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Frozen adipose-derived mesenchymal stem cells maintain high capability to grow and differentiate ☆

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ABSTRACT

In recent years, there has been a shift toward tissue-engineering strategies using stem cells for plastic and reconstructive surgical procedures. Therefore, it is important to develop safe and reproducible protocols for the extraction of adipose-derived stromal cells (ASCs) to allow cells to be stored in liquid nitrogen for future needs.

The aspirated liposuction obtained from healthy donors were immediately processed after the suction using a protocol developed in our laboratory. The resulting stromal vascular fraction (SVF) was then characterized by the presence of adipose-derived stromal cells, at later stage frozen in liquid nitrogen. After that, cells were thawed and again characterized by adipose-derived stromal cells, cellular survival, differentiation ability and Colony Forming Unit-Fibroblast like colonies (CFU-F).

Extraction and freezing of cells contained in the stromal vascular fraction demonstrate that thawed cells maintain the full capability to grow and differentiate in culture.

The advent of adipose-derived stromal cells use in tissue engineering will assume a wide role in esthetic restoration in plastic surgery. It is thus important to develop clinically translatable protocols for the preparation and storage of adipose-derived stromal cells. Our results show that adipose-derived stromal cells in serum free can easily be frozen and stored in liquid nitrogen with retention of 85% of cell viability and 180,890 cell/g yield plus normal proliferative capacity and differentiation potential compared with fresh controls. These observations set the basis for adipose-derived stromal cells banking.

Introduction

The importance and the role of adipose tissue has been lately greatly re-evaluated after the discovery that adipose tissue is the largest endocrine organ, which is able to interact with all major organs via production of a wide range of hormones and cytokines [25]. Furthermore, many groups working independently have shown that adult stem cells derived from white adipose tissue can differentiate along multiple pathways raising great hope in regenerative medicine, considering that adipose tissue can be an abundant source of therapeutic cells [17].

Mesenchymal stem cells (MSCs) were first isolated from bone marrow and then turned out to be able to regenerate rudiments of bone and support hematopoiesis in vivo [8]. They also provided an hemopoietic microenvironment in vitro [3,16] and circulated in the blood between tissues [15,14,7]. Plastic adherent populations isolated from bone marrow were proved to be functionally heterogeneous and fibroblast colony-forming unit-derived colonies were made up of undifferentiated stem cells and progenitor cells. These cells were multipotent and they were able to differentiate into mesenchymal cells types, including osteoblasts, chondrocytes, and adipocytes. Because of the fact that MSCs are generated from the stromal component of bone marrow, they were later renamed as multipotent mesenchymal stromal cells (with the same acronym) to reflect their origin and biological properties [6]. MSCs are found in many tissues, including bone marrow, umbilical cord, placental tissue and adipose tissue. However, adipose

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tissue-derived stems cells (even called adipose-derived stromal cells, ASCs) for autologous therapies are easier to obtain than MSCs from other tissue sources, such as bone marrow, opening the door for potential Advanced Therapy Products [17].

Recently, human ASCs were successfully reprogrammed into embryonic stem cell-like colonies (induced pluripotent stem cell, iPS) faster and more efficiently than adult human fibroblasts [20,1], using the strategy developed by Yamanaka and co-workers.

ASCs cells are also increasingly appreciated in the plastic and reconstructive surgical procedures, where the shift toward tissue-engineering strategies using stem cells is now apparent [22]. Currently available reconstructive surgery using synthetic materials or autologous fat transplants are often unsatisfactory, which is also due to the long-problems of volume maintenance. Transplanted ASCs may overcome these problems via real stem cell-based regeneration of the tissues and thus introducing the development of clinically translatable protocols for the preparation and storage of ASCs for tissue engineering.

In this report we validate a safe and reproducible protocol to extract and freeze ASCs from lipo-aspirated and we demonstrate that ASCs can be frozen and thawed without damaging or compromising their stem cell properties.

Materials and methods

Surgical techniques and adipose tissue sampling

Liposuction was performed during surgical esthetic procedures. Women older than 18 years (range 18–53 years) in good health and HIV (Human Immunodeficiency Virus), HCV (Hepatitis C Virus) and HBV (Hepatitis B Virus) negative were included in this study after obtaining their written informed consent.

Liposuction procedure started with a preemptive analgesia: Calecoxib 200 mg per os (400 mg for patients whose weight is over 50 kg) about 1 h before surgery. Before going to the operating room, we administered an intravenous infusion with 100 ml of NaCl 0.9%, Ranitidine 50 mg, Ondansetron 4 mg, Desametason 8 mg, Cefazolin 2 g and a sedation with Midazolam 1 mg bolus I.V. Sedoanalgesia was performed with Sufentanil bolus I.V. (0.05 μg/kg) and Propofol continuous infusion.

The access points of the cannula were infiltrated with a physiologic solution containing 0.1% lidocaine and 1:100,000 adrenalin. The composition and the quantity of the infiltrated solution depended on the volume of the adipose tissue to be removed and it corresponded to a 1:1 proportion with the aspirated amount. A negative pressure of 400 mm Hg was applied to the cannula connected to a 60 ml syringe for aspiration.

Isolation of stromal vascular fraction (SVF)

The isolation of the SVF was performed by means of a protocol we developed in our laboratories [2]. This isolation protocol is based on the use of a 100 ml syringe (Omnifix 100 ml with Luer Adaptor, B. Braun AG, Melsungen, Germany) as a separation funnel (Patent pending). The protocol is based on the fact that adipose tissue and hydrophilic fluids spontaneously separate in two phases with no need of centrifugation. The piston of the syringe is used to take in or to expel the solutions used to wash the sample, to dissociate the suctioned fat, or to extract the cells from the dissociated adipose tissue. The syringe is hold in a vertical position using a laboratory apparatus stand with support rings. Therefore, all the necessary manipulations for the extraction of ASCs are performed inside the syringe and last about 70 min. The first step is to wash the sample with 40 ml Dulbecco's PBS (DPBD, with Ca^{2+} and Mg^{2+}, PAA Laboratories, Pasching, Austria) by gentle agitation. The syringe is hold vertically in the support stand for a few minutes to allow the separation of the phases, then the lower aqueous phase is discarded by pushing the piston. The sample is washed twice. To free the cells in the aqueous phase the washed adipose tissue must be digested with the appropriate amount of Liberase MTF-S (Roche Applied Science, Basel, Switzerland) at a final concentration of 0.28 Wünsch U/ml diluted in 10 ml DPBS (with Ca^{2+} and Mg^{2+}). The sample is incubated for 45 min at 37 °C under constant but gentle agitation. Enzymatic reaction is stopped by aspiration of 30 ml of injectable 5% human albumin solution (CSL Behring AG, Bern, Switzerland) in the syringe. The syringe is then put back in vertical position to allow the separation of the phases. The lower layer, which contains now the SVF cells, is carefully poured out into a conical 50 ml centrifuge tube (TPP, Trasadingen, Switzerland). The extracted adipose tissue is washed again with 40 ml 5% human albumin solution to increase cell yield. Finally, after filtration through 100 and a 40 μm sieve (Cell Strainer, BD Falcon, Basel, Switzerland), SVF is centrifuged 400*g*, 5 min RT and the pellet suspended another time in DPBS (without Ca^{2+} and Mg^{2+}, PAA Laboratories, Pasching, Austria) or in tissue culture medium.

The SVF is then analyzed for cell count and number of nucleated cells using an electronic cell counter (Hemocytometer – AxonLab ABX Micros60).

SVF characterization by FACS analysis

The cells of the SVF were characterized by cytofluorimetric analysis using a 10 channel Navios cytometer (Beckman Coulter, "BC", Nyon, Switzerland), as earlier [21]. Briefly, roughly 500,000 cells from fresh SVF preparation were taken and centrifuged 5 min at 400*g*. The pellet was re-suspended in 220 μl of PBS without Ca^{2+}/Mg^{2+} (Eurobio, CS1PBS01) with 1% human converted AB serum (PAA, C11-021). 100 μl of cell suspensions were put into 2 test tubes and stained with control antibodies IgG2a-PE (BC, A12695), IgG1-KRO (BC, A96415), IgG1-APC-A750 (BC, A71120) and Syto 40 (Invitrogen, S11351) for the control tube and CD146-PE (BC, A07483, CD146 is a cell adhesion antigen present mainly in endothelial cells), CD45-KRO (BC, A96416, CD45 is a common leukocyte antigen found on all leukocytes), CD34-APC-A750 (BC, A89309, CD34 is an hematopoietic progenitor cell antigen found on hematopoietic stem cells), 7-AAD and Syto 40 for the positive tube, respectively. All antibodies were used accordingly to the manufacturer's instructions. After 20 min of incubation, erythrocytes were lysed with 1 mL of VersaLyse (BC, A09777). Before acquisition, 100 μl Flow-Count Fluorospheres (BC, 7547053) were added to the test tube. Post-acquisition, the data were analyzed with the Kaluza software (BC). Briefly, the DNA marker Syto 40 was used to exclude cellular debris (i.e. negative) and 7-amino-actinomycin D (7-AAD) was used for dead and live cell discrimination and therefore for assessing the cellular viability [10,18]. ASCs were identified in the CD45 and CD146 negative and CD34 positive fraction [6,21]. Finally, Flow-Count Fluorospheres were used to directly determinate the absolute number of ASCs by applying the formula: Absolute Count (cells/μl) = (Total Number of Cells Counted/Total Number of Fluorospheres Counted) × Flow-Count Fluorospheres Assayed Concentration.

Colony-Forming Unit (CFU-F) assay

The CFU-F assay was performed as already described elsewhere and used to evaluate the frequency of mesenchymal progenitors in the SVF fraction. Therefore, freshly extracted nucleated cells were plated at two cell concentrations (5000 and 10,000 cells) in standard 100 × 20 mm tissue culture dishes (growth area 58.95 cm^2, BD Falcon, Basel, Switzerland) and cultured in MEM/5% converted human serum/1% antibiotics for 14 days. The plates were then