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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

washed with DPBS, fixed in 2% formaldehyde (Sigma–Aldrich, Buchs, Switzerland)/0.2% Glutaraldehyde (AppliChem, Darmstadt, Germany) for 5 min and stained with crystal violet solution (Sigma–Aldrich, Buchs, Switzerland) for 10 min. After washing the plates with water, the number of colonies were counted. A colony consisting of more than 50 cells was defined as a CFU-F.

Cryopreservation and thawing of SVF

Fresh SVF cells were centrifuged 5 min at 400*g*, re-suspended in 25 ml ice-cold solution of injectable 5% human albumin solution with 5% ME_2SO (Dimethylsulfoxide, WAK-Chemie Medical GmbH, Steinbach, Germany) and transferred into a freezing 25 ml cryobag (Pall Europe Ltd., Portsmouth, England). Cells were frozen by means of a programmable freezer (Consartic GmbH, Schoellkrippen, Germany) under the following "controlled-rate" conditions: from 4 °C to 0 °C in 6 min, then hold for 15 min at 0 °C. From 0 °C to −2 °C in 9 min and then hold for 2 min at −2 °C. From −2 °C to −35 °C in 25.5 min and finally from −35 °C to −100 °C in 13 min.

For what regards thawing, the cryobag was immersed in a 37 °C water bath for 2–3 min. Immediately after being thawed, the cells were carefully aspirated, mixed with an equal volume of injectable 5% human albumin solution in a 50 ml TPP conical tube and centrifuged at 400*g* for 5 min.

Cell culture and differentiation

Adipose-derived mesenchymal stem cells were cultured until passage 2 in basal Ham's F12/IMDM (1:1) medium (Cell Culture Technologies, Gravesano, CH) supplemented with various growth factors and referred as to serum free medium (Patent pending). Cells were then plated at a density of $3 \times 10^3/cm^2$ onto multi wells plates (PureCoat ECM Mimetic Cultureware, BD Biosciences, Bedford, USA) for induction. Half of the wells cells were cultured in the conditions specified here above, i.e. serum free medium (basal Ham's F12/IMDM (1:1) medium supplemented with growth factors) and referred as non-induced cells, whereas in the remaining wells cells were induced to osteoblasts, adipocytes and chondrocytes by means of different induction media. For osteoinduction we used the serum free medium supplemented with 3 mM Sr^{2+} and 10–200 nM Vitamin D. Cell differentiation was confirmed at day 21 by Alizarin Red staining. Briefly, the cells were fixed in 10% formalin for 30 min RT and incubated 30 min RT in Alizarin Red staining. The formation of red calcium deposits is a marker of osteogenic differentiation. For adipogenic induction serum free medium was supplemented with Epidermal Growth Factor (EGF, cyt-217, ProSpec-Tany Technogene Ltd., East Brunswick, USA) and Rosiglitazone (Sigma–Aldrich, Buchs, Switzerland). Adipogenesis was assessed by Oil Red staining. Briefly, cells fixed in 10% formalin for 30 min RT were incubated in fresh Oil O Red water solution for 5 min RT. Induced cells were visible as cells containing consistent red deposits in vacuoles. Chondrogenic differentiation was assessed by induction of ASCs using the micro mass method. Briefly, ASCs were gently centrifuged in a 15 ml conical tube to form small pellets and then cultured for 21 days in the serum free medium supplemented with sodium pyruvate, Bone Morphogenic Protein 6 (BMP6), Transforming Growth Factor Beta 3 (TGF-beta3), Fibroblast Growth Factor beta (beta-FGF) and Prostaglandin E2 (PGE2). Chondrogenic pellets were fixed in 10% formalin for 30 min RT. Samples were then embedded in paraffin and sections stained with Alcian Blue. Control cells did not retain a spheroid shape and showed no specific staining while induced cells showed a strong blue signal.

Results

We analyzed the adipose-derived stromal vascular fraction of more than 130 liposuction procedures. We show here the obtained data from $N = 44$ adipose tissue samples before cell culture. On average, we obtained 75.3 g of fat tissue per sample and 180,890 total nucleated cells/g. The procedure developed in our laboratory allows the extraction of nucleated cells in a safe and the reproducible way by showing an average cell viability of 85.05% as measured by 7-AAD stain (Table 1 and Fig. 1, left panel).

ASCs cells were characterized by FACS analysis and considered to be CD45 and CD146 negative and CD34 positive. On the 44 samples considered we found an average of 26.44% of ASCs, following the characterization by FACS method (Fig. 2).

ASCs were then checked for the ability to form CFU-F colonies. The average value for colony formation in fresh samples was 5.8×10^{-3} colonies, where a colony was defined to have more than 50 clonal cells (Table 1). We also checked the CFU-F after thawing by seeding cells at three different concentrations for three different

Table 1
Characterization of SVF in $N = 44$ adipose tissue samples.

Sample	Volume	Cells/g	Viability (%)	ASCs (%)
1	50	22,600	60.88	7.94
2	50	93,950	64.46	15.84
3	100	85,830	74.04	17.49
4	100	79,500	76.75	16.83
5	50	104,000	72.64	30.10
6	40	176,600	79.71	32.37
7	50	100,300	68.18	20.67
8	40	127,800	60.19	20.25
9	50	61,400	68.65	8.03
10	50	288,708	94.77	8.68
11	50	170,905	88.14	18.09
12	50	39,235	75.41	6.71
13	50	35,882	65.73	12.99
14	150	15,265	85.52	16.77
15	150	118,110	68.93	40.00
16	150	163,333	86.05	35.28
17	150	310,396	93.00	43.18
18	150	6,033	93.00	23.48
19	48	49,875	94.60	31.00
20	30	66,500	94.60	31.00
21	48	302,083	82.93	17.43
22	52	182,692	84.09	18.92
23	122	259,016	80.00	28.12
24	40	87,500	82.31	29.47
25	40	50,000	91.58	25.44
26	80	452,500	83.00	42.91
27	40	140,000	94.73	43.04
28	30	45,800	97.23	43.79
29	80	138,750	98.28	13.17
30	40	146,625	98.15	22.02
31	104	49,279	89.65	32.48
32	30	250,000	95.95	45.64
33	82	595,122	94.00	44.31
34	89	547,890	88.67	35.70
35	60	193,500	98.62	30.85
36	158	6,582	84.50	17.09
37	80	91,875	98.20	24.79
38	50	105,000	94.00	33.60
39	50	175,000	87.08	25.57
40	136	218,750	93.49	39.82
41	180	464,667	89.89	10.23
42	68	491,912	97.64	36.50
43	50	420,000	86.75	22.95
44	50	428,400	86.21	42.98

Fresh adipose tissue samples were processed for ASCs extraction as described. Volume, cells/g, viability and percentage of ASCs are reported in Table. (SVF, stromal vascular fraction; ASCs, adipose-derived stem cells.)

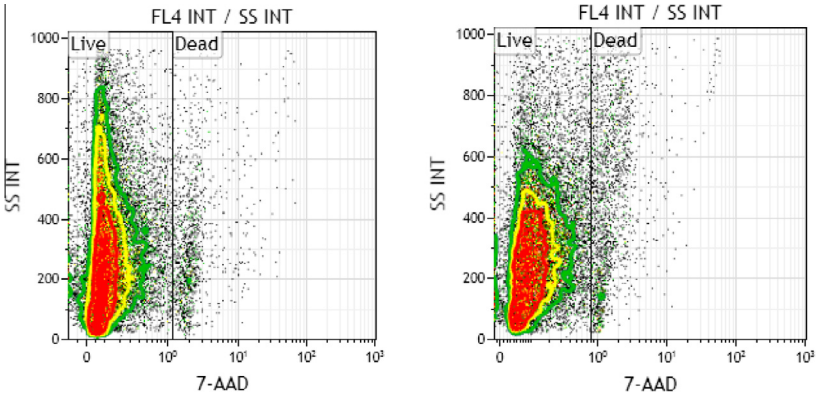

Fig. 1. Viability of stromal vascular fraction cells before and after a freeze/thaw cycle. FACS analysis for 7-AAD stain in fresh extracted SVF cells (left) and the same sample of SVF cells thawed after a freeze cycle (right). Dead cells are gated at the right of both panels. (7-AAD, 7-amino-actinomycin; SS, side scatter.)

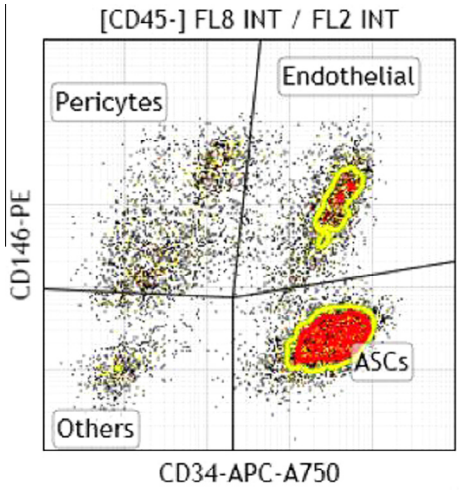

Fig. 2. FACS analysis of fresh adipose-derived SVF cells. FACS analysis for the characterization of ASCs in fresh extracted SVF. ASCs cells are CD45−, CD34+ and CD146− and are gated in the right lower panel. (FACS, fluorescent activated cell sorter; ASCs, adipose-derived stem cells; SVF, stromal vascular fraction; CD, cluster of differentiation.)

samples and the results showed that the freezing protocol do not affect the clonogenic ability of ASCs. Conversely, the CFU-F number was shown to increase after thawing (data not shown).

The fresh SVF cells were successively challenged by a freezing and thawing cycle. $N = 15$ samples were used in the freezing/thawing procedure as described above. SVF samples ranging from 6.66×10^5 to 3.94×10^6 total cells were taken in consideration. Table 2 shows the results of these experiments. Cell samples were kept frozen for periods ranging from 14 to 193 days. Viability of SVF cells was measured by FACS analysis and gave an average value of 89.6% ranging from 81% to 98%. The total ASCs content of each fresh sample ranged from 237,938 to 1,092,925 with an average value of 587,753 cells. After thawing, cells were counted for ASCs number and viability. We could demonstrate the viability results over 15 samples, ranging from 71.7% to 98.3% and average recovery rates of 79.82% of living ASCs after the freeze/thaw procedure.

Alive cells after a freezing/thawing cycle are important because the freezing process prolongs cells' life and makes them available for future therapies based on expanded ASCs. To check whether the thawed cells can grow and differentiate again after the freezing/thawing cycle, we cultivated and differentiated 3 samples of thawed SVF-cells in 0.1% human serum supplemented medium. The results are showed in Fig. 3. Three different samples were plated at 3000 cells/cm^2 and cultured for 20 days. Cells showed a classical growth pattern with an early lag-phase in the first 7 days and a subsequent exponential growth. After 20 days in culture, cells reached a concentration of 42,550 cells/cm^2 i.e. a 13× expansion of the initial seeded number.

The same cells were induced to differentiate into adipocytes, osteocytes and chondrocytes and representative results are shown in Fig. 4. Cells were clearly inducible to the specified phenotypes. Oil Red staining evidenced adipoinduction by red deposits in vacuoles (Fig. 4, panel A: induced and D: control), whereas Alizarin-S staining was used for osteoinduction and showed the formation of red calcium deposits as a marker of osteogenic differentiation (Fig. 4, panel B: induced and E: control). Sections of chondro-induced samples stained with Alcian Blue showed a strong blue signal (Fig. 4, panel C, induced and F, control). We found all tested samples to be inducible for the differentiation of adipocytes, osteocytes and chondrocytes.

Discussion

Tissue engineering keeps promise for the restoration of the soft tissue esthetic function and for the treatment of known diseases that have currently no therapy option [22]. In this regard, the storage of ASCs is still for long time the initial step for future cell therapies using ASCs for regenerative purposes. There are actually more than 60 clinical trials worldwide involving ASCs in the treatment of human disease (clinicaltrials.gov [9]). Current standard clinical strategies for soft tissue augmentation primarily include the use of synthetic implants and fillers. However, various complications derived from the foreign body, such as capsular contracture or displacement, lead to implant removal or replacement at a relatively high rate.

Free fat transfer gives unpredictable results, where graft reabsorption can vary between patients, although it seems to work well for small defects correction [4].

Mixing autologous ASCs with a portion of suctioned fat and injecting subcutaneously back into the target site is another strategy which is recently used to overcome these problems and to provide a "living scaffold" for stem cells [27].

It has become crucial to develop safe and reproducible protocols for the extraction and storage of ASCs that can adhere to the strict European regulation concerning the Advanced Therapy Medicinal Products (ATMPs). Storage of the SVF could be seen as an intermediary GMP product to be needed in the future for many differentiation protocols to be developed. One step in this direction is the possibility to store frozen cells for long periods of time in liquid

Table 2
Post-thaw characterization of SVF in $N = 15$ adipose tissue samples.

Sample no.	Pre-thaw			Post-thaw		
	Total SVF cells	Total ASCs	Viability (%)	Total ASCs recovered	ASCs recovery (%)	Viability (%)
1	1,681,603	726,185	93.00	691,000	95.15	88.25
2	840,801	363,093	93.00	265,000	72.98	91.65
3	852,300	237,938	98.00	179,000	75.23	76.56
4	1,704,600	475,876	98.00	363,000	76.28	91.80
5	1,704,600	475,876	98.00	344,000	72.29	77.05
6	666,667	288,084	93.00	197,000	68.38	88.75
7	666,667	288,084	93.00	190,000	65.95	89.86
8	3,949,910	1,092,925	89.00	1,228,000	112.36	98.29
9	1,265,923	334,800	81.25	252,000	75.27	93.06
10	835,625	276,250	89.67	229,000	82.90	82.90
11	3,949,910	1,092,925	89.00	919,000	84.09	75.72
12	3,949,910	1,092,925	89.00	874,000	79.97	71.72
13	3,180,288	868,269	79.60	539,000	62.08	88.16
14	3,180,288	868,269	79.60	539,000	62.08	88.16
15	1,265,923	334,800	81.25	376,000	112.31	84.08

SVF samples were characterized for total cell content, total ASC content and viability and frozen in 10% DMSO in liquid nitrogen following the protocol described in Materials and Methods. Post-thawing results are shown for ASCs recovery and percentage of viability recovered after the freeze cycle.

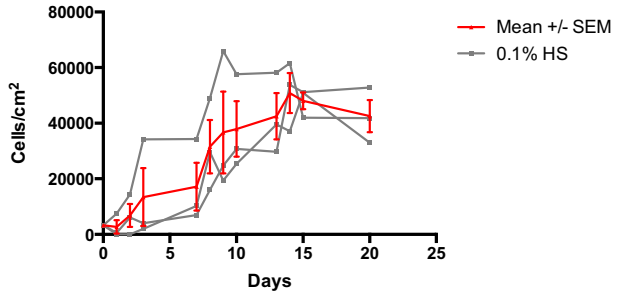

Fig. 3. Growth curve of frozen/thawed ASCs. $N = 3$ samples (gray lines) of adipose-derived mesenchymal stem cells (ASCs) submitted to a freeze/thaw cycle were cultured for 21 days in a medium supplemented with 1% human serum. (ASCs, adipose-derived stem cells; red line = mean ± SEM). (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

nitrogen and to be able to use them after thawing, i.e. for cell amplification and/or differentiation.

We show here that SVF extracted cells can be frozen and thawed without losing their ability to grow and differentiate in mesenchymal-specific lineages.

Only a few studies examined the role of frozen storage of adipose tissue. One of them has recently described the storage of entire adipose tissue at various temperatures for periods longer than 1 year to see whether the tissue was still capable of adipogenic differentiation. Cells isolated from the tissue proved to be a reliable source of human ASCs and adipocytes [11]. Early research studies described a domestic −18 °C storage of adipose tissue for 2 weeks. Injection of fat tissue in nude mice demonstrated the survival of this tissue as compared to a control group of non-frozen tissue [19]. A simple freezing technique was recently used by storing fat tissues at −196 °C in liquid nitrogen for up to 8 days demonstrating a good maintenance of mitochondrial metabolic activity in the frozen grafts [12]. Remarkably, in both experiments fat tissue samples were frozen without the addition of a cryopro-

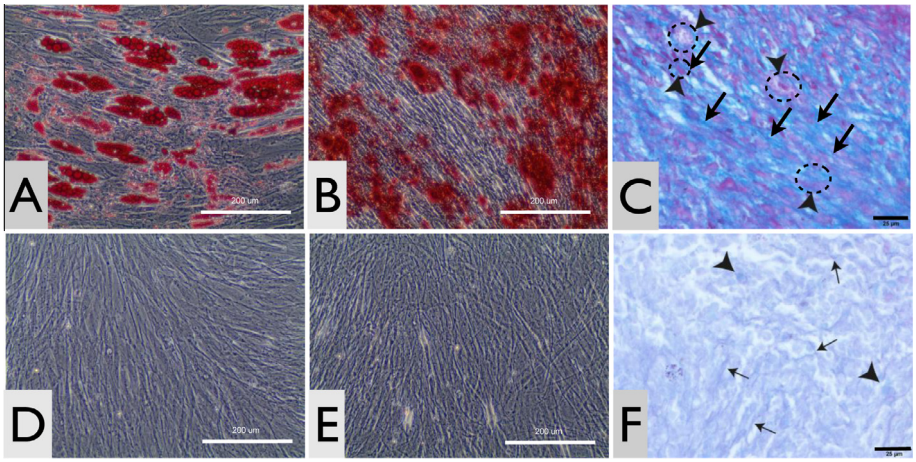

Fig. 4. Differentiation assay of thawed ASCs. Cells were grown confluent to 70% and induced for 9 days to differentiate to osteo-, chondro- or adipo-lineages. Staining was performed as described in Materials and methods, where, induced and non-induced samples were treated the same way. Oil-O-Red staining for adipocytes, Alizarin-S staining for osteocytes and Alcian Blue staining for chondrocytes were used. Induced cells incubated in fresh Oil-O-Red were visible as cells containing consistent red deposits in vacuoles (Panel A: induced cells; panel D: control); osteocytes induction, evaluated with Alizarin-S staining, is shown by the formation of red calcium deposits (Panel B: inducted cells, Panel E: control) and chondrocyte induction, as evaluated by Alcian Blue staining, show in Panel C (induced cells) robust fiber tracts of collagen matrix (black arrows) and several chondrocyte-like cells (dashed circles at arrowhead points) as compared to Panel F (control non-induced) where only marginally developed collagen-like fibers can be evidenced (black arrows), chondrocyte-like cells cannot be clearly identified and Alcian Blue stained areas are rather sparse (arrowheads). (ASCs, adipose-derived stem cells.)

tective agent. Another study reported the use of a cryoprotective agent to better save and keep viable tissues after thawing [26].

Nevertheless, we have to consider that adipose tissue is the source of ASCs responsible for the biological effect observed in regenerative medicine. Thus, for long conservation purposes we should only consider the stromal vascular fraction (SVF) by isolating it from the carrier tissue. Indeed, the vast majority of studies report the separation, growth and differentiation of the SVF and all clinical trials to date using ASCs have been designed on this particular fraction of cells, where a large number of stem cells have been found.

Published studies about the cryoconservation of human SVF-cells extracted from adipose tissues are rare (for a review see [24]). Recently, it has been described a method for liquid nitrogen storage of SVF-cells [5], where thawed SVF-cells has been shown to differentiate into adipocytes and endothelial cells. Unfortunately, this study used a freezing medium containing fetal bovine serum thus avoiding the possibility to use cells as an Advanced Cell Therapy Product.

The presence of serum in the freezing medium was also challenged in another study and reported to be not necessary by the authors. They suggested indeed that post-thaw ASCs viability, adipogenic and osteogenic differentiation can be maintained even when ASCs cells are frozen in the absence of serum but with a minimal concentration of 2% ME_2SO in DMEM [23], which represents a step forward to the use of these cells as therapeutic agents. Other reagents like sericin, a protein hydrolysate very rich in serine, has been used in the freezing medium and found to be effective on the survival of ASCs and in their differentiation potential [13].

MSCs are pluri-potential cells and can thus give rise to many target tissues, like bone, tendons, cartilages, heart and nerves, opening the door to the real world of Advanced Therapy Products that, in a first time, will be autologous-based but could in the near future be engineered to everyone's need.

We designed and validated a protocol to extract and freeze SVF stem cells from adipose tissues that allows thawed cells to maintain their growth and differentiation potential. Overall, our data show that the SVF can be easily frozen following defined standard conditions for cell freezing. The yield after the procedure, in terms of cell survival number and percentage of viable cells, is high enough to be safely used for banking purposes.

These results need further confirmation and we are actively working on the GMP-validation of the whole process to be able to store SVF-cells as a real medicinal drug, allowing thus the patient to dispose of his own cells for cell therapies in the near future.

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