod, säger Katarina Edbom.

Studien är gjord med hjälp av ett instrument som automatiskt kan utvinna stamceller från fettväv, en procedur som endast tar en förmiddag. Denna procedur har också utvärderats, och bedöms ha en god potential för att användas inom sjukvården.

- Detta är dock en väldigt liten studie, och forskningen om stamceller från just fettväv är ännu i sin linda, poängterar Katarina Edbom. För att dra några slutgiltiga slutsatser krävs fler och större studier, men det är spännande att spekulera om framtiden redan nu.

Om donationsbanker med celler från fettväv eller behandlingar med eget fett blir verklighet återstår att se. Denna studie väcker dock intresset för att vidare följa forskningen inom detta område.

För mer information kontakta:
Katarina Edbom, med. kand., Örebro Universitet, katedv111@studentmail.oru.se
Karin Johansson, PhD, Laboratriemedicinska enheten, Universitetssjukhuset Örebro, karin.johansson11@regionorebrolan.se